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# ISOLATION OF NUCLEI FROM RED AND WHITE SKELETAL MUSCLES OF THE ADULT RAT

I. R. HELD, R. T. RODRIGO, H. C. YEOH and H. TONAKI

*Neurosciences Research Laboratory, Veterans Administration Hospital, Hines, IL 60141 and Loyola University Stritch School of Medicine, Maywood, IL 60153, USA*

SUMMARY

The distinct procedures required for the isolation of nuclei from red and white skeletal muscles of the adult rat in reproducible yields of 15 and 20%, respectively, are described. The purity and preservation of the isolated nuclei were evaluated by phase contrast and electron microscopy, by analyses of RNA and protein content and by RNA polymerase assays.

Our understanding of the physiologic alterations which may influence the genetic control of protein synthesis in skeletal muscle is much less advanced than in the case of other mammalian tissues. This is probably due to the unique difficulties of obtaining pure nuclear material from skeletal musculature.

The well established procedures for the isolation of nuclei from various soft tissues such as liver, pancreas, kidney and brain [1, 2] and even from heart [3–5] cannot be successfully applied to skeletal muscle. Although various modifications for the preparation of nuclei from hind-limb muscles of the adult rat have been described, the low recoveries of nuclei, ranging from 3–15%, are not always reproducible and are frequently contaminated with myofibrillar debris. In addition, various agents which would be deleterious in further studies on the endogenous metabolic activity of the isolated nuclei were employed [5–8].

In order to recover nuclei from different types of muscle such as the red, slow twitch soleus and the white, fast twitch extensor digitorum longus (EDL), we find that different critical modifications in procedure are required for each muscle. The recovery of nuclei from soleus and EDL is then reproducibly $15.5\pm0.7$ and $20.3\pm0.9\%$, respectively, using only solutions with sucrose and $MgCl_2$. Phase contrast and electron microscopic examination of nuclear fractions and measurements of RNA and protein content indicate very little myofibrillar or cytoplasmic contamination. In addition, the isolated nuclei exhibited relatively high levels of DNA-dependent RNA polymerase activities.

## MATERIALS AND METHODS

### *Isolation of muscle nuclei*

The soleus and extensor digitorum longus (EDL) muscles were obtained from male, albino, Wistar rats of 90 to 150 days of age. These muscles were removed from the hind-limbs within several minutes after sacrifice of the rats by decapitation and then immediately

| SKELETAL MUSCLE | PROCEDURE |
|---|---|
| | 1. *Homogenate (H)* |
| | Prepared with 0.32 M sucrose−3 mM $MgCl_2$, pH 6.8. Volume used (in ml)= g minced muscle×31. |
| EDL | Virtis setting 3 for 30 sec, 3 times. |
| Soleus | Virtis setting 5 for 30 sec, 5 times. |
| | 2. *Filtrate (F)* |
| | Vacuum filtration with stainless steel screens: |
| EDL | 20, 50, 100, and 150 mesh, successively. |
| Soleus | 20, 50, and 100 mesh, successively. |
| | 3. *Myofibrillar—Nuclear Pellet (MNP)* |
| | Centrifuge F at 800 g, 15 min, 2°C. |
| | 4. *Purified Nuclear Pellet (PNP)* |
| | Ultracentrifuge suspensions of MNP at 52800 $g_{ave}$, 45 min, 5°C, in Beckman Type SW 27 rotor. |
| EDL | Prepare suspension of MNP=21–27 mg muscle per ml in 1 mM $MgCl_2$ and 54.3–54.8 % sucrose determined by refractometry. Layer suspension over 5 ml of 56.0 % sucrose−1 mM $MgCl_2$. |
| Soleus | Re-homogenize MNP in volume=g muscle×4 with a Potter-Elvehjem tube and Teflon pestle, clearance 0.13–0.18 mm, taking 5 strokes at 500 rpm. Prepare suspension of MNP=24–34 mg muscle per ml in 1 mM $MgCl_2$ and 55.5–56.0 % sucrose determined by refractometry. Layer suspension over over 5 ml of 58.5 % sucrose−1 mM $MgCl_2$. |

*Fig. 1.* Outline of differences in the procedures used to isolate nuclei from the EDL and soleus muscles of the rat.

placed in ice-cold homogenizing media (0.32 M sucrose−3 mM $MgCl_2$, pH 6.8). For one experiment, usually twenty muscles of each type were pooled. Dissection of tendon and connective tissue, weighing and mincing finely with a curved scissor were done in a cold room (3°C). The remaining steps are given in fig. 1 which illustrates the minor but critical variations which need to be taken to successfully recover nuclei from soleus and EDL muscles. The pelleted nuclei (step 4) were resuspended in 0.25 M sucrose−1 mM $MgCl_2$, pH 6.5.

## *Microscopic procedures*

Suspensions of nuclei in 0.25 M sucrose−1 mM $MgCl_2$ were examined by phase contrast microscopy and also fixed with 3% glutaraldehyde in Millonig buffer (diluted 1 : 1 with double distilled water) as the preliminary step for preparation of the sample for electron microscopic observation. In addition, some soleus and EDL muscles were bathed in situ with the same fixative, dissected, minced into pieces of 1–2 $mm^3$ and further fixed for 4 h. Following fixation, all samples were rinsed twice in the diluted Millonig buffer for 15 min, treated with 1 % buffered osmium tetroxide for 1 h and rinsed again with the same buffer. After dehydration through a graded series of ethanol, the samples were treated with propylene oxide and then infiltrated with a 1 : 1 mixture of Araldite (Ciba) and propylene oxide overnight. Conical BEEM capsules were half-filled with the infiltrated suspensions of isolated nuclei, centrifuged and finally filled with an equal volume of undiluted Araldite. The infiltrated muscle tissue blocks were simply centrally oriented at the bottom of truncated BEEM capsules which contained undiluted Araldite mixture. Polymerization of all samples was done at 60°C for two days. An Ivan Sorval, Porter-Blum ultramicrotome MT-2 was used to section the specimens. Electron microscopic observations were made with an RCA, EMU-3H instrument after staining the specimens with 10% uranyl acetate in methanol and lead citrate.

## *Biochemical analyses*

The Schmidt-Thannhauser procedure as modified by Fleck & Munro [9] was employed to extract RNA, DNA and protein from the homogenates, filtrates, myofibrillar-nuclear and purified nuclear pellets. The macromolecular components were precipitated and washed once with ice-cold 0.2 N perchloric acid (PCA). The precipitate was then incubated in 0.3 N KOH at 37°C for 1 h. DNA and protein were re-precipitated with cold 0.2 N PCA and the hydrolysed RNA was assayed by ultraviolet absorption at 260 nm [9]. The precipitate was next incubated in 1.2 N PCA at 70°C for 15 min to hydrolyse the DNA. This incubation was repeated once and the two hydrolysates were pooled and analysed for DNA by the modified diphenylamine reaction of Burton [10]. The final precipitate was dissolved in 0.5 N NaOH and analysed for protein content by the method of Lowry [11]. The bovine serum albumin, yeast RNA and calf thymus DNA which were used as reference standards were obtained from Miles Laboratories, Elkhardt, IN 46514, USA. The bovine serum albumin was in a 9.3±0.1 g% solution based upon Kjeldahl nitrogen by the supplier.

Table 1. *Recovery of DNA in various fractions during the isolation of nuclei from rat skeletal muscles*[a]

| Fraction[b] | Soleus | EDL |
|---|---|---|
| H | 100.0 | 100.0 |
| F (6) | 92.6±2.7 | 100.4±4.3 |
| MNP (6) | 83.3±1.0 | 84.5±1.8 |
| PNP (14) | 15.5±0.7 | 20.3±0.9 |

[a] Means±S.E.M. in the table are expressed as the % of the initial DNA in H.
[b] Fractions are described in fig. 1. The number of preparations is given in parentheses.

Table 2. *DNA content of red and white muscles of the adult rat*

| Parameter | Soleus[a] | EDL[a] | $\frac{\text{Soleus}}{\text{EDL}} \times 100$ |
|---|---|---|---|
| Wet wt (mg) | 178± 6 | 203± 5 | 88[b] |
| μg DNA/gm wet wt | 571±25 | 348±14 | 164[b] |
| μg DNA/muscle | 101± 4 | 70± 4 | 144[b] |

[a] Results are expressed as means±S.E.M. of ten groups of rats (90–150 days of age) in which the soleus and EDL muscles were assayed simultaneously.
[b] Based upon *t*-test analyses, the difference between the means is highly significant ($P<0.01$).

Based upon our determinations of the phosphorus content after hot acid hydrolysis [12], the purity of the yeast RNA was 93.8% and of the calf thymus DNA was 77.5%. Results of protein, RNA and DNA analyses have been appropriately corrected for the purity of the respective standard.

### *RNA polymerase assays*

For the $Mn^{2+}+(NH_4)_2SO_4$-activated enzyme, the incubation media contained the following components: Tris (hydroxymethyl)-aminomethane · HCl, pH 7.5, 100 mM; $MnCl_2$, 4 mM; $(NH_4)_2SO_4$, 300 mM; ATP, CTP, GTP, 0.8 mM; NaF, 5 mM; cysteine, 20 mM; and UTP-5-$^{14}$C, 0.042 mM (spec. act. 60 μCi/μmole, Amersham Searle Corp.). The incubation media was the same for the $Mg^{2+}$-activated enzyme assay except that the Tris-HCl buffer was pH 8.5, $MgCl_2$, 5 mM, was used instead of $MnCl_2$ and the $(NH_4)_2SO_4$ was omitted. After the addition of nuclear suspensions containing 15–35 μg DNA, the reaction mixture in a final volume of 0.5 ml was incubated for 20 min at 37°C in a water bath. The reaction was terminated by placing the tubes in ice and adding 2 ml of 10% trichloroacetic acid (TCA) with 0.01 M $Na_2P_2O_7$. Then 0.6 mg of yeast RNA in 0.3 ml was added as a carrier. Zero time reactions were performed by the addition of TCA before the nuclear suspension. After chilling the tubes for 30 min, the precipitate was collected by centrifugation at 700 g for 10 min at 2°C and then washed three times with 5% TCA with 0.01 M $Na_4P_2O_7$. The precipitate was dissolved in 1.5 ml of Soluene 350 (Packard Corp.) and transferred into scintillation fluid in a counting vial. The radioactivity incorporated into RNA was then determined by scintillation spectrometry in a Packard Tri-Carb Model 3380.

## RESULTS AND DISCUSSION

### *Isolation of nuclei*

The critical steps in the isolation of nuclei from EDL and soleus muscles of the adult rat and the different modifications required for each of these muscles are listed in fig. 1. After trial and error we found that hard, fibrous tissue such as the EDL and soleus muscles can be well homogenized with a blade-type homogenizer (like the Virtis 45) with very little, if any, destruction of nuclei when there is a large volume of homogenizing media in proportion to muscle (31 : 1) and the homogenizing process is adjusted for apparent differences in the ease of disruption of different types of muscle. It is necessary, however, to dissect tendinous connective tissue from the muscles before homogenization. Our homogenization procedure is similar to that described by Sobel & Kaufman [6] for rat soleus except that mercaptoethanol and KCl are not used in the homogenizing media.

The filtration step is required to remove the larger bundles of muscle fibers which would otherwise trap nuclei during ultracentrifugation. The EDL homogenates can be rapidly filtered through finer screens than the more vigorously obtained soleus homogenates which still contain a greater number of large fibers (fig. 1). Since 93 and 100% of the initial DNA in the homogenates are recovered in the filtrates of soleus and EDL, respectively (table 1), the homogenization process with only 0.32 M sucrose −3 mM $MgCl_2$ media adequately releases the nuclei from muscle fibers for filtration. By the method recently reported by Kuehl [7] about 75% of skeletal muscle nuclei were lost during filtration, probably because of incomplete homogenization by the Potter-Elvehjem procedure even with Triton X-100 detergent in the media and a tendency for nuclear-myofibril material to adhere to cloth filters.

After low speed centrifugation of the filtrate (fig. 1), 83–85% of the initial DNA is recovered in the myofibrillar-nuclear

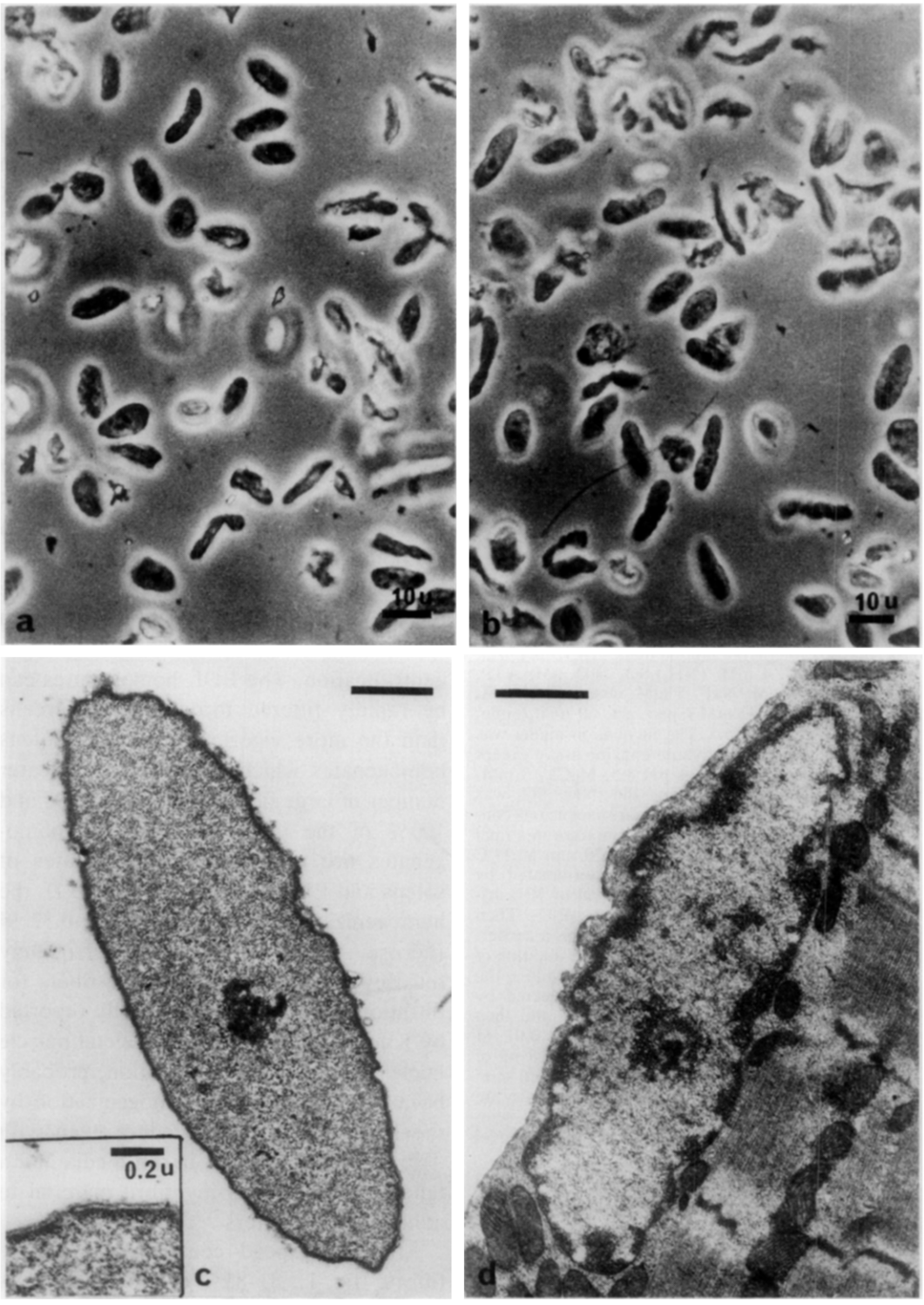


*Fig. 2.* (*a*), (*b*). Phase contrast micrographs of resuspensions of nuclei isolated from soleus and EDL muscles, respectively; (*c*), (*d*) electron micrographs of isolated and in situ nuclei from soleus muscle as described in "Methods".

Table 3. *Protein and RNA relative to DNA in the purified nuclear pellets from rat skeletal muscles*

| Ratio | Soleus[a] | EDL[a] |
|---|---|---|
| Protein/DNA | 1.78±0.08 | 2.32±0.08 |
| RNA/DNA | 0.45±0.03 | 0.47±0.02 |

[a] Results are expressed as means±S.E.M. of 14 nuclear preparations.

pellet (table 1). It appears, therefore, that there is no significant destruction of nuclei during homogenization and filtration. No correction is made for mitochondrial DNA in the homogenate.

The yield of nuclei after ultracentrifugation of myofibrillar-nuclear suspensions is very much influenced by the sucrose density of the suspension. We find that it is necessary to check each suspension before ultracentrifugation with a hand-type refractometer (Bausch & Lomb). Ultracentrifugation of myofibrillar-nuclear suspensions from soleus at a sucrose density of only 0.5% lower than our recommended range of 55.5–56.0% (fig. 1) will result in variable yields of nuclei with significant myofibrillar contamination. In addition (fig. 1), the concentration of myofibrillar-nuclear material, which is based on the original weight of the muscle, and a second homogenization of the soleus material, but not necessarily the EDL, is also important to facilitate the sedimentation of nuclei during ultracentrifugation.

The myofibrillar-nuclear suspensions were also layered over a more dense sucrose solution as described in fig. 1 so that fine myofibrils would collect at the interface during ultracentrifugation rather than sedimenting with the nuclei. The solution below the interface remains clear. Although most of the fibers along with nuclei form a thick pellicle at the top of the ultracentrifuge tube, some fibers are suspended throughout the tube to the interface, particularly with the EDL samples. When a preparation of pure nuclei is obtained, a white, ring-like pellet is seen around the bottom of the tube.

*Yield of nuclei*

Our recovery of nuclei from soleus is 15.5± 0.7% and from EDL is 20.3±0.9%, based on the DNA content of the homogenate (table 1). Since the total DNA content of soleus, however, is 44% greater than EDL (table 2), there may be more muscle cell nuclei in soleus compared with EDL. The contribution made by mitochondria [13] or satellite cell nuclei to the total DNA content would not be sufficient to account for this difference. Although it has been reported that the number of satellite cells in soleus is almost threefold greater than in EDL, these are only about 5% of the total soleus nuclei [14].

*Microscopic observations*

Our re-suspensions of nuclear pellets appear uniformly turbid. Routine examination of these by phase contrast microscopy revealed little cytoplasmic or myofibril contamination as shown in fig. 2*a, b*. By electron microscopy the majority of the isolated nuclei appeared moderately well preserved with intact double nuclear membranes underlined with a thin layer of dense chromatin except by the nucleopores, densely staining nucleoli and homogeneously dispersed, loose chromatin as seen in the representative, isolated soleus nucleus in fig. 2*c*. The in situ soleus nucleus in fig. 2*d*, however, has a thicker layer of dense chromatin along the inner nuclear membrane and numerous foci of dense chromatin throughout the less dense nucleo-

Table 4. *Comparison of the composition and recovery of nuclei isolated by various methods*

| Ref. | Source of | Components in sucrose isolation media | Protein / DNA | RNA / DNA | %Recovery of tissue DNA |
|---|---|---|---|---|---|
| Held et al. [this paper] | Rat soleus | $MgCl_2$ | 1.8 | 0.45 | 15.5 |
| | Rat EDL | $MgCl_2$ | 2.3 | 0.47 | 20.3 |
| Sobel & Kaufman [6] | Rat soleus | $MgCl_2$, mercaptoethanol, KCl | 4.2 | – | 15.0 |
| Kuehl [7] | Rat hind-limb | $MgCl_2$, Triton X-100, bovine serum albumin | 2.4 | 0.14 | 10.5 |
| Edelman et al. [8] | Rat femoral | $MgCl_2$, KCl | 6.1 | 0.30 | 2.8 |
| Ibid [8] | Rat femoral | Muscle pre-treated with Krebs-Ringer media and *N*-ethylmaleimide | 3.4 | 0.10 | 14.0 |
| Marchok & Wolff [15] | Chick leg | $MgCl_2$ | 1.6 | 0.24 | 10–15 |
| Zak et al. [5] | Rat heart | $MgCl_2$, KCl, EGTA, $Na_4P_2O_7$ | 1.5 | 0.19 | – |
| Widnell et al. [4] | Rat heart | $MgCl_2$ | 4.3 | 0.23 | 43 |
| Tata & Baker [16] | Rat liver | $MgCl_2$, KCl, Tris : HCl | 2.0 | 0.27 | 83 |
| McEwen & Zigmond [2] | Rat brain regions | $MgCl_2$, Triton X-100, $KH_2PO_4$ | 2.2–7.5 | 0.08–0.45 | 38–61 |

plasm. The relative difference in density of the homogeneously dispersed, loose chromatin in the isolated and in situ nuclei suggest that the nucleoplasm was not remarkably extracted during our isolation procedure but primarily re-distributed.

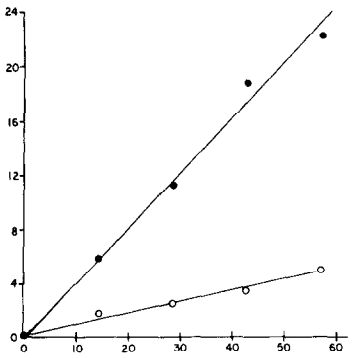


*Fig. 3. Abscissa:* μg DNA incubated; *ordinate:* RNA polymerase activity (pmoles $^{14}$C-UMP incorporated in 20 min).

The linear relationship between the level of RNA polymerase activity and the number of isolated soleus nuclei in the incubation media under two assay conditions; i.e., with ●—●, $Mn^{2+}$ and $(NH_4)_2SO_4$ and ○—○, with $Mg^{2+}$ as described in "Methods".

*Biochemical analyses of purity*

Since it is most difficult to avoid myofibrillar contamination during the isolation of skeletal muscle nuclei, the ratio of protein to DNA is most indicative of the purity of the nuclear preparation. The lower ratio of 1.78 (table 3), which was obtained for soleus nuclei, compared with 2.32 for EDL nuclei, probably reflects a greater degree of purity rather than any real difference in the protein content of these nuclei. These ratios are lower than any previously reported for limb muscles of the rat, but comparable to some obtained with isolated nuclei of other tissues (table 4). The comparatively higher RNA to DNA ratios, however, suggest that more RNA is associated with these muscle nuclei (tables 3, 4). Since Triton X-100 is not used in our isolation procedure, the double nuclear membrane is retained along with some endoplasmic reticulum (fig. 2*c*) which would contribute to the level of RNA. This detergent was avoided since it has been reported to promote the degrada-

tion of nuclear RNA and would obliterate further studies of amino acid incorporation [2].

*Enzymatic activity of nuclei*

The different classes of DNA-dependent RNA polymerase (nucleoside triphosphate: RNA nucleotidyltransferase, EC 2.7.7.6) which are discretely localized in eukaryotic nuclei can be differentiated under appropriate assay conditions [17]. The activity of the nucleolar RNA polymerase I (or A) is stimulated by $Mg^{2+}$ at low ionic strength while the nucleoplasmic RNA polymerase II (or B) is stimulated by $Mn^{2+}$ at high ionic strength. Under these conditions the incorporation of [$^{14}$C]UTP into acid-insoluble material by soleus nuclei isolated according to our procedure was linearly dependent upon the amount of DNA, or presumably the number of nuclei, added to the incubation media (fig. 3). The enzyme activity with $Mn^{2+}$ at high ionic strength is approximately fivefold that of $Mg^{2+}$ at low ionic strength. These levels are comparable to those reported by Sobel & Kaufman [6] for soleus nuclei. Further characterization of RNA polymerase activities in muscle nuclei will be reported in a subsequent paper [18].

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