TRANSCRIPTION ASSAY DEVELOPMENT WITH NUCLEI ISOLATED FROM PORCINE SKELETAL MUSCLE

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ABSTRACT

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A system for isolation of nuclei from porcine skeletal muscle (SM) and for *in vitro* RNA transcription was established in order to investigate the regulatory mechanism of developmental changes in SM protein. In addition, SM alpha-actin and beta-tubulin mRNA abundance for pigs of different ages were determined by Northern blot analysis. SM nuclei were isolated from longissimus dorsi muscle (LD) of 1- and 28-day old pigs by using a modification of a method used to isolate nuclei from cardiac muscle (Surk et al.,1974). Results from the tritiated UTP incorporation assay indicate that these nuclei preparations have the capacity to synthesize RNA and attain maximum incorporation after 40-45 minutes. SM nuclei from 1-day-old pigs synthesized more RNA than SM nuclei from 28-day-old, but the difference was not significant ($P>0.25$). The appropriate condition of the transcription assay was determined to be $3x10^7$ nuclei in a 400 ul assay volume. All nascent tRNA, rRNA and mRNA in the nuclei were elongated since [^{3}H]-UTP

incorporation was reduced after addition of 0.05 ug/ml alpha-amanitin in the transcription mixture. Transcription assay results indicated that more ($P<0.02$) SM alpha-actin hnRNA was synthesized in the 28-day-old pig SM nuclei than in the 1-day-old pig SM nuclei. Northern blot analysis indicated that there are developmental increases in alpha-actin mRNA ($P<0.03$) and no changes in beta-tubulin mRNA ($P>0.1$) from one- to twenty eight-day-old pigs. A decreasing trend of beta tubulin was actually observed. These results indicate that the relative increase in SM alpha-actin mRNA observed was due, at least in part, to an increase in the transcriptional activity of the SM alpha-actin gene.

This is dedicated to my family.

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LITERATURE REVIEW

In this study, we are focusing on the transcriptional regulation of muscle protein synthesis using Northern blot analysis and a transcription assay *in vitro*. Therefore, the following concepts are described in this introduction:

1. General information on transcription in eucaryotes.
2. General information on regulation of skeletal muscle protein synthesis.

1. GENERAL INFORMATION ON REGULATION OF GENE TRANSCRIPTION IN EUCARYOTES

DNA is the hereditary material which replicates and is transferred to progeny with high fidelity. In the nucleus of all eurcaryotic cells, DNA serves as a template to be transcribed to primary RNAs by RNA polymerases. These primary RNAs undergo post-transcriptional processing and modification by various nucleases, ligases and other modifying enzymes. Following the post-transcriptional changes, these transcripts become mature messenger RNA (mRNA), transfer RNA (tRNA) and ribosomal RNA (rRNA). The mature transcripts are then transported out of the nucleus to the cytoplasm, and ribosomal complexes composed of mRNA, tRNA, rRNA and ribosomal protein are assembled. These complexes function to translate codons into their

corresponding polypeptides. These polypeptides are modified by post-translational processing and fold to become functional proteins.

The transcriptional process includes initiation, elongation and termination. The major difference in transcription between eucaryotes and procaryotes is that transcription of mRNA, tRNA and rRNA in procaryotes is performed by a single RNA polymerase, but in eucaryotes there are multiple forms of RNA polymerase, each responsible for the transcription of a different RNA species. General characteristics of eucaryotic RNA polymerases are summarized in Table 1. RNA polymerase II has at least four subunits to form a pentameric holoenzyme which can carry out mRNA syntheses, and unlike procaryotic RNA polymerase, it does not recognize the promoter on the DNA sequence by itself. There are different transcription factors (TFs) required for promoter selection to begin the initiation process of transcription. These TFs are involved in initiation and regulation of transcription. TFs can be divided into two classes based on their functions, one composed of general factors that are required for the transcription of all genes transcribed by polymerase II, and the other class of sequence specific factors that are required for optimal or enhanced transcription of only one gene or subset of genes (Manley et al., 1980; Berk et al., 1990; Tjian et al., 1990).

In order to achieve basal level transcription from

Table 1. Characteristic of eucaryotic RNA polymerase.

	polymerase I	polymerase II	polymerase III	Mitochondria
native M.W KDa	7,500 KDa	7,500 KDa	7,500 Kda	140 Kda
location	nucleus	nucleoplasm	nucleoplasm	mitochondria
relative activity[a]	50%-75% activity	20%-40% activity	10% activity	1% activity for 100% mitochondria synthesis
role	produce rRNA 5.8S 18S, 28S	produce hnRNA and SnRNA	produce tRNA and rRNA 5S	produce all RNA in M.t genome
response to alpha amanitin	not inhibited by alpha-amanitin	inhibited by alpha-amanitin	inhibited by alpha-amanitin	not inhibited by alpha-amanitin

[a]Activity relative to total transcriptional activity performed by RNA polymerases.

polymerase II *in vivo*, promoters require at least four distinct general TFs for activity. These general TFs include TFIIA, TFIIB, TFIID, and TFIIE/F (Roeder et al., 1988[a]). The first step in the assembly of the transcription complex at the promoter is when TFIID binds to the TATA box promoter element. This complex then regulates the expression of most eucaryotic genes transcribed by polymerase II (Sharp et al., 1989). DNA binding studies suggest that several transcription factors might contact TFIID (Roeder et al., 1985, 1988[a], 1988[b], 1988[c]). After TFIID binds to the promoter TFIIA then binds to the promoter, followed by the incorporation of TFIIB, RNA polymerase II and TFIIE/F into the initiation complex. This complex then functions to direct the basal level of transcription (Greenblatt et al., 1990; Roeder et al., 1990; Tjian et al., 1989, 1990). TFIID is also thought to be the binding site for several upstream transcription factors (enhancers) such as USF, ATF,and GAL4 (Roeder et al., 1988[b], 1988[c]). However, the relationship between upstream regulators and TFIID remains unclear. Recently, Ptashne and Gann (1990) indicated that in addition to TFIID which is now defined by the cloned gene (Pei et al. 1990), the chromatographic fraction of TFIID contains some component or components, all of which are necessary for a response from any activating factors. Ptashne and Gann (1990) also concluded that all activators (upstream regulators) exert their effects by interacting with TFIID. Acidic activators

work directly and hence universally, whereas other activators such as Oct1, SP1, E1a, perform activation via intermediaries bearing the acidic activating regions. Walsh and Schimmel (1987) conducted DNA-binding assays to indicate that two nuclear factors compete for the chicken skeletal muscle actin gene. Their conclusion was that one complex is ubiquitous and the second appears to be controlled in a cell-type-specific manner.

Many characteristics of RNA differ between eucaryotes and procaryotes. Almost all the major types of RNA synthesized by cellular DNA-dependent RNA polymerase undergo changes before they can carry out their functions. Two types of changes are usually distinguished. The first is modification, which involves additions to or alterations of existing bases or sugars. The other is processing, which involves phosphodiester bond cleavage and loss of certain nucleotides (Burgess et al., 1988). All three classes of RNA (mRNA, tRNA and rRNA) have precursors in eucaryotes; however, in procaryotes the mRNA is a mature transcript, and ready for translation. Only the rRNA and tRNA have precursors. These precursors must undergo modifications and processing before they become functional types of RNA. A summary of RNA modification and processing is presented in Table 2.

The precursor of eucaryotic mRNA, heterogeneous nuclear RNA (hnRNA), is synthesized by RNA polymerase II. The early kinetic labeling experiments to determine the relationship

Table 2. Summary of RNA Modification and Processing

	mRNA		rRNA	tRNA
	Procaryotic	Eucaryotic		
precursor	None	hnRNA	Pre-rRNA	Pre-tRNA
Modification	None? Poly-adenylation?	Capping, Methylation, polyadenylation	Methylation	Many modified bases
Processing	In some case specific cleavage by endoribo-nucleases	In most case excision introns	Specific cleavage	Specific cleavage by endo-nucleases, trimming by exo-nucleases, CAA addition, removal of intervening sequences in eucaryotes
Products	mRNAs	mRNAs	16S,23S,5S in Pro. 18S,28S,5.8S in Eu.	Mature tRNA

between hnRNA and mRNA were ambiguous and indicated that not more than 10-20% of hnRNA of higher organisms migrates to the cytoplasm after processing (Lewin et al., 1975[a], 1975[b]). These data suggested that degradation of hnRNA occurs in the nucleus and the majority of the degradation products return to an acid-soluble pool (Houssais and Attardi, 1966; Warner et al., 1966). Due to difficulties encountered in conducting effective pulse-chase experiments and the inability to fractionate hnRNA from mRNA, the studies on relationship between hnRNA and mRNA have resulted in conflicting data (Naora, 1977; Adams et al., 1986). The estimated half-life of hnRNA varies greatly, depending on the species, cell types and the method of measurement. For instance, Gefter and Mory (1977) state that hnRNA is characterized by a high turnover rate, 5% of the genome corresponds to hnRNA and about 35% of hnRNA undergo post-transcription processing to mature mRNA.

It is now established that eucaryotic mRNA is the product of the maturation of longer precursors which arises, at least in part, from the high molecular weight nuclear RNA known as the hnRNA. There is a several fold difference in the size of hnRNA and mRNA. HnRNA can have a length of up to 50,000 base pairs (bp) whereas mRNAs, in general, are not larger than 7,000 bp. Almost all mammalian mRNAs fall within the size range of 400 to 4,000 nucleotides. In order to become mature mRNA, eucaryotic hnRNA must be altered by a process composed of three main steps: 5'-capping, 3'-

polyadenylation and the removal of introns. CAP addition occurs in the nucleus immediately after the 5′-end of the polyribonucleotide chain has been synthesized. It is an addition of a guanylate (GMP) through a 5′-5′ triphosphate linkage to the first nucleotide, with methylation on the N^7 position of the additional guanosine to become CAP 0 ("zero"). Further methylation on the 2′-O position of the ribose of the first two nucleotides become CAP 1 and CAP 2, respectively (Adams et al., 1986). Polyadenylation occurs when polymerase II proceeds to the cleavage signal (AAUAAA) and a specific ribonuclease that can recognize the signal then cleaves 10 to 30 nucleotides downstream of this signal. Poly A polymerase catalyzes the addition of a 200 to 250 adenylate (AMP) residues to the 3′ end. This poly A polymerase does not require a DNA template to synthesize the poly A tail (Burgess et al., 1988, Edmonds, 1990).

Intron excision and splicing involves a RNA-protein complex called a spliceosome. This protein-RNA complex composed of five small nuclear RNAs (snRNAs), four small nuclear riboprotein complexes (snRNPs) called U1, U2, U5 and U4/6, is assembled with hnRNA. After assembly of the spliceosome, the reaction occurs in two steps: the first step is lariat formation and cleavage at the 5′ splice site of intron and the second step is cleavage at the 3′ splice site, and subsequent exon ligation (Burgess et al., 1988; Mattaji et al., 1990; Siebel and Rio, 1990; Brow and Guthrie, 1990). The whole process occurs in the nucleus,

starting with capping addition, followed by initiation of intron excision and splicing, and then addition of the poly A tail and completion of intron excision and splicing. Mature mRNA is transported from the nucleoplasm to the cytoplasm where the mRNA is translated into protein on ribosomes. The abundance of mRNA, in general, is about 2-5% of total RNA which is the lowest abundance of the three types of RNAs. The variety of different mRNAs, however is the greatest of all the three types of RNA. That is due to a triple code required for one amino acid signal.

2. GENERAL INFORMATION ON REGULATION OF SKELETAL MUSCLE PROTEIN SYNTHESIS

Thick and thin myofilaments make up myofibrils in skeletal muscle fiber. On the surface of a fiber there is a lipoprotein bilayer, and nuclei are located along the muscle fiber underneath the plasmalemma called sarcolemma. Satellite cells are located between sarcolemma and basal lamina (basement membrane). Basement membrane is composed of proteoglycan and belonged to connective tissue (collage type 4). The sarcolemma of individual muscle fibers is surrounded by the endomysium, which is a delicate connective tissue. Approximately 20-40 muscle fibers, and the associated endomysium, are grouped into primary bundles. Several primary bundles are grouped together to form larger bundles known as secondary bundles. Both primary and secondary bundles are ensheathed in the perimysium, which is

a sheath of collagenous connective tissue. Finally, several secondary bundles are grouped together to form a muscle, which is itself contained in a connective tissue sheath called the epimysium (Judge et al. 1989). The cellular structure and composition of skeletal muscle presents inherent difficulty in isolating viable nuclei (Figure 1).

Muscle cell growth is a combination of muscle protein accretion and myogenic cell proliferation. Myogenic cell proliferation includes embryonic cell proliferation and satellite cell proliferation. Muscle protein accretion is the net difference between protein synthesis and protein degradation (Figure 2). A complete discussion on muscle cell growth is beyond the scope of this review. In this study we are focusing on muscle protein synthesis specifically at the level of transcription (Figure 2).

Protein accretion in postnatal muscle growth results from muscle hypertrophy which is synthesis of muscle specific protein. Muscle hypertrophy includes increases in myofiber length which involves an increase in sarcromere length, as well as sacromere number. The synthesis of muscle specific protein involves the synthesis of myosin heavy chains, myosin light chains and several isoforms of actin (Minty et al., 1981, 1982). Generally, from birth to maturity, muscle cell proliferation is low and myofiber number only increases slightly but nuclei number and total DNA increase 5- to 10-fold (Allen et al., 1979); however, DNA and RNA concentration (mg/g) decreases with increasing

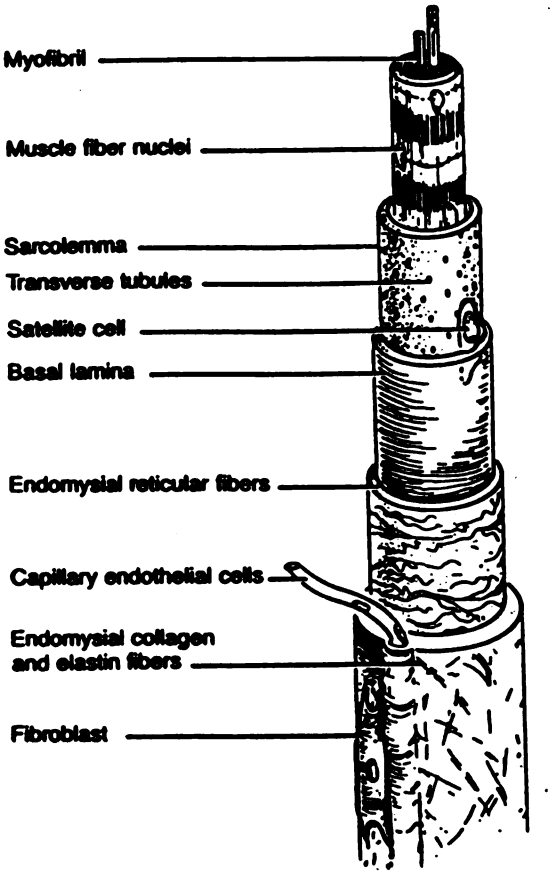


Figure 1. Schematic representation of the structure of skeletal muscle fibers. This figure was taken from "Muscle as Food" 1986 (Edited by Dr. P.J. Bechtel). The location of skeletal muscle nuclei is beneath the sarcolemma.

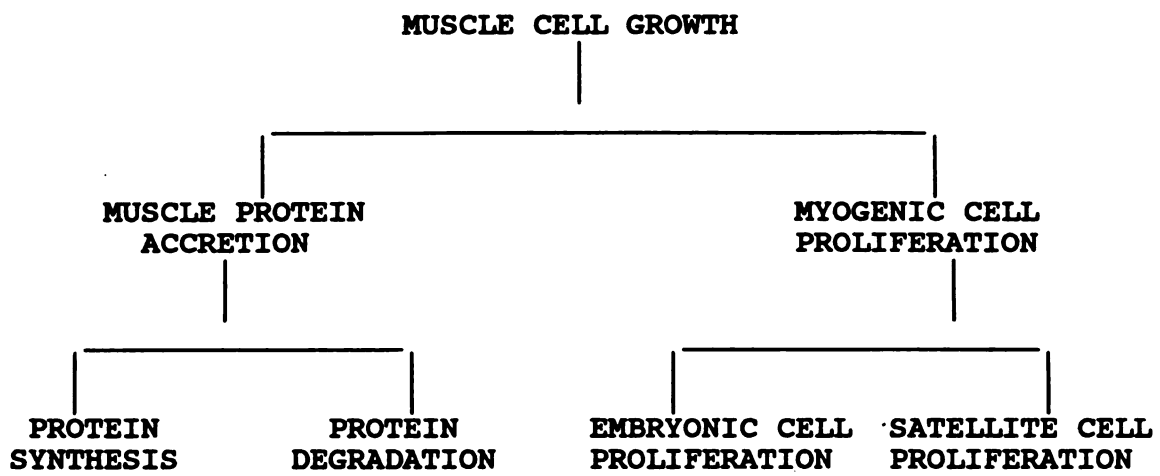


Figure 2. A general scheme outlining cellular processes involved in muscle growth. Muscle protein accretion is a function of intracellular protein synthesis and protein degradation, and myogenic cell proliferation encompasses processes involved in the replication of muscle precursor cells in prenatal and postnatal muscle (satellite cells) (From Allen, Merkel, and Young, Michigan State University, J. Anim. Sci. 49, 116).

age.

Using a cell-free translation assay, Devlin and Emerson (1979) demonstrated that the coordination of protein synthesis in culture was regulated at the level of mRNA. Hastings and Emerson (1982) demonstrated that gene sets in muscle development suggested by cloned cDNA should reveal whether transcriptional or post-transcriptional mechanisms determine contractile-protein mRNA level. Shani et al. (1981) have shown using well characterized cDNA probes to skeletal muscle alpha-actin, myosin heavy chain (MHC), and myosin light chain (MLC) that there is a strong correlation between the abundance of mRNA and the synthesis of these proteins during myogenesis in rat primary skeletal muscle cell cultures. These data suggest that a common mechanism may regulate gene expression in muscle based on coordinated regulation of muscle gene sets. Furthermore evidence for transcriptional regulation of muscle genes as a primary control was provided by Affara et al. (1980[a]) by comparison of the DNAse 1 hypersensitive site between myoblasts and myotubes in muscle cell culture. An open chromatin structure, sensitive to the activity of DNase 1, is correlated with the potential for a gene to be transcribed. Further indirect evidence for transcriptional regulation was provided by transfecting a putative sequence of muscle genes linked to a reporter gene such as CAT (chloramphenicol acetyl transferase) into myoblasts or myotubes in culture (Buckingham, 1989).

Medford et al. (1983) conducted a transcription assay with nuclei isolated from rat L6E9 muscle cell culture using a nonspecific myosin heavy chain probe to show that regulation is at the transcription level. However, the mechanism(s) by which synthesis of different proteins characteristic of muscle is turned on and off during myogenesis and during further maturation of muscle fibers remains to be determined. Factors which regulate the specific initiation, elongation, termination and subsequent processing of muscle-characteristic RNAs and the mechanisms that control the activation of muscle gene expression are also unclear. Since muscle gene activation during development results in the accumulation of specific mRNAs, Pearson and Epstein (1982) had suggested that the regulation of these genes occurs primarily at the level of transcription. Pearson and Epstein (1982) indicated that knowledge of the processing of nascent transcripts is also essential for understanding of active mRNA production.

The current studies will focus on the molecular mechanism(s) related to protein synthesis of SM alpha-actin which has been used as a myofibrillar protein marker. Alpha-actin represents 22% of the myofibrillar proteins in skeletal muscle (Yates et al., 1983). Skeletal muscle alpha-actin (Garfinkel et al., 1982; Helferich et al. 1990; Zakutetal et al., 1982) and myosin light chain (Garfinkel et al., 1982; Smith et al. 1989) has been used successfully as markers of myofibrillar proteins in skeletal muscle.

Although myosin accounts for 43% of myofibrillar proteins (Yates et al., 1983) there are up to 12 isoforms (Buckingham, et al., 1984, 1985; Humphries et al., 1981) which originate from different genes. Furthermore, the rate of expression of these genes may be altered the rate of expression during the differentiation of myoblasts to myotubes in the embryo. Thus, alpha-actin was selected over myosin as a marker of SM myofibrillar proteins accretion in growing, food-producing animals for several following reasons. First, actin is the second-most abundant protein in muscle representing 22% of myofibrillar proteins. Secondly, there is only one isoform of alpha actin (Barton et al., 1987; Caravatli et al., 1982; Ponte et al., 1983) in postnatal skeletal muscle originating from one gene in the species studied (,unlike the 12 isoforms of the multigene myosin family). Finally, actin is an excellent marker of myogenesis during growth and development and has been demonstrated to be an early indicator of myogenesis (Vandekerckhove et al., 1978, 1979, 1987; Sassoon et al., 1988) in both skeletal and cardiac muscle. Since accumulation of muscle proteins is a highly synchronous process (Affara, et al., 198^{b}; Obinata et al., 1981), following the synthesis and gene expression of a single marker (SM alpha-actin) will permit the determination of metabolic events in the assembly of myofibrils in muscle.

Bergen et al. (1987) have conducted experiments to determine general changes in fractional synthesis rate (FSR)

of total muscle protein, total RNA, and total DNA during development in growing pigs. FSR (defined as the percent of the total muscle protein pool synthesized per unit time) decreases from 20% per day in the neonate to 3% per day in the mature rats. These results are consistent with data obtained in young and old male rats (Waterlow et al., 1978). Fractional synthesis rate (FSR) of total protein is approximately 12% in the neonate (3d of age) and 3% in the mature (180d of age) pig mainly due to an increase in the muscle protein pool size. However, early results from our laboratory indicated that relative mRNA abundance for SM alpha-actin, increases 3- to 4-fold during this same period (skjaerlund et al 1988). In these investigations concentration of RNA and DNA decreased with age, whereas RNA:DNA ratios remained similar regardless of age. Muscle cell proteins (mostly myofibrillar proteins) per unit of DNA increases proportionally as muscle mass increases. Since FSR is calculated based on pool size, it is important to distinguish between FSR (% of pool size/day) and absolute protein synthesis rate (g/d). The myofibrillar proteins (including SM alpha-actin) pool size is much greater in older animals due to increased muscle mass. It is likely that absolute synthesis rates of individual myofibrillar proteins may not follow the general FSR described above. In fact, absolute synthesis rates of myofibrillar proteins including alpha-actin may increase as the animal grows from 1 day to 28 days of age to support the accretion and

turnover of protein in this large protein pool. In view of the large quantities of myofibrillar protein mRNA which must be transcribed in the muscle cell nucleus, Young and Allen (1979) pointed out that these muscle nuclei possess a mechanism whereby RNA polymerases have a preference to interact with the myofibrillar protein genes to increase their transcription. In this study, we focus on regulation at the transcriptional level of muscle protein synthesis to investigate the relative transcription rate of certain genes by using skeletal muscle nuclei to conduct a transcription assay *in vitro*.

MATERIALS AND METHODS

The first portion of the materials and methods section of this thesis will discuss the background for the methods used in this thesis.

I. BACKGROUND FOR METHODS UTILIZED

Isolation of RNA is essential for both Northern blot analysis and transcription assay. During isolation RNA must be protected throughout the procedure from nuclease degradation, strong shearing force, high temperature, excessive alkalinity and acidity, and excessive ionic strength. Enzymic degradation can be minimized by performing all operations between 0^{o} and 4^{o}, and by adding ribonuclease inhibitors (Chirgwin et al., 1979). Ribonuclease activity can be minimized during the isolation process by including a suitable inhibitor such as macaloid, heparin or some anionic detergent. The secondary structure of RNA is dependent on total ionic strength and pH of the extraction solution. When the ionic strength is low many RNA species undergo a certain degree of denaturation, whereas high ionic strength results in aggregation and precipitation of RNA.

Guanidinium, sodium sarcosinate, phenol, chloroform and sodium dodecylsulfate (SDS) all function as dissociating and deproteinizing agents during the dissociation and

deproteinization to release RNA. A dissociating agent usually is an anionic detergent which is used to lyse organelles and release RNA from protein and lipoprotein structure, as well as to inhibit ribonuclease activity. Deproteinizing agents can precipitate proteins that have been released from the protein-nucleic acid complex. RNA is further purified by ethanol precipitation at pH between 5 to 6 (Woo et al., 1975; Poulson, 1977; Wallace, 1987).

Northern blot analysis is a process by which the isolated RNA is separated by size on a denaturing agarose gel and transferred to a solid phase such as a nitrocellulose membrane. The RNA of interest is then hybridized to a radioactive-labeled single strand of cDNA. The basic concept involved in Northern analysis is that the denaturation of these nucleic acids (RNA and cDNA) is followed by annealation between complementary polynucleotide sequence. One of the simplest ways to denature nucleic acids is by heating. The extend of denaturation at any temperature can be readily measured by the change of in absorbance at 260 nm of a solution of nucleic acids. The absorbance of a DNA solution remains constant until an elevated temperature is reached. A substantial rise in absorbance (hyperchromic shift) accompanies the transition from native to denatured state. At the denaturing temperature the absorbance is increased approximately 40% over a narrow range. This rise in temperature coincides with the disruption of the regular base-paired structure,

and the two polynucleotide strands separate from one another. The sharpness of the disruption of the regularly hydrogen-bonded base-paired native structure may be caused by the melting point of a pure organic compound (Marmur and Zubay, 1988). The melting temperature (T_m) of DNA is defined as the temperature at the midpoint of the absorption increase. Also at this temperature, the double helix retains 50% of the original helical structure. The midpoint of denaturation (T_m) of naturally occurring DNAs is precisely correlated with the average composition of the DNA; the higher the mole percent G-C base pairs, the higher the T_m. This is because the G-C base pair contains three hydrogen bonds, whereas the A-T base-pair contains only two bonds. The T_m is directly proportional to G-C mole percent by the following equation (Doty et al., 1962):

$$T_m = 69.3 + 0.41\ (G+C)$$

Nucleic acid hybridization takes place between any two complementary single-stranded polynucleotides when they are annealed at T_m-25^{o}C for a prolonged period of time to form heteroduplexes. This is an annealed duplex structure which does not show perfect complementarity. Since the rate of duplex formation depends on the concentration of the interacting complementary strands, hybridization can be used to measure the abundance of a specific nucleic acid in a mixture (Southern, 1979; Marmur and Zubay, 1988). In Northern blot hybridization, RNA is separated by agarose gel electrophoresis under denaturing conditions, immobilized

onto nitrocellulose membrane and is then hybridized to a particular radioactively labeled single-stranded probe. The membrane is washed under suitable conditions which depend on the degree of homology between the two strands of the heteroduplex. The homology need not be 100% as hybridization may be between a variety of related but not identical sequences. After hybridization, the washing is usually performed 2 to 12°C below the T_m, and the effect of ionic strength (u) on the melting temperature is given by the following equation (Davidson et al., 1962):

$$T_{m2}-T_{m1} = 18.5 \log_{10}(u_2/u_1)$$

Thus, to allow for the fact that the T_m of duplex nucleic acid decreases by 1°C per 1-1.5% mismatch, the stringency of the hybridization can be decreased by lowing the temperature of the wash and/or by increasing the ionic strength of the washing buffer (Adams et al., 1986). These blotting techniques can be used to determine the size and abundance of immobilized RNA.

The transcription assay is generally used to measure the relative transcription rate of specific genes. This assay is performed using isolated nuclei since the nucleus is the major site for the regulation of transcription. Nuclear RNA is synthesized by incubating nuclei in the presence of radiolabeled UTP, since UTP is only incorporated into RNA but not DNA. The nuclear RNA is purified and hybridized to specific gene sequences which have been immobilized on a membrane such as nitrocellulose. In this

in vitro system, RNA synthesis consists of enlongation and completion of previously initiated RNA molecules with a negligible amount of new initiation (Reeder and Roeder, 1972). Therefore, the transcriptional reaction in this *in vitro* system is the completion of polyribonucleotide synthesis. Within the isolated nucleus the chromatin (the nucleoprotein fibers of eucaryotic chromosome) is maintained in its native state. Nuclei are incubated with all four ribonucleoside triphosphates, one of which is radiolabeled in the transcription mixture which contains all the essential components for RNA synthesis. The amount of a particular RNA can be quantitated by RNA-DNA hybridization using appropriate DNA probes. The most convenient method is to immobilize the DNA probes as dots on a nitrocellulose membrane. The membrane containing immobilized DNA is then incubated in a hybridization solution with the radiolabeled RNA transcripts. After washing to remove nonspecific hybridization, the amount of hybridized RNA is determined either by autoradiography followed with densitometer scan or by excising the radiolabeled spots and quantifying by liquid scintillation counting.

There are certain points which need to be clarified, first is that the supercoiled plasmid DNA will not efficiently bind to nitrocellulose, thus plasmid DNA containing the specific cDNA sequence must be linearized using an appropriate restriction enzyme. Second, the DNA probe should be at least 1.0 Kb in size to ensure

quantitative binding to the nitrocellulose membrane. Third, the amount of cDNA on the nitrocellulose membrane must be in excess. It is essential that the amount of RNA hybridized should not be limited and the quantity of DNA must be in molar excess. In general, 5 ug plasmid containing of DNA insert of interest/dot or 1 ug of excised DNA insert/dot is sufficient to assure that the quantity of DNA is not limiting. Finally, the plasmid DNA lacking the cDNA sequence of interest must be used a negative control to monitor for nonspecific binding (Marzluff and Huang, 1984).

The isolation of nuclei consist of disruption of cell structure under relatively isosmotic conditions in the presence of divalent cations to stabilize the nuclei. The major modification we made is the physical strength used to break down muscle structure. After several low speed centrifugation and washing steps, nuclei are recovered by ultracentrifugation through a cushion of sucrose of high density. Nuclei are the most dense of the organelles in the cell and will pellet through the sucrose cushion (Gornall et al., 1972; Surks et al., 1974; Liew et al., 1980, 1983; Oppenheimer et al., 1981, 1987; Marzluff and Huang, 1984; Hanson et al., 1985; Jump et al., 1988). This allows for separation of nuclei from other cellular organelles and debri.

II. MATERIALS AND METHODS

(1) Relative abundance of alpha-actin and beta-tubulin mRNA in LD muscle

Animals Four 1-day-old pigs and four 28-day-old pigs were selected from the Michigan State University swine research center. Animals were immobilized and then slaughtered by exsanguination and approximately 3 grams of longissimus dorsi (LD) muscle were collected from each pig, quickly frozen by submerging in liquid nitrogen, and stored at -80^{o}C until RNA extraction.

RNA extraction Total RNA was extracted from LD muscle by a method modified from the acid guanidinium thiocyanate-phenol-chloroform extraction procedure of Chomczynski et al. (1987). Briefly, 1 g muscle was homogenized using a Polytron homogenizer in 10 ml homogenization buffer consisting of 4 M guandine isothiocyanate (Boehringer Mannheim Biochem., Indianapolis, IN), 1 M sodium citrate (Sigma Chem. Co., St. Louis, MO), 0.1 M mercaptoethanol (Mallinckrodt, Inc., Paris, KY), 10% sodium sacrosyl (Boehringer Mannheim Biochem., Indianapolis, IN). Sequentially, 1 ml 2 M sodium acetate (Sigma Chem. Co., St. Louis, MO), pH 5, 10 ml water saturated phenol and 2 ml of a chloroform (Fisher Scientific Co., Fair Lawn, NJ) : isoamyl alcohol (Sigma Chem. Co., St. Louis, MO) mixture (24:1) were added to the homogenate and mixed after the addition of each reagent. The final suspension was vigorously shaken and

placed on ice for 15 minutes. The aqueous and the organic phase was separated by centrifugation at 10,000 x g for 20 minutes. Under these conditions where the pH in the aqueous phase is between 5 to 6, RNA would be soluble in the aqueous phase, whereas DNA and protein would be present in the interphase or organic phase (Wallace, 1987). The aqueous phase was transferred to a 30 ml Corex tube, mixed with an equal volume isopropanol (Mallinckrodt, Inc., Paris, KY), and stored at -20^{o}C for a minimum of 2 hours to precipitate RNA. Sedimentation of RNA was conducted by centrifugation at 10,000 x g for 20 minutes and the pellet was dissolved in 750 ul of a pH 7 solution which was composed of 7 M guanidine-hydrochloride (Research Organics Co., Cleveland, OH), 0.2 M sodium acetate, 1 mM dithiothreitol (Boehringer Mannheim Biochem., Indianapolis, IN), 1 mM EDTA (disodium EDTA Boehringer Mannheim Biochem., Indianapolis, IN) adjusted to pH 7 with glacial acid, and then transferred to a 1.5 ml microfuge tube. RNA was precipitated with 75 ul 2 M sodium acetate and 450 ul ethanol then placed at -20^{o}C for 1 hour. The mixture was centrifuged at 12,000 g for 3 minutes and pellet was sequentially washed with 300 ul 3.0 M sodium acetate, 10 mM iodoacetamide (Boehringer Mannheim Biochem., Indianapolis, IN), 300 ul 30 mM sodium acetate, 0.1 mM iodoacetamide, 66% ethanol and finally with 300 ul absolute ethanol (-20^{o}C). Each wash consisted of resuspension and sedimentation procedures, discarding the supernatant after each step. The RNA pellet was dried and

resuspended in 100 ul TE (10 mM Tris/1 mM EDTA, pH 8) buffer. The concentration of RNA was determined by absorbance at 260 nm and used for Northern blot analysis (Thomas et al., 1980). Complete details of this procedure are given in appendix A.

Northern blot analysis Messenger RNA (mRNA) abundance of skeletal muscle alpha-actin and beta-tubulin was determined in the LD muscle by Northern blot analysis. Northern blot analyses were conducted as described by Jump et al. (1988) with the following modification. Aliquots of total RNA were denatured and electrophoretically separated in a 1.2% agarose gel containing 2.2 M formaldehyde. RNA was transferred to nitrocellulose paper (Schleicher and Schuell, Keene, NH) with 10X SSC (25X SSC is equivalent to 3.75 M sodium chloride, 0.375 M sodium citrate). Blots were prehybridized for 2 hours at 42^{o}C in 50% formamide (Boehringer Mannheim Biochem., Indianapolis, IN), 5X SSC, 5X Denhardt's solution: 100X Denhardt's solution is equivalent to 2% bovine serum albumin (from fraction V, essentially fatty acid free, Sigma Chem. Co., St. Louis, MO), 0.1% SDS (sodium dodecyl sulfate, Boehringer Mannheim Biochem., Indianapolis, IN), 1 mM EDTA (disodium EDTA, Fisher Scientific Co., Fair Lawn, NJ), 50 mM sodium phosphate buffer (pH 6.5), and 0.5 mg/ml tRNA (Boehringer Mannheim Biochem., Indianapolis, IN). The hybridization solution was identical to the prehybridization solution, except that the hybridization solution contained 1X

Denhardt's and 2 million cpm of appropriate cDNA probe/ml.

The human full length alpha-actin cDNA subcloned into the Pst 1 and Pvu II sites of a modified pBR322 plasmid (Okayama and Berg, 1982) was obtained from Dr. Kedes at University of CA, Los Angeles (Gunning et al., 1983). A mouse beta-tubulin cDNA (m-beta-5 tubulin) subcloned into the EcoRI site of pUC9 was obtained from Dr. Donald Cleveland at John Hopkins University (Sullivan and Cleveland, 1986). The cDNAs were randomly primed (Boehringer Mannheim Biochemicals) using [^{32}P] dCTP (3000 Ci/mmol) at a specific activity of 3.4 X 10^8 cpm/ ug DNA for the alpha-actin probe and 2 X 10^8 cpm/ug DNA for the beta-tubulin probe. After hybridization at 42^{o}C for 16 hours, blots were washed with 2X SSC and 0.1% SDS at room temperature for 10 minutes, then washed three times for 45 minutes in 0.1X SSC and 0.1% SDS at 65^{o}C for the alpha-actin blots and in 0.2X SSC and 0.1% SDS at 55^{o}C for the beta-tubulin blots. After the last wash, the nitrocellulose membrane was dried and exposed to x-ray film (XAR-5; Eastman Kodak, Rochester, NY) at -80^{o}C with two intensifying screens (DuPont, Wilmington, DE). Following autoradiography, hybridization of cDNA to RNA was quantified by densitometry. The blot that contained adult pig RNA was stripped with 0.01X SSC and 0.5% SDS for 2 hours at 75^{o}C to remove the radiolabeled beta-tubulin cDNA, then re-hybridized with the alpha-actin cDNA probe. Blots that contained four 1-day-old and four 28-day-old pig LD muscle RNA samples were stained

with methylene blue as described by Maniatis et al. (1982) to visualize the 18S and 28S ribosomal bands in order to confirm that equal amounts of RNA are loaded onto the gel and that the transfer efficiency was similar among lanes. Details of the Northern blot analysis are presented in appendix B.

Statistical Analysis All data were statistically analyzed using the General Linear Models Procedures of SAS (1987). Contrasts between means were analyzed by Bonferonni t-test as outlined by Gill (1978).

(2) Transcription Assay

Animals Four 1-day-old and four 28-day-old pigs were randomly selected from the Michigan State University swine research center.

Nuclei isolation Thirty grams LD muscle from 1-day and 28-day old pigs was collected immediately following exsanguination and ground through a fine plate (4.5 mm) meat grinder at 4°C. The samples were mixed with buffer A (0.32 M sucrose, density gradient grade, ribonuclease free, (Schwarz/Mann, Organgeburg, NY), 3 mM $MgCl_2$, 1 mM KH_2PO_4), then gently homogenized with a Polytron homogenizer. The homogenate was diluted with 1 volume buffer A and filtered through 1 layer of cheesecloth. Nuclei and cellular debris were pelleted by centrifugation at 700 g for 10 minutes. The pellet was gently homogenized with 0.25% triton X-100 to remove an outer nuclear membrane without further disruption

of the nuclei (Blobel and Potter, 1966) and centrifuged at 700 g. After two washes with buffer A, the pellet was resuspended into buffer B (2.4 M sucrose, 3 mM $MgCl_2$) and ultracentrifuged at 105,500 g for 80 minutes to separate nuclei from other cellular organelles (Figure 3). Nuclei were resuspended in storage buffer [pH 7.5, 40% glycerol (Mallinckrodt, Inc., Paris, KY), 75 mM HEPES (4-[2-Hydroxyethyl]-1-piperazine-ethanesulfonic acid, Boehringer Mannheim Biochem., Indianapolis, IN), 60 mM KCl, 15 mM NaCl, 0.15 mM spermidine (Sigma Chem.Co., St.Louis, MO), 0.5 mM spermine (Sigma Chem.Co., St.Louis, MO), 0.5 mM dithiothreitol (Boehringer Mannheim Biochem., Indianapolis, IN), 0.1 mM EDTA and 0.1 mM EGTA], examined under a phase-contrast microscope, and quantified at 260 nm. Concentration of nuclei were determined by converting 1 absorbance unit at 260 nm equal to 5 X 10^3 nuclei/ul. Overall transcriptional activity was determined using [^{3}H]-UTP incorporation into RNA. Tritiated UTP incorporation assays were conducted immediately after nuclei isolation. Various nuclei concentrations were used to determine viability of nuclei and appropriate conditions for the transcription assay. Details of nuclei isolation are presented in appendix C.

Tritiated UTP Incorporation Assay The [^{3}H]-UTP incorporation assay (Marzluff and Huang, 1984; Liew and McCully, 1988) was conducted at different time points and at various nuclei concentrations to determine appropriate assay

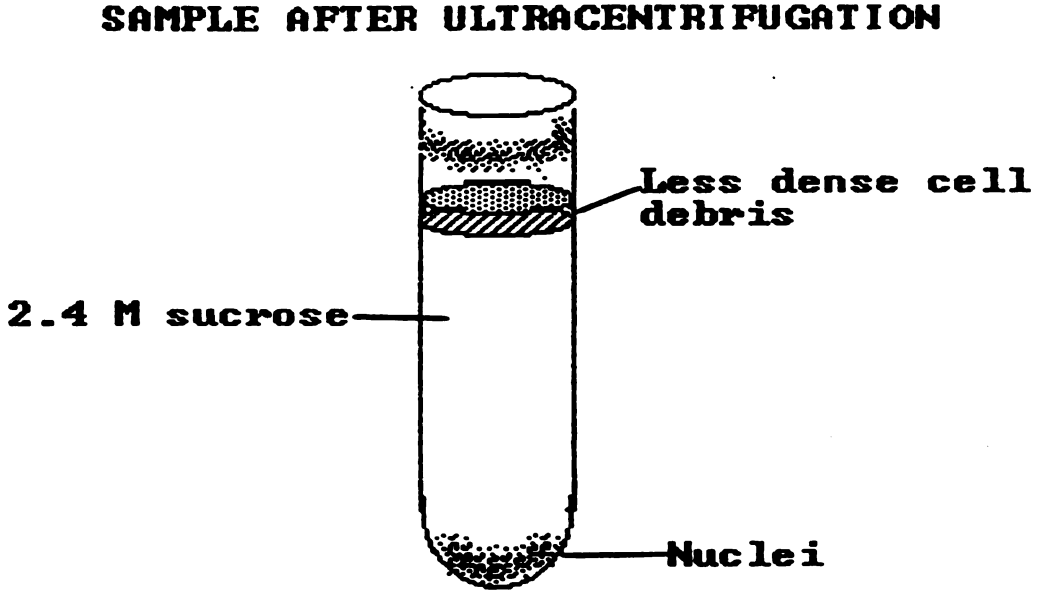


Figure 3. Schematic picture of nuclei separated from other cellular organelles after ultracentrifugation at 105,500 g for 80 minutes with 2.4 M sucrose buffer.

conditions. Three nuclei concentrations, $1X10^7$, $2X10^7$, and $3X10^7$, and 7 time points were evaluated in assays conducted at 26°C. The composition of transcription mixture is described in Table 3. One hundred microliters of nuclei preparation at three different nuclei concentrations listed above were added to 300 ul of the transcription mixture and incubated for 0, 5, 10, 15, 20, 30, and 60 minutes. Following the incubation, triplicate samples aliquots (17 ul) of the reaction mixture (transcription mixture with nuclei) were transferred to 13 X 100 mm test tubes containing 100 ul 1 mg/ml tRNA. The reaction was stopped with 100 ul 20% TCA (trichloroacetic acid, J.T.Baker Inc., Phillipsburg, NJ) which disrupts the nuclear membrane and precipitates the RNA at each sampling time point. Each tube was rinsed three times with cold 10% TCA and the suspension was subsequently filtered through glass microfiber filters using a vacuum manifold (FH224VM, Hoefer Scientific Instruments). After the filtration procedure was complete, filters were placed into scintillation vials containing 10 ml of an aqueous scintillation solvent. Radioactivity was determined using a Beckman, LS-100C liquid scintillation counter. Inhibition of RNA polymerase II was tested by including 0.05 ug/ml alpha-amanitin at $3X10^7$ nuclei concentration in the reaction mixture to conduct the experiment. Details of tritiated UTP incorporation assay are given in appendix D.

Statistical Analysis All data were statistically

Table 3 Composition of transcription mixture for tritium incorporation assay

glycerol	21.1%	HEPES	39.75 mM
NaCl	7.95 mM	spermine	0.265 mM
EDTA	0.053 mM	EGTA	0.053 mM
DTT	0.265 mM	KCl	31.8 mM
spermidine	0.0795 mM	ATP	0.5 mM
CTP	0.5 mM	UTP	0.5 mM
GTP	0.5 mM	^{3}H-UTP	60 uCi[a]
s-adenosyl methionine	0.25 mM	RNase-inhibitor	1 U/ul

[a]For transcription assay 100 uCi [^{32}P]-UTP were utilized instead of [^{3}H]-UTP.

analyzed using the General Linear Models Procedure of SAS (1987). A split-split-plot with repeated measurements design was utilized (Gill, 1987). Significance of age effect (factor A) was tested using variation of pigs within age (P/A as error 1). Significance of nuclei concentration effect (factor B) and age-nuclei concentration interaction (A X B) were tested using variation of interaction between nuclei concentration and pigs within animal (B X P/A as error 2). Significance of sampling time effect (factor C) and age-sampling time interaction (A X C) were tested using variation of interaction between sampling time and pigs within age (C X P/A as error 3). Significance of interaction between nuclei concentration and sampling time (B X C), and significance of interaction among age, nuclei concentration and time point (A X B X C) were tested using variation of interaction among nuclei concentration, time point and pigs within age (B X C X P/A as error 4). Effect of nuclei concentration was analyzed by Bonferonni t-test. Effect of sampling time was analyzed by Stepwise-regression (Gill, 1978).

Transcription in vitro The transcription assay was developed as a modification of that described by Jump et al. (1988) and Marzluff and Huang (1984). The transcription mixture composition is the same as that used in the tritiated UTP incorporation assay (Table 3) except that 100 uCi of [^{32}P]-UTP were used instead of [^{3}H]-UTP. Nuclei number of 3X10^{7} was incubated in a 1.5 ml microfuge tube at 26^{o}C for 20 minutes in a 400 ul volume of transcription mixture. Two microliters DNase I (30 U/ul, final concentration equal to 1 unit/ml) (Boehringer Mannheim Biochem., Indianapolis, IN) were added to stop the reaction by digesting DNA at 26^{o}C for 3 minutes. At the same time, 12 ul 10 mg/ml tRNA were added to increase the amount of RNA for RNA extraction. Subsequently, 4 ul 0.2 M EDTA, 14 ul 10% SDS, and 12 ul 25 mg/ml proteinase K (Boehringer Mannheim Biochem., Indianapolis, IN) were added and the incubation continued for 2 hours at 65^{o}C to disrupt the nuclear membrane and digest the nuclear proteins. RNA isolation began by adding 600 ul phenol: chloroform:isoamyl alcohol (25:24:1) and centrifuging at 12,000 g for 3 minutes. The aqueous phase was collected and mixed with 600 ul chloroform:isoamyl alcohol (24:1) and centrifuged at 12,000 g. The aqueous phase was transferred to a microfuge tube containing 80 ul 5 M ammonia acetate (pH 5) and 1 ml ethanol and allowed to stand at -20^{o}C for at least 1 hour. RNA precipitates were recovered by centrifugation and resuspend into 100 ul TE buffer, pH 8 in a microfuge tube.

Another DNase-1 digestion was conducted at 37^{o}C for 10 minutes and stopped with 3 ul 0.2 M EDTA. The reaction mixture was incubated at 65^{o}C for 5 minutes chilled on ice and the RNA was sequentially precipitated with ammonia acetate/isopropanol for 20 minutes at -20^{o}C for three cycles. Then the pellet was washed with 200 ul 70% ethanol and vacuum dried. Synthesized RNA was quantified and equal amounts of ^{32}P RNA transcripts were hybridized to immobilized plasmid cDNA on nitrocellulose paper at 42^{o}C for 72 hours. The immobilized plasmid cDNAs on nitrocellulose membrane were prehybridized in 50% formamide, 5X SSC, 5X Denhardt's, 0.1% SDS, 1 mM EDTA, 50 mM sodium phosphate buffer (pH 6.5) and 0.5 mg/ml tRNA for 16 hours at 42^{o}C. The hybridization solution was identical to the prehybridization solution, except that the hybridization solution contained 1X Denhardt's solution and [^{32}P] labeled transcripts. Blots were washed at 65^{o}C with 0.1 X SSC and 0.1% SDS for three cycles and dried for autoradiography. Hybridization was quantified by densitometry. In addition, the dots containing cDNA were cut and radioactivity was determined by a scintillation counting. Details of the transcription assay are presented in appendix E.

Results

In order to determine whether skeletal muscle alpha-actin and beta-tubulin mRNA change in relative abundance Northern blots were conducted. Preliminary tests using Northern blot analysis of RNA isolated from 1-day-old (n=1), 28-day-old (n=1) and adult pigs (n=1) indicated that mRNA abundance for SM alpha-actin mRNA increases with age in the pig (Figure 4), and there is larger difference in actin mRNA between 1-day-old and 28-day-old pigs compared to that between 28-day-old and adult pigs (Table 4). On the contrary, beta-tubulin mRNA abundance decreases with age in the pig (Figure 4), and there is larger difference between 28-day-old and adult pigs compared to that between 28-day and 1-day-old pigs (Table 4). In addition, the Northern blots containing only 1-day-old and 28-day-old LD muscle RNA show that there is a developmental increase of SM alpha-actin mRNA abundance and a developmental decrease of beta-tubulin mRNA abundance between these two ages (Figure 5). Densitometer scans of these autoradiograms indicated a 2.2-fold increase ($P<0.03$) in alpha-actin mRNA and no significant change ($P>0.1$) in beta-tubulin mRNA between 1 and 28-day-old pigs (Table 5). Methylene blue staining visualized the 18S and 28S ribosomal bands which indicated that equal amounts of total RNA were loaded and transferred

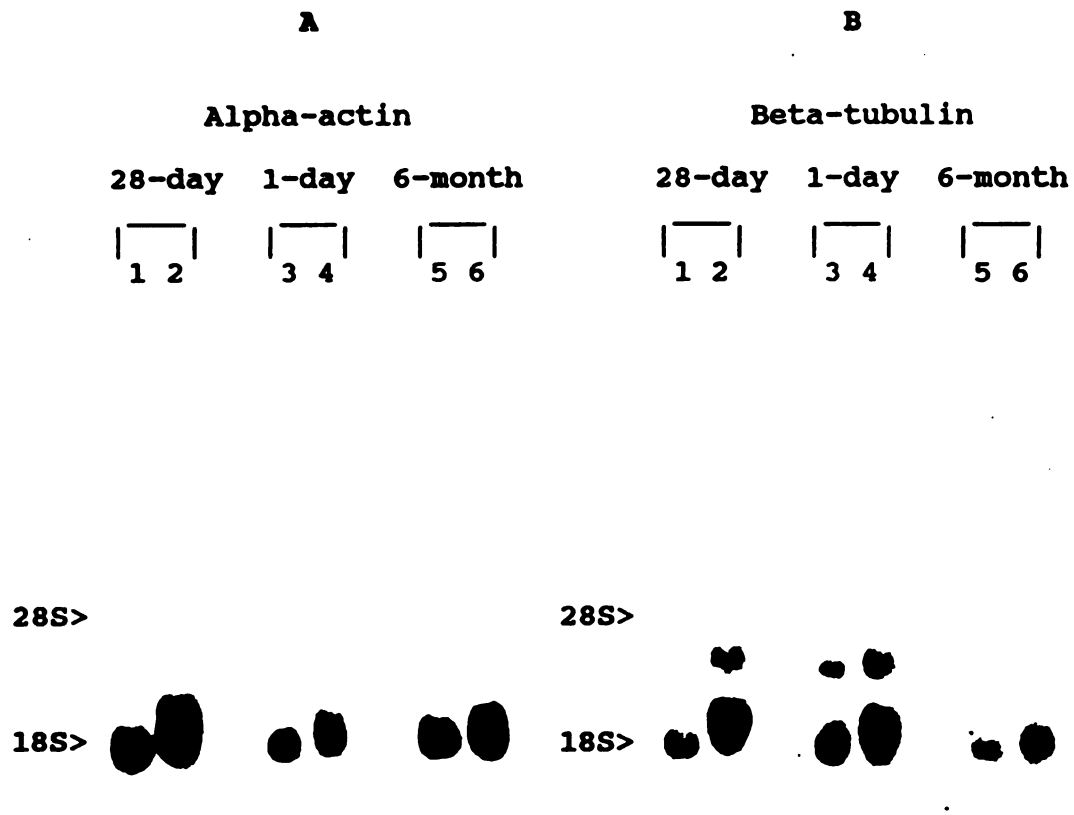


Figure 4. Autoradiogram of alpha-actin (A) and beta-tubulin (B) mRNA abundance in LD muscle from 1-day-old, 28-day-old and adult pigs. RNA was isolated and analyzed via a Northern blot. The mouse Mß5 tubulin cDNA hybridized to mRNA species of 1800 and 2800 nucleotides (B), then the blot was stripped with 0.01X SSC and 0.1% SDS for 2 hours at 70°C and re-hybridized with a human alpha-actin cDNA. A single band of 1650 nucleotide was observed (A). Northern blot of beta-tubulin (B) was washed with 0.2X SSC and 0.1% SDS at 55°C and exposed for 24 hours. Northern blot of alpha-actin was washed with 0.1X SSC and 0.1% SDS at 65°C and exposed for 30 minutes. Lane 1: 10 ug 28-day-old pig LD RNA, lane 2: 20 ug 28-day-old pig LD RNA; lane 3: 10 ug 1-day-old pig LD RNA, lane 4: 20 ug 1-day-old pig LD RNA; lane 5: 10 ug adult pig LD RNA, lane 6: 20 ug adult pig LD RNA.

Table 4. Relative abundance of alpha-actin and beta-tubulin mRNA among 1-day-old, 28-day-old and adult pigs. Data was obtained by densitometry scan of autoradiograms shown in figure 3.[a]

	Total RNA loaded			
Age	10 ug	20 ug	10 ug	20 ug
	alpha-actin		beta-tubulin	
1-day-old	1.589	2.504	4.258	10.785[b]
28-day-old	4.145	10.291	1.437	10.134[b]
Adult	4.454	10.774	0.555	1.699

[a]Abundance of alpha-actin mRNA is expressed as densitometer units. RNA was isolated from three animals, one animal per each age.

[b]These bands were out of the linear portion of the densitometer.

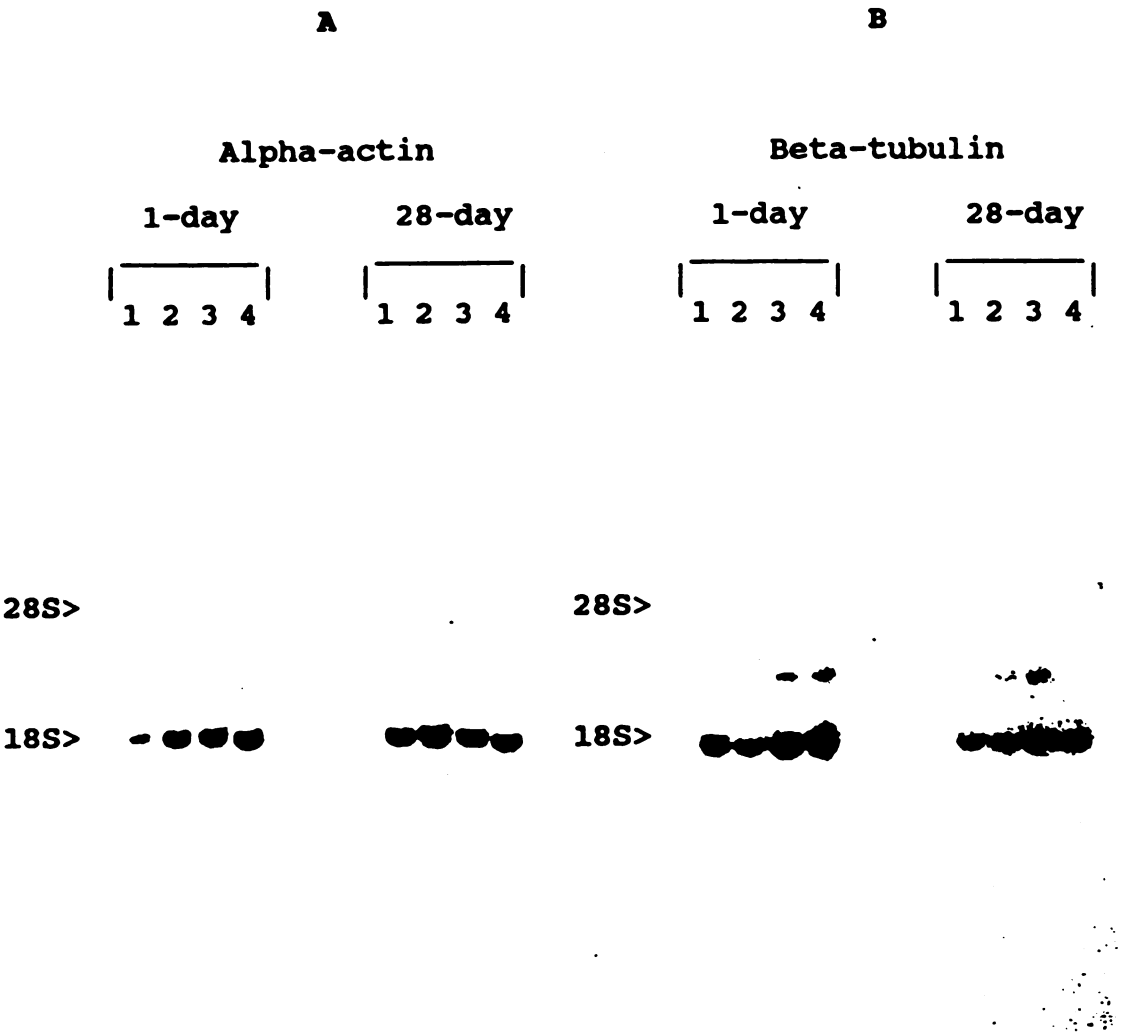


Figure 5. Autoradiograms of alpha-actin and beta-tubulin mRNA abundance in LD muscle from four 28-day-old and four 1-day-old pigs. Each lane contains 10 ug of RNA isolated from LD muscle from each animal. Animals are number 1 to 4 for each age group evaluated. The human alpha-actin cDNA hybridized to mRNA species of 1650 nucleotides (A). The Northern blot of alpha-actin was washed with 0.1X SSC and 0.1% SDS at 65^{o}C then exposed for 30 minutes. The mouse Mß5 tubulin cDNA hybridized to mRNA species of 1800 and 2800 nucleotides (B), Northern blot of beta-tubulin (B) was washed with 0.2X SSC and 0.1% SDS at 55^{o}C then exposed for 24 hours.

Table 5. Relative abundance of alpha-actin mRNA and beta-tubulin mRNA in LD muscle from 1-day-old and 28-day-old pigs.[a] Data were obtained by densitometry scan of autoradiograms shown in figure 4[b]

	mRNA abundance			
Protein	1-day-old	SEM	28-day-old	SEM
Alpha-actin[c]	2.247	0.655	4.493	0.321
Beta-tubulin[d]	3.459	0.649	2.523	0.769

[a]Total number of animals is 8 (n=4 for each age group).
[b]Northern blot of alpha-actin was exposed for 30 minutes. Northern blot of beta-tubulin was exposed for 24 hours. These autoradiograms were scanned by densitometry.
[c]Age effect for change of alpha-actin is significant ($P<0.03$).
[d]Age effect for change of beta-tubulin is not statistically significant ($p>0.1$).

to nitrocellulose membrane from each lane (Figure 6).

Visual inspection at 400X magnification under the microscope indicated that the nuclei containing an intact nuclear membrane were isolated from 1- and 28-day-old pigs (Figure 7). Total transcriptional activity was determined by conducting [^{3}H]-UTP incorporation assays. The results of the microscopic evaluation (Figure 7) and tritiated UTP incorporation data (Table 6) indicated that isolated nuclei were of high quality and able to synthesize RNA (Figure 8). Larger quantities of total RNA appeared to be synthesized by 1-day-old pig SM nuclei than 28-day-old pig SM nuclei (Table 6, Figure 8); however, the difference was not statistically significant ($P>0.25$) between 1-day-old and 28-day-old pig SM nuclei (Table 7). Both the effects of nuclei concentration ($P<0.0001$) and sampling time point ($P<0.0001$) have significant effects on synthesis of total RNA as indicated from the [^{3}H]-UTP incorporation data (Table 7). Therefore, the effect of nuclei concentrations must be tested conditionally upon sampling time points. At 5 minutes, there was a significant difference ($P<0.02$) between nuclei concentration of $1X10^7$ in contrast to $3X10^7$, but there was no difference ($P>0.05$) between either $1X10^7$ in contrast to $2X10^7$ or $2X10^7$ in contrast to $3X10^7$. At 10, 15 and 20 minutes, there were significant differences ($P<0.01$) between nuclei concentration both $1X10^7$ in contrast to $2X10^7$ and $1X10^7$ in contrast to $3X10^7$, but there were no differences ($P>0.05$) between $2X10^7$ in contrast to $3X10^7$. At 30 and 60

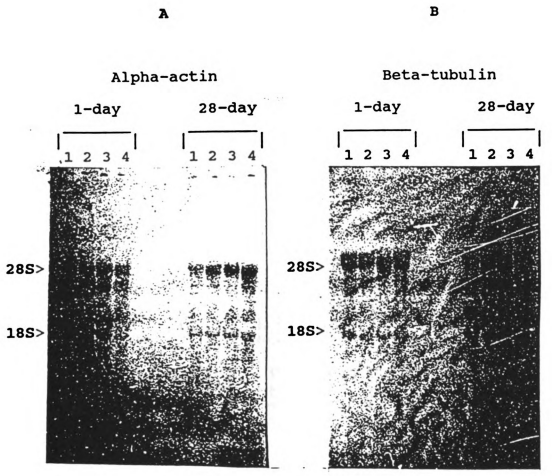


Figure 6. Northern blots of alpha-actin and beta-tubulin stained with methylene blue to visualize the 18S and 28S ribosomal bands.

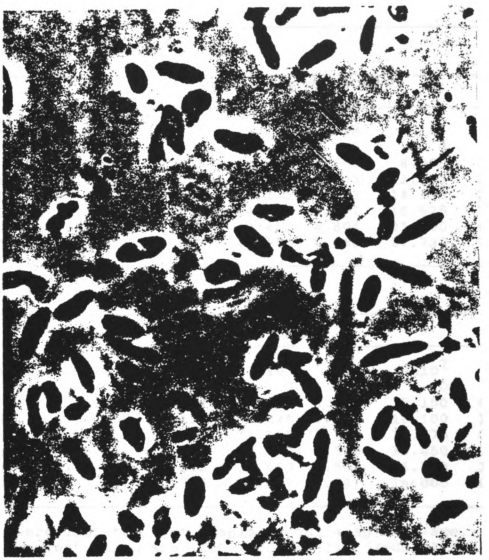


Figure 7. Photograph of Nuclei (400X magnification) using a phase-contrast microscope. Nuclei were isolated by differential centrifugation as described in Materials and Methods.

Table 6. Total RNA synthesized by isolated skeletal muscle nuclei. Tritiated UTP incorporated into RNA (tritium incorporation assay) was conducted as described in Materials and Methods.[a] Data are expressed as CPM X 10^3 for each time point

1-day-old pig SM Nuclei

	Nuclei Concentration[b]					
Time(min)	$1X10^7$	SEM	$2X10^7$	SEM	$3X10^7$	SEM
0	0	0	0	0	0	0
5	3.925	1.539	6.925	2.395	8.000	1.096
10	6.250	2.661	13.075	5.393	11.233	2.598
15	7.425	2.436	14.175	5.322	14.225	2.301
20	8.775	3.612	14.150	3.992	16.925	3.957
30	10.650	4.471	15.450	4.804	21.200	4.887
60	13.733	5.985	19.550	5.462	27.725	7.004

28-day-old pig SM Nuclei

	Nuclei concentration[b]					
Time(min)	$1X10^7$	SEM	$2X10^7$	SEM	$3X10^7$	SEM
0	0	0	0	0	0	0
5	2.475	0.309	5.400	0.187	8.150	0.202
10	4.200	0.430	8.225	0.743	10.100	1.125
15	6.033	0.418	11.150	1.282	10.375	0.557
20	6.433	0.367	12.050	1.596	13.450	0.932
30	6.800	1.278	12.600	1.568	16.750	1.050
60	7.167	0.767	16.867	1.487	18.000	0.569

[a]Nuclei were isolated from 8 animals and 4 animals/age.
[b]Nuclei concentration are $1X10^7$/400 ul, $2X10^7$/400 ul and $3X10^7$/400 ul.

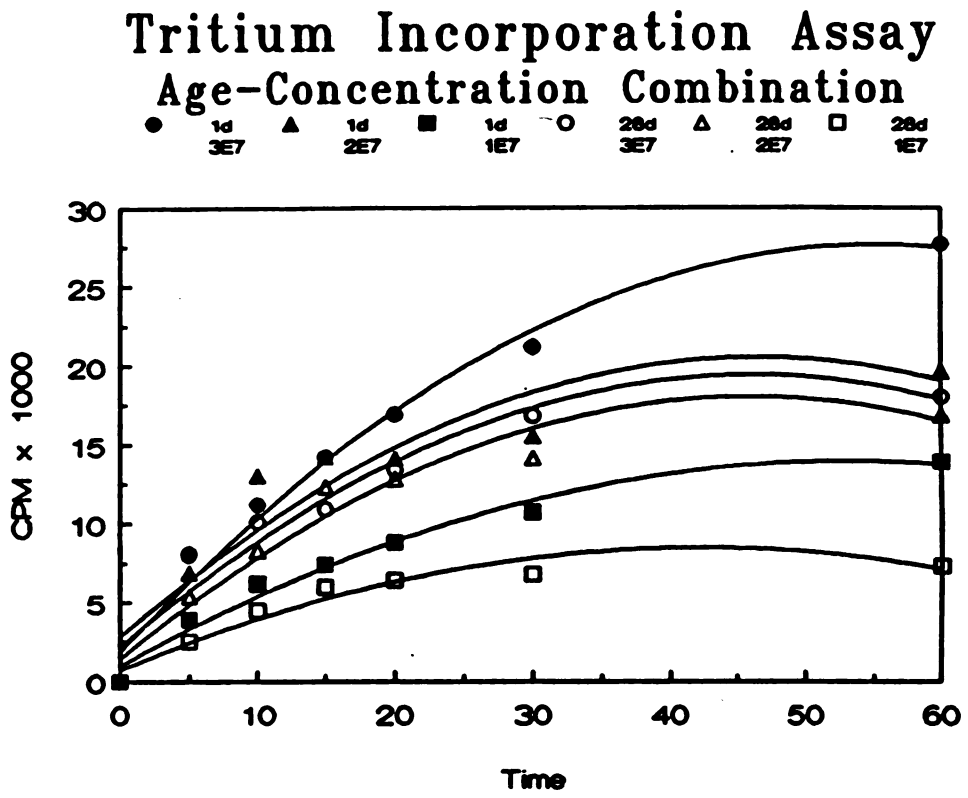


Figure 8. Effects of age and nuclei concentration on transcription <u>in vitro</u> (Tritiated UTP incorporated into RNA). Incorporation assay was conducted as described in Materials and Methods. Skeletal muscle nuclei isolated from 1-day-old and 28-day-old pig LD muscle and 3 different nuclei concentrations (total nuclei number equal to $3X10^7$, $2X10^7$ and $1X10^7$ nuclei per 400 ul assay volume) for each age group were evaluated.

Table 7. ANOVA of tritium incorporation assay[a]. The experimental design is a split-split-plot with repeated measurements design

Source of Variation	DF	Mean Squares	F Value	Prob.
Age of pig (A)[b]	1	290.397	0.73	>0.25
Pigs/age (P, E1)	6	395.175		
Nuclei Concentration (B)[c]	2	630.604	29.25	<0.0001
Age*Conc (AB)[d]	2	4.404	0.20	>0.5
Conc*Pigs/age (PB, E2)	12	21.556		
Sampling Time (C)[e]	6	646.100	28.87	<0.0001
Age*Time (AC)[f]	6	18.197	0.81	>0.5
Time*Pigs/age (PC, E3)	36	22.379		
Conc*Time (BC)[g]	12	37.126	11.50	<0.0001
Age*Conc*Time (ABC)[h]	12	4.783	1.48	>0.1
Conc*Time*Pigs/age (PBC, E4)	62[i]	2.780		

[a]Experiment was conducted using 8 animals, 4 animals/age. Nuclei concentration and sampling time were described as in Material and Method.
[b]Age effect is not statistically significant (P>0.25).
[c]Effect of nuclei concentration is highly significant (P<0.0001).
[d]Effect of age*concentration interaction is not significant (P>0.5).
[e]Effect of sampling time is highly significant (P<0.0001).
[f]Effect of age*sampling time interaction is not significant (P>0.5).
[g]Effect of concentration*time interaction is highly significant (P<0.0001).
[h]Effect of age*concentration*time interaction is not statistically significant (P>0.1).
[i]DF decrease from 72 to 62 because there were 10 missing data of 168 data set.

minutes, there are significant difference ($P<0.02$) among all contrasts (Table 8). These polynomial curves (Figure 9) of nuclei concentration and sampling time effects were calculated by Stepwise-regression. Tritiated UTP incorporation is represented by Y and sampling time is represented by X, the equation for the $1X10^7$ nuclei concentration is $Y=0.748+0.444X-0.005X^2$ and R square equals to 0.976. The equation for the $2X10^7$ nuclei is $Y=2.270+0.691X-0.007X^2$ and R square equals to 0.909. The equation of $3X10^7$ nuclei is $Y=1.873+0.855X-0.008x^2$ and R square equals to 0.973. The maximal value of Y for equation 1 and 2 is 45 minutes and for equation 3 is 40 minutes. The appropriate assay condition for maximum incorporation is 45 minutes incubation time using a nuclei concentration of $7.5x10^4$ nuclei/ul in a 400 ul assay volume ($3x\ 10^7$ nuclei per assay) (Table 8, Figure 9). The isolated nuclei were sensitive to alpha-amanitin, a polymerase II inhibitor. Using a nuclei concentration of $3X10^7$/400 ul, there is a decreased trend of the synthesis of total RNA after addition of alpha-amanitin to a final concentration equal to 0.05 ug/ml (Figure 10). No hybridization was detected using transcription assay when the assay was conducted in the presence of alpha-amanitin (data not shown). These results indicate that RNA polymerase II was functional in these nuclei preparations.

Determination of relative transcriptional rate was evaluated by hybridization of the [^{32}P]-RNA synthesized by

Table 8. Effects of nuclei concentration and sampling time on total RNA synthesized in vitro[a]. Data are expressed as CPM X 10^3 for each sampling time point

Time	Nuclei Concentration[b]		
	$1X10^7$	$2X10^7$	$3X10^7$
0	0	0	0
5	3.20 ± 0.78^{x}	6.16 ± 1.15^{xy}	8.08 ± 0.52^{y}
10	5.23 ± 1.31^{x}	10.65 ± 2.68^{y}	10.59 ± 1.17^{y}
15	6.83 ± 1.34^{x}	12.66 ± 2.60^{y}	12.30 ± 1.32^{y}
20	7.77 ± 1.99^{x}	13.10 ± 2.03^{y}	15.19 ± 1.99^{y}
30	9.00 ± 2.56^{x}	14.03 ± 2.40^{y}	19.72 ± 3.24^{z}
60	10.45 ± 3.07^{x}	18.40 ± 3.02^{y}	23.56 ± 4.23^{z}

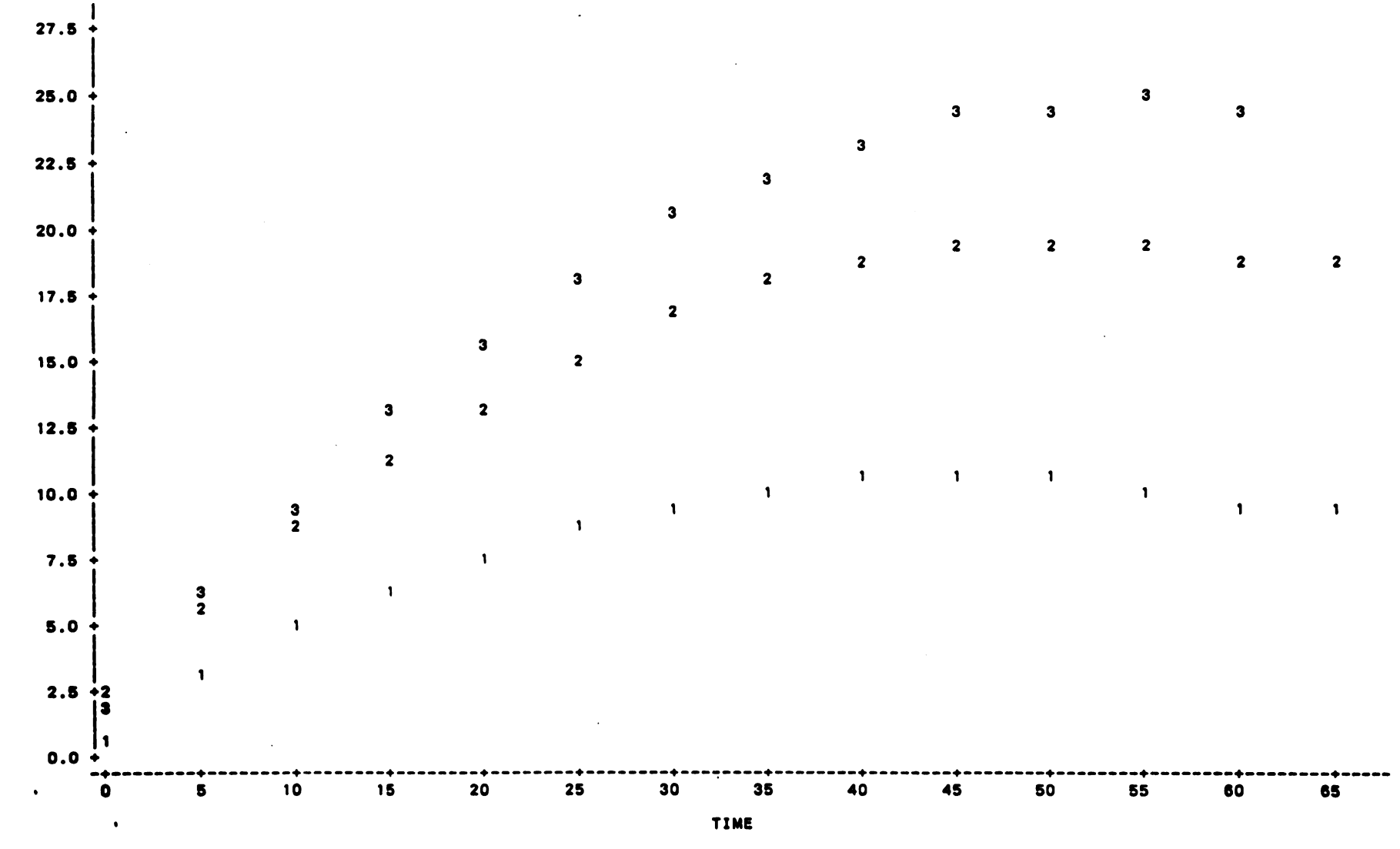
[a]This experimental design is Split-Split-Plot with Repeated Measurements (V_3=5.8, f_3=37.5) and using Bonferroni-t test to compare these means. Within a row means followed by the different letter (x, y, z) indicated that the contrast is different.

[b]Nuclei concentration are $1X10^7$/400 ul, $2X10^7$/400 ul and $3X10^7$/400 ul.

Figure 9. Effects of nuclei concentration and sampling time on total RNA synthesized *in vitro*. Polynomial curves were calculated by Stepwise-regression. Equation of $1X10^7$ nuclei is $Y=0.748+0.444X-0.005X^2$ and R Square=0.976. Equation of $2X10^7$ nuclei is $Y=2.270+0.691X-0.007X^2$ and R Square=0.909. Equation of $3X10^7$ nuclei is $Y=1.873+0.855X-0.008X^2$ and R Square=0.973. Tritium incorporation is represented by Y and sampling time is represented by X. The maximum of Y for equation 1 and 2 is 45 minutes and the maximum of Y for equation 3 is 40 minutes.

PLOT OF TIME(X) VS. RESPONSE(V)

PLOT OF RESP1*TIME1 SYMBOL USED IS 1
PLOT OF RESP2*TIME2 SYMBOL USED IS 2
PLOT OF RESP3*TIME3 SYMBOL USED IS 3



NOTE: 39 OBS HAD MISSING VALUES 37 OBS HIDDEN

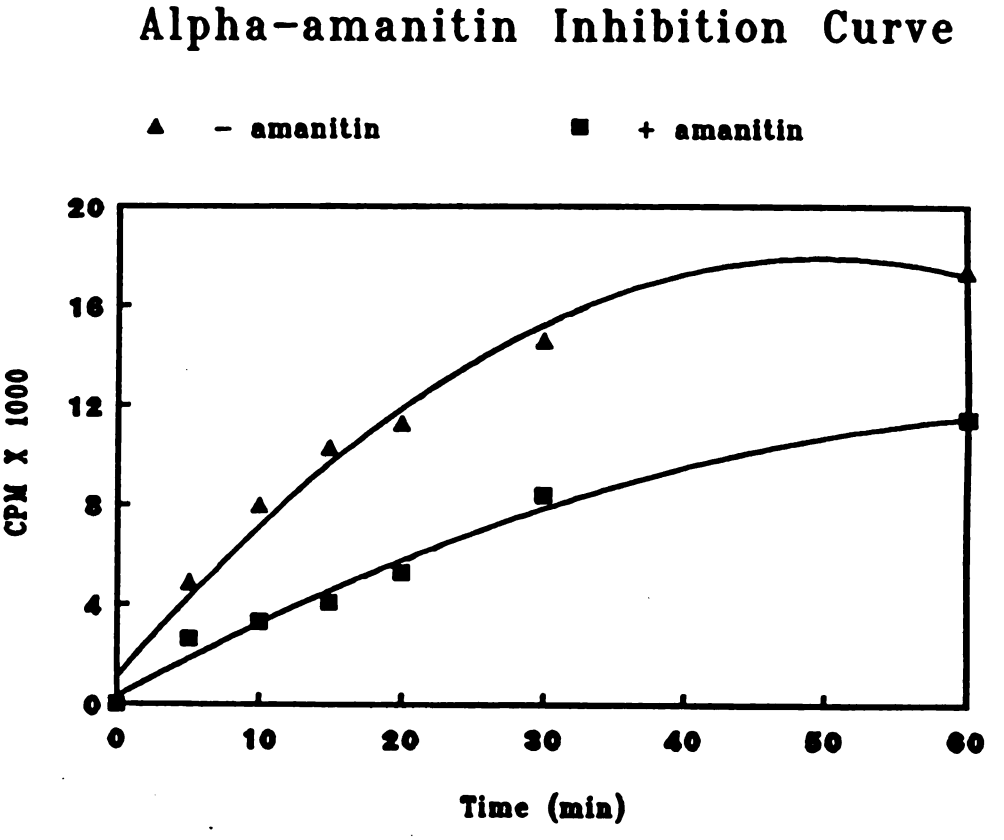


Figure 10. Effect of alpha-amanitin inhibition on transcription in vitro was evaluated as described in Materials and Methods. Experiments were conducted using nuclei isolated from 28-day-old pig LD muscle at nuclei concentration equal to $3X10^7$ in a 400 ul assay volume.

the nuclei to immobilized cDNAs (alpha-actin and beta-tubulin). Transcriptional assay and hybridization results indicate that greater ($P<0.02$) SM alpha-actin hnRNA was synthesized in the 28-day-old pig SM nuclei than in the 1-day-old pig SM nuclei (Figure 11). There was a 1.9-fold increase ($P<0.02$) in the ability of nuclei to synthesize alpha-actin hnRNA from 1 to 28 days. Hybridization was quantified by densitometry of the autoradiogram (Table 9). Beta-tubulin hnRNA synthesis was not detected using the current condition of this assay (Figure 11). Nonspecific hybridization, measured with immobilized plasmid pBR322 DNA onto nitrocellulose paper, was negligible (Figure 11).

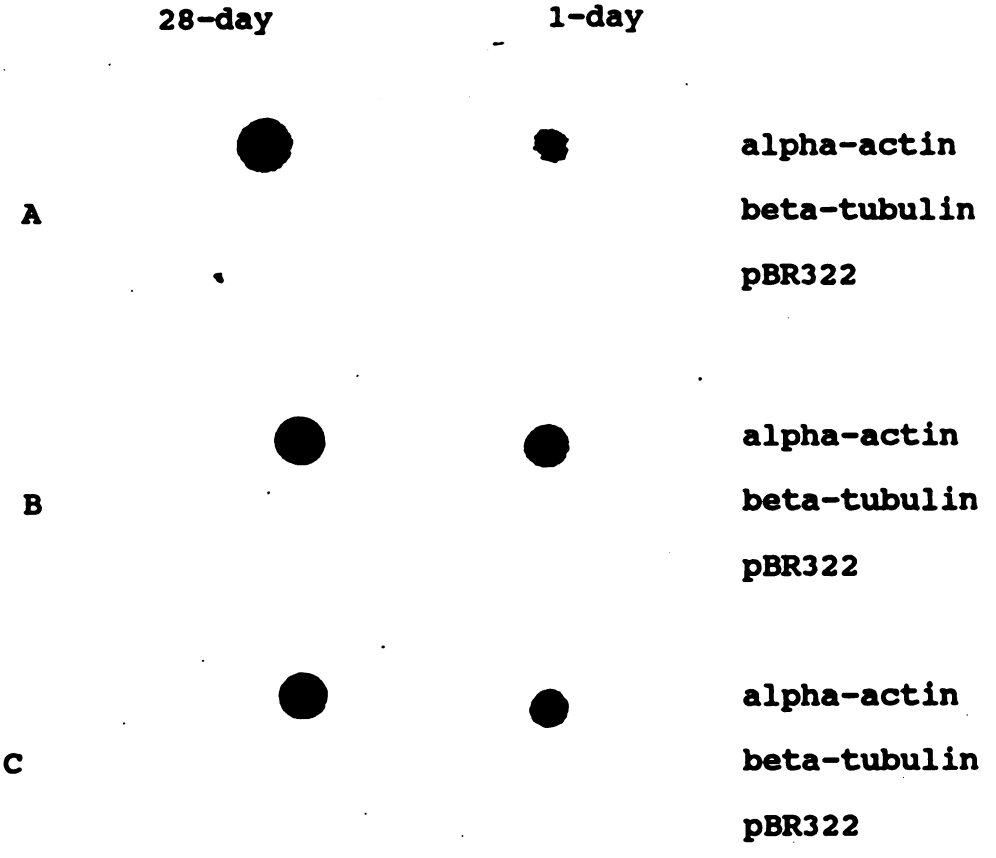


Figure 11. Autoradiograms of alpha-actin hnRNA abundance and beta-tubulin hnRNA abundance synthesized by 1-day-old and 28-day-old pig SM nuclei. RNA was synthesized and isolated via a transcription assay as described in Materials and Methods. Plasmid pBR 322 was included as a negative control. Panel A, B, and C each represent analysis of 1- and 28-day-old pigs.

Table 9. Relative abundance of alpha-actin hnRNA synthesized by 1-day-old and 28-day-old porcine SM nuclei.[a] Data are expressed as densitometry unit from the autoradiograms shown in figure 10

	Age			
	1-day-old	SEM	28-day-old	SEM
Alpha-actin hnRNA[b]	4.979	0.581	9.327	1.002

[a]Beta-tubulin hnRNA and nonspecific hybridization to pBR322 were not detectable either by scintillation counter or densitometry.
[b]Age effect is significant ($P<0.02$).

DISCUSSION

In the present study, we investigated the regulatory mechanism(s) underlying the synthesis of skeletal muscle proteins in pigs. We have demonstrated that there is a developmental increase in skeletal muscle alpha-actin mRNA abundance as the pig ages from one to twenty eight days (Figure 5, Table 5). These results are consistent with preliminary results (Figure 4, Table 4) from our laboratory suggesting that the mechanism to increase muscle protein mass during development is due, at least in part, to change in mRNA abundance for skeletal muscle protein. This increase in mRNA for SM alpha-actin is due either to an increase in transcriptional rate and/or changes in stability of the transcript. Results of experiments from this study demonstrate that the abundance of skeletal muscle alpha-actin mRNA increases ($P<0.03$) and beta-tubulin mRNA abundance is unchanged ($P>0.1$). These data suggest that message abundance of specific proteins varies during growth and development. Gunning et al. (1987) found that individual patterns of transcript accumulation could be grouped into broad categories and each gene investigated had its own unique determinants of transcript accumulation and no two mRNAs were identically expressed. The muscle cell culture studies of Gunning et al.(1987) and Kedes et al.

(1988) indicated that abundance of mRNA parallels the levels of myofibrillar protein synthesis and that genes corresponding to each transcript are regulated on an individual basis. Gunning et al. (1987) suggest that the quantity of mRNA is regulated by transcriptional rate; however stability of mature mRNA or processing of pre-existing pre-mRNA have not been elucidated. Recently, Cox et al. (1990) reported transcriptional regulation of actin genes during differentiation in a mouse muscle cell line (C2/7). Their study indicated that mRNA of skeletal alpha-actin and cardiac alpha-actin was not detectable in myoblasts and these genes were cotranscribed in a developmental manner throughout the time-course of differentiation. Northern blot analysis indicated that skeletal alpha-actin mRNA increased 4.3-fold from 24 hours to 48 hours and continuously increased to 21-fold from 48 hours to 120 hours. The present study was conducted to evaluate the contribution of transcription of the skeletal muscle alpha-actin gene to the relative increase in skeletal muscle alpha-actin mRNA abundance in 1- and 28-day-old pigs.

In order to determine the relative transcriptional rate of the alpha-actin hnRNA synthesis in porcine skeletal muscle, experiments using isolated nuclei from longissimus dorsi muscle and a radioactive nucleotide were performed. We established a protocol for isolation of nuclei from longissimus dorsi muscle of young pigs. The isolated nuclei retained the capacity to perform a number of reactions as

they occur in vivo, and provide a useful system for studying several aspects of RNA metabolism.

In this in vitro system RNA synthesis consists of elongation and completion of previously initiated RNA molecules with a negligible amount new initiation (Reeder and Roeder, 1972). Incorporation of tritiated UTP into initiated RNA indicated these isolated nuclei were capable of transcript elongation. Experiments using alpha-amanitin demonstrated a decreasing trend in total RNA elongation, which indicates that all the nascent RNA (pre-tRNA, pre-rRNA and hnRNA) were elongated during the transcription reaction, because alpha-amanitin at low concentration is a specific inhibitor for RNA polymerase II.

Our results also indicate that an appropriate nuclei concentration is $3X10^7$ nuclei for transcriptional analysis. Similar concentration were obtained by McCully and Liew (1988) using myocardial-cell nuclei for their transcription reaction. We also found maximal incorporation of [^{3}H]-UTP into RNA between 40 to 45 minutes, which is consistent with the observations of Ridgway et al. (1985). However, incubation times from 5 up to 60 minutes have been used with success in nuclei isolated from a variety of tissues (Hanson et al., 1982, 1985; Jump et al., 1988; Manley et al., 1980; Oppenheimer et al., 1981, 1987; Rall et al., 1986; Ridgway et al., 1985, Casero et al., 1989). The linear portion of the tritiated UTP incorporation study is from 5 to 45 minutes. During this interval, the ratio of various gene

transcripts at different time points should be constant. Overall transcriptional activity was estimated by measuring the incorporation of [^{3}H]-UTP into RNA from isolated skeletal muscle nuclei. We found that 1-day-old pig SM nuclei synthesized a larger amount of RNA than did 28-day-old pig SM nuclei (Table 6, Figure 6), but the difference was not statistically significant ($P>0.25$). Mulvaney and Gore, (1988) reported greater incorporation of [^{3}H]-UTP into RNA of fetal bovine skeletal muscle nuclei in response to cortisol treatment than control. In addition, they suggested that [^{3}H]-UTP incorporation into total RNA decreases with age. It is possible that there is an overall decrease in transcriptional activity and a relative increase in mRNA abundance for myofibrillar proteins as indicated by the relative increase of alpha-actin mRNA. Results of the transcription assay using nuclei isolated from skeletal muscle indicated that nuclei from 28-day-old pigs are capable of synthesizing 1.9-fold ($p<0.02$) more alpha-actin hnRNA than nuclei from 1-day-old pigs (Figure 11, Table 9). Transcription of beta-tubulin was not detected under the condition used in this study. The reason for the inability to detect beta-tubulin hnRNA may be due to technical difficulties in detecting high abundance (alpha-actin) messages and low abundance (beta-tubulin) messages from one incubation on one blot.

CONCLUSION

These results indicated that pig skeletal muscle alpha-actin mRNA abundance is greater in 28-day-old than 1-day-old pigs and there are different developmental changes between alpha-actin and beta-tubulin mRNA abundance between these two age groups. Also, nuclei can be isolated from skeletal muscle of young pigs (1-day-old and 28-day-old pigs). Further modification should enable us to isolate viable skeletal muscle nuclei from older animals; however, our preliminary studies were unsuccessful (data not shown). Furthermore, total RNA synthesized by the SM nuclei from 1-day and 28-day-old pigs were not significantly different ($p>0.25$). The relative transcriptional rate of the SM alpha-actin gene increased 1.9-fold in the isolated nuclei from 1-day to 28-days of age. These results indicate that the relative increase in mRNA abundance for SM alpha-actin from 1-day to 28-day-old pigs is due, at least in part, to an increase in transcriptional activity of the SM alpha-actin gene.

APPENDICES

APPENDIX A

ACID GUANIDINE PHENOL CHLOROFORM RNA EXTRACTION METHOD

(modofied from the protocol of Chomczynski and Sacchi, 1987)

REAGENTS:

1. 4 M Guanidine thiocyanate:

guanidium thiocyanate (Kodak)	94.60 g.
sodium citrate	0.74 g.
B-mercaptoethanol (14 M)	1.42 ml
10% Na sarcosyl	10.0 ml

Total volume of 200 ml: use sterile H_2O.
Adjust pH to 7.0 with 1 M HCl or 1 N NaOH.
Filter through 0.45 um millipore.
Store in sterile brown bottle at room temperature.

2. Buffer II: 7 M Guanidine Hydrochloride

guanidine-HCl (Sigma, practical grade)	668.6 g.
Na-acetate (trihydrate)	2.7 g.
dithiothreitol	150.0 mg.
iodoacetamide	1.84 mg.
0.2 M EDTA (pH 8.0)	5.0 ml.

Add GD-HOH to 900 ml.
Dissolve all reagents by heating in a 50°C water bath.
When all reagents are dissolved, adjust to pH 7.0 with glacial acetic acid or 5 N NaOH.
Adjust final volume to 1 l.
Filter through 0.45 um millipore.
Store in sterile bottle in the dark at room temperature.

3. Buffer III: 2 M Na-acetate, pH 5.0

Dissolve 272 g. Na-acetate (trihydrate) in 600 ml sterile GD-HOH.
Adjust to pH 5.0 with glacial acetic acid.
Adjust final volume to 1.0 l.
Filter through 0.45 um millipore.
Store at 4°C. in sterile bottle.

4. Buffer VI: 3 M Na-acetate, pH 5.0

Na-acetate (trihydrate)	409.8 g.
iodoacetamide	1.84 g.

Dissolve reagents in 300 ml glacial acetic acid and 300 ml sterile H_2O. Adjust to pH 5.0 with glacial acetic acid. Adjust final volume to 1.0 l. Filter through 0.45 um millipore filter and store in sterile bottle at 4°C.

5. Buffer VII: ETOH-Na-acetate, pH 5.0.

Absolute Ethanol	660 ml.
3 M Na-acetate, 10 nM iodoacetamide, (pH 5.0)	11 ml.

Filtration of this solution is not necessary, store in sterile bottle at -20°C.

6. TE-8.0:

1.0 M Tris-HCl, pH 8.0 (20 C)	10.0 ml.
0.2 M EDTA, pH 8.0	5.0 ml.

Adjust final volume to 1.0 l. with sterile H_2O.
Store at room temperature.

7. 10% Na-sarcosyl:

Dissolve 10 g. Na-sarcosyl (ICN) in 100 ml HOH, filter through 0.45 millipore and store at room temperature.
Note: sarcosyl is a carcinogen, so take the appropriate precautions.

THE EXTRACTION PROTOCOL:

1. Use sterile glassware and plasticware throughout the procedure.

2. Add 1 g frozen pig LD muscle to a sterile 30 ml Corex tube containing 10 ml 4 M guanidine thiocyanate homogenization buffer.
Homogenize using a Polytron at a setting of 6 for 20 seconds or until the tissue is completely homogenized.

3. Add 1 ml Buffer III, stopper, and vortex.
Add 10 ml H_2O-saturated phenol, vortex.
Add 2 ml chloroform:isoamyl (24:1).
Mix vigorously, put on ice for 15 minutes.

4. Centrifuge at 10,000 x g for 20 minutes at 4°C.
Remove the aqueous phase with a sterile Pastier pipet and put it into a sterile 30 ml Corex tube.

5. To the organic phase, add 2 ml 4 M guanidine thiocyanate and 0.2 ml Buffer III and vortex.
Add 2 ml H_2O-saturated phenol and 0.4 ml chloroform: isoamyl.
Vortex and put on ice for 15 minutes.

6. Centrifuge at 10,000 x g for 20 minutes at 4°C.
Add this aqueous phase to the previously collected aqueous phase.

7. Extract the aqueous composite with 5 ml H_2O-saturated phenol and 5 ml chloroform: isoamyl.

8. Centrifuge at 10,000 x g for 20 minutes at 4°C. Remove the aqueous layer and put it into a sterile 30 ml Corex tube.

9. Add 1 volume of isopropanol to the aqueous layer, cover the tube with Parafilm and mix. Store at -20°C for at least 2 hours to allow precipitation of RNA.

10. Centrifuge at 10,000 x g for 20 minutes at 4°C.

11. Transfer pellet to a 1.5 ml microfuge tube with 750 ul Buffer II. Add 75 ul Buffer III and 450 ul absolute ethanol and allow RNA to precipitate at -20°C for 1 hour.

12. Microfuge at 12,000 x g for 5 minutes. Decant and drain.

13. Resuspend the pellet in 300 ul of Buffer VI. Sediment and drain as described above.

14. Resuspend the RNA pellet in 300 ul of Buffer VII and sediment and drain as described above.

15. Resuspend the RNA pellet in 300 ul of absolute ethanol and sediment and drain as described above.

16. Resuspend the RNA pellet in 100 ul of TE-8 buffer.

17. Dilute 5 ul of RNA solution to 1 ml with TE-8 and scan in a spectrophotometer from 320 to 220 nm. Record the optical density at 260 and 280 nm. The ratio of 260/280 should be greater than 1.8.
RNA concentration of solution in step 16 (ug/ul) is equal to the optical density at 260 nm multiplied by 200 (dilution factor) and divided by 25 (RNA extinction coefficient).

18. RNA samples should be labeled and stored at -80°C.

APPENDIX B

NORTHERN BLOT ANALYSIS
(modification of Jump et al. 1988)

Reagents

1. GEL RECIPE:

10X-MAE, pH 7.0	25 ml
LE-Agarose	3.0 g equals 1.2% agarose
MG-H_2O	181 ml
deionized formaldehyde	44 ml

(10X-MAE, pH 7.0: 0.4 M MOPS, pH 7.0; 100 mM Na-acetate; 10 mM EDTA, pH 8.0)

NOTE: FORMALDEHYDE IS TOXIC, IN SOME INSTANCES CARCINOGENIC. TAKE THE APPROPRIATE PRECAUTIONS WHEN WORKING WITH FORMALDEHYDE. WEAR GLOVES, WORK IN THE FUME HOOD.

Dissolve agarose in 10X-MAE and water by heating on high in the microwave for three minutes. Cool melted agarose to 60°C. Transfer melted agarose to fume hood, add formaldehyde slowly while mixing, then pour gel. The gel should be in the hood.

2. ELECTRODE BUFFER:

10X-MAE, pH 7.0	100 ml
MQ-HOH	720 ml
deionized formaldehyde	180 ml

Mix just before use, keep in the fume hood.

3. SAMPLE PREPARATION:

Samples should contain total RNA in a total volume of 5.5 ul. Use TE-8 as a diluent.
Denaturing Mix (for 20 samples)

10X-MAE, pH 7.0	20 ul
deionized formaldehyde	70 ul
deionized formamide	200 ul

Add 14.5 ul of denaturing mix to each RNA sample, cap microfuge tube and heat denature for 5 minutes at 60°C.

Add 5 ul of 4X-dye mix and load all of the sample on the gel. (4X-RNA dye: 50 % glycerol; 0.4 % bromophenol blue; 6.4 % zylene cyanol; 1 mM EDTA)

Gel electrophorosis

Load samples onto the gel. Cover the gel with Saran wrap to prevent loss of formaldehyde.
Electrophoretically separate RNA for 16 hours at 40 volts or 65 volts for 4 hours. The entire unit should be in the fume hood.

TRANSFER TO NITROCELLULOSE:

Transfer the gel to MQ-H_2O and rinse briefly. Transfer the gel to 500 ml 50 mM NaOH and soak for 30 minutes.

Cut the marker lanes off. Stain the marker lanes with TBE buffer and EtBr (0.5 ug/ml) for two hours, then destain with MQ-H_2O.

After soaking gel in NaOH, transfer gel to 500 ml 0.1 M Tris, pH 7.5, and soak for 30 minutes.

Soak nitrocellulose paper cut to the size of the gel in water for ten minutes, then soak nitricellulose paper, wicks, and 3 small pieces 3 MM paper (cut to the size of the gel) in 10X SSC for 15 minutes.

Put upper surface of gel next to the wicks and use a pipet to remove any bubbles. Put nitrocellulose paper on top of the gel and remove any bubbles with a pipet. Put 3 pieces 3 MM paper on top of the nitrocellulose paper. Seal the edges of the gel with Saran wrap and put a stack of paper towels on top. Place a 500 g weight (a book works well) on the stack of towels. Transfer overnight.

Remove the paper towel and 3 MM paper. Turn over gel with nitrocellulose and use a pencil to mark each well.

Soak nitrocellulose in 2X SSC briefly, dry under a heat lamp, and dry in an 80°C vacuum oven for two hours.

Prehybridization and Hybridization

Prehybridize for at least two hours at 42°C.

Prehybridization solution:

formamide	10.0 ml
25X SSC	4.0 ml
100X Denhardt's	1.0 ml
10% SDS	0.2 ml
0.2 M EDTA	0.1 ml
1 M $NaPO_4$	1.0 ml
MQ-H_2O	2.7 ml
	19.0 ml
tRNA (boil)	1.0 ml
	20.0 ml

Random prime cDNA insert during the prehybridization.

Hybridize overnight at 42°C.

Hybridization solution:

formamide	10.0 ml
25X SSC	4.0 ml
100X Denhardt's	0.2 ml
10% SDS	0.2 ml
0.2 M EDTA	1.0 ml
MQ-H_2O	3.5 ml
	19.0 ml
*tRNA (boil)	1.0 ml
	20.0 ml

*The tRNA should contain enough probe so that the hybridization solution contains probe at 2 million cpm/ml.

Wash blot with 500 ml of 2X SSC 1% SDS at room temperature for 10 minutes. Transfer blot to 0.1 or 0.2X SSC with 0.1% SDS at 55°C or 65°C. Wash for 45 minutes and repeat twice.

APPENDIX C

SKELETAL MUSCLE NUCLEI ISOLATION PROTOCOL

(modification of cardiac muscle nuclei isolation method from Oppenheimer et al., 1974)

REAGENTS

1. Buffer A

0.32 M Sucrose	109.44 g.
3 mM $MgCl_2$	6.609 g.
1 mM KH_2PO_4	0.136 g.

Dissolve KH_2PO_4 in 20 ml MQ-H_2O, and add 2 ml of this KH_2PO_4 solution to 900 ml MQ-HOH containing the other chemicals. Adjust pH to 7.5 using HCl, then bring up to 1 l. with steriled H_2O. Filter through 0.45 um millipore filter, then autoclave this solution.

2. Buffer B

2.4 M Sucrose	820.8 g.
3 mM $MgCl_2$	0.609 g.

Dissolve sucrose and $MgCl_2$ in MQ-H_2O to make one liter. Filter through ordinary filter paper (No. 1).

3. Storage Buffer

40% glycerol	400 ml.
75 mM HEPES	17.9 g.
60 mM KCl	4.5 g.
15 mM NaCl	0.88 g.
0.15 mM Spermidine	1.2 ml.
0.5 mM Spermine	2.0 ml.
0.5 mM dithiothriotol	5.0 ml.
0.1 mM EDTA	0.5 ml.
0.1 mM EGTA	0.5 ml.

Melt 0.15 mM spermidine and 0.5 mM spermine in a 40^{o}C water bath. Add chemicals to MQ-H_2O to make 500 ml. Filter through 0.45 um millipore filter, and add glycerol. Adjust pH to 7.5 with HCl and add sterile H_2O to 1 liter.

NUCLEUS ISOLATION PROTOCOL

1. Collect 20 g longissimus dorsi muscle in saline solution after exsanguiation.

2. Grind with a meat grinder (some of the pieces cannot go through the knives; cut these with scissors in Buffer A).

3. Separate the ground muscle into 10 orange-topped tubes which each contain 10 ml Buffer A (2 g tissue/10 ml Buffer A).

4. Homogenize with the Polytron tissue homogenizer at a very low speed for 20-30 seconds.

5. Dilute the homogenized sample with 1 volume of Buffer A.

6. Filter through 1 layer cheesecloth into a polypropylene bottle.

7. Spin at 700 x g for 10 minutes (in the G.S.A. rotor, 2.2 scale).

8. Decant supernatant, add 100 ml 0.25% X-100, resuspend pellet by gentle homogenization.

9. Spin at 700 x g for 10 minutes.

10. Decant supernatant, add 100 ml Buffer A. Resuspend pellet by gentle homogenization.

11. Spin at 700 x g for 10 minutes.
Repeat the Buffer A washing step one more time.

12. Decant the supernatant (removing as much of the supernatant as possible).
Add approximately 170 ml Buffer B, resuspend pellet by gentle homogenization.

13. Spin at 105,500 x g for 80 minutes in ultracentrifuge at $4^{o}C$.

14. Recover the pellet with storage buffer (using 0.5 ml storage buffer at one time).

15. Collect the nuclei in storage buffer, put into an Eppendorf tube.

16. Spin at 7,000 x g for 3 minutes (in Beckman Microfuge, at speed 10).

17. Resuspend nuclei into 1-2 ml storage buffer.

18. Check with a microscope (using 1 ul nuclei in 10 ul Rice stain).

19. Take 10 ul storage buffer contained nuclei mix with 900 ul TE-8 and 100 ul 10% SDS.

20. Scan with spectrophotometry from 320 nm to 220 nm, record the absorbance at 260 nm. DNA concentration of 1 ug/ul equal to $1X10^{5}$ nuclei/ul.

21. Aliquate nuclei to $3X10^{7}$, $2X10^{7}$ and $1X10^{7}$ nuclei per tube, take one tube from each concentration to conduct

tritium incorporation assay. The other nuclei aliquates store at -80^{o}C until transcription assay.

APPENDIX D

TRITIUM INCORPORATION ASSAY PROTOCOL

REAGENTS:

1. Solution A
   - 10 ul nucleotide mixture (50 mM ATP, 25 mM GTP, 25 mM CTP, 25 uM UTP)
   - 10 ul S-adenosyl-L-methionine (1 mM SAM in 1 mM H_2SO_4)
   - 80 ul sterile H_2O
   - 60 ul [^{3}H] UTP ===> Vacuum dry first

2. Soluiton B

0.6 M KCl	8.95 g.
12.5 mM Mg Acetate	0.536 g.

   Dissolve into 200 ml sterilized water, adjust pH 7.5 using KOH or acetic acid, filter through 0.45 um millipore filter paper then autoclave.

3. Transcription Mixture

Storage Buffer	112.7 ul
RNase Inhibitor (55 units/ul)	7.3 ul
Solution B	80.0 ul
Solution A	100.0 ul

Tritium Incorporation Assay

1. Warm the transcription mixture at 26^{o}C for 1 minute.

2. Add 100 ul nuclei aliquate ($3X10^7$, $2X10^7$, and $1X10^7$) to the transcription mixture. IMMEDIATELY pipette out three equal amounts (17 ul) of the reaction mixture (as 0 minute) and add to glass tubes which contained 100 ul 1 mg/ml tRNA.

3. Add 100 ul 20% TCA to each glass tube .

4. Take out sample at 5, 10, 15, 20, 30, and 60 minutes as described in step 2 and 3.

5. Add about 2 ml 10% TCA to each glass tube and vortex. Filter this solution through glass microfiber filter.

6. Transfer these filters to snitillation vials. Add 10 ml safety solvent and count with scnillation counter.

APPENDIX E

TRANSCRIPTION ASSAY IN VITRO

REAGENTS

1. 0.4 M NaOH/1 mM EDTA
   400 ul of 5 M NaOH and 25 ul of 0.2 M EDTA, bring volume to 5 ml with 4575 ul of GD-H_2O.

2. Prehybridization Solution

formadine	2.5 ml.
25x SSC	1.0 ml.
100x Denhartt's	0.25 ml.
10 % SDS	0.05 ml.
1.0 M $NaPO_4$	0.25 ml.
0.2 M EDTA	0.025 ml.
H_2O	0.675 ml.
Total	4.75 ml.

3. Hybridization Solution

formadine	2.5 ml.
25x SSC	1.0 ml.
100x Denhartt's	0.05 ml.
10% SDS	0.05 ml.
1.0 M $NaPO_4$	0.25 ml.
0.2 M EDTA	0.025 ml.
H_2O	0.875 ml.
total	4.75 ml.

4. Solution A
   10 ul nucleotide mixture
   (50 mM ATP, 25 mM GTP, 25 mM CTP, 25 uM UTP)
   10 ul S-adenosyl-L-methionine
   (1 mM SAM in 1 mM H_2SO_4)
   70 ul sterile H_2O
   10 ul [^{32}P] UTP

5. Soluiton B

0.6 M	KCl	8.95 g.
12.5 mM	Mg Acetate	0.536 g.

   Dissolve into 200 ml sterilized water, adjust pH 7.5 using KOH or acetic acid, filter through 0.45 um millipore filter paper then autoclave.

3. Transcription Mixture

Storage Buffer	112.7 ul
RNase Inhibitor (55 units/ul)	7.3 ul
Solution B	80.0 ul
Solution A	100.0 ul

PROCEDURE

IMMOBOLIZE cDNA

1. linearize plasmid cDNA:
   4 ul a-actin plasmid cDNA (2.5 ug/ul)
   2 ul psT-1
   1 ul Buffer H
   3 ul sterile H_2O

   2.7 ul b-tubulin plasmid cDNA (3.75 ug/ul)
   2 ul EcorI
   1 ul Buffer H
   4.3 ul sterile H_2O

   10 ul plasmid pBR322 (1 ug/ul)
   5 ul psT-1
   7 ul Buffer H
   3 ul sterile H_2O
   Incubate at 37°C water bath for 3 hours.

2. Spin the Eppendrof tube for 3 minutes, add 5 ul 0.2 M EDTA, pH 8,

3. Add 10 ul of TE-8 and bring volume up to 25 ul (Not for pBR322 digestion).

4. Add 1.25 ml 0.4 M NaOH/1 mM EDTA and heat at 65°C for 10 minutes.

5. Cool on ice and add 312.5 ul 5 M NH_4OAc.

6. Soak filter paper and nitrocellulose paper in H_2O for 10 minutes, then transfer to 6X SSC for 10 minutes.

7. Assemble the minifold filter and rinse each well with 300 ul 6X SSC several times.

8. Filter the denatured plasmid DNA through nitrocellulose paper.

9. Heat under a heat lamp, then bake at 80°C in a vacuum oven for 2 hours.

10. Boil 0.25 ml of 1 mg/ml tRNA for 10 minutes. Prehybridize at 42°C for 17 hours.

Transcription assay

1. Warm up transcription mixture for 3 minutes at 26°C, then mix with 100 ul nuclei ($3X10^7$).

2. Incubate at 26°C for 20 minutes. (Recommond for 42 minutes).

3. Add 12 ul 10 mg/ml tRNA and 2 ul DNase I (30 U/ul, final conc.=1 unit/ml).

4. Incubate at 26°C for 3 minutes.

5. Add 4 ul 0.2 M EDTA, 14 ul 10% SDS, and 12 ul 25 mg/ml proteinase K.

6. Incubate at 65°C for 2 hours.

7. Add 600 ul phenol:chloroform:isoamyl alcohol (25:24:1) to the Eppendorf tube.

8. Vortex mix and spin for 3 minutes.

9. Remove aqueous layer to a new Eppendorf tube. Add 600 ul chloroform:isoamyl alcohol (24:1).

10. Vortex and spin for 3 minutes.

11. Remove the aqueous layer to a new Eppendorf tube. Add 80 ul 5 M NH_4OAc and 1000 ul absolute ethanol. Percipitate at -20°C for at least 1 hour.

12. Spin for 3 minutes. Resuspend pellet in 100 ul TE-8.

13. Add 100 ul 2x T/S-buffer, 2 ul RNase Inhibitor, 1 ul DNase I, 10 ul tRNA. Digest at 37°C for 10 minutes.

14. Stop DNase I digestion with 3 ul 0.2 M EDTA. Add 20 ul tRNA.

15. Heat at 65°C for 5 minutes, then cool on ice.

16. Add 105 ul 5 M NH_4OAc and 700 ul isopropanol. Percipitate at -20°C for 20 minutes.

17. Spin for 3 minutes and resuspend pellet in 100 ul TE-8.

18. Add 40 ul 5 M NH_4OAc and 500 ul isopropanol. Precipitate at -20°C for 20 minutes.

19. Spin for 3 minutes and wash pellet with 200 ul 70% ethanol.

20. Drain and dry the pellet. Resuspend in 50 ul TE-8.

21. Heat at 65°C for 5 minutes.

22. Take 1 ul transcripts, quantitated.

23. RNA-DNA hybridization at 42°C for 72 hours.

24. Wash dot blot with 0.1 SSC and 0.01 % SDS at 65°C for 45 minutes, three times.

25. Dry dot blot under heat lamp briefly.

26. Expose to X-ray film at -80°C for 24 hours or shorter time.

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LITERATURE CITED

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