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Immunological localization of ribosomes in striated rat muscle

Evidence for myofibrillar association and ontological changes in the subsarcolemmal:myofibrillar distribution

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Ribosome distribution in skeletal-muscle fibres was investigated immunohistochemically by using polyclonal antibodies raised against large-ribosomal-subunit proteins isolated from rat liver. Immunoblot analysis showed the antibodies to recognize five major proteins of the large subunit; these were identified as L4, L6, L7, L15 and L17 by two-dimensional electrophoresis. Immunohistochemistry of frozen rat skeletal-muscle sections showed staining of both the sub-sarcolemmal and intermyofibrillar cytoplasm. A distinct banding pattern was observed, and when peroxidase and phase-contrast images of the same field were compared by image analysis the anti-ribosome staining was found to correspond to the A-bands. These results suggest that a proportion of muscle ribosomes are present in the myofibrillar cytoplasm in a regular fashion, possibly associated with myosin. Densitometric analysis of the peroxidase immunostaining showed that the ratio of myofibrillar to sub-sarcolemmal ribosomal material was lower in muscle from 51-day-old rats compared with those from 14-day-old animals.

INTRODUCTION

The biosynthesis of muscle structural proteins and their subsequent organization into a formal and complex arrangement within the myofibre presents unique logistic problems. These problems are highlighted both by the need for large quantities of actin and myosin to be synthesized during periods of muscle growth and also by the turnover of actomyosin, which requires that the newly synthesized proteins are incorporated into the contractile apparatus (Millward, 1980). Such potential difficulties may be mitigated if synthesis of the myofibrillar proteins occurs close to the myofibrils. It is thus of importance to identify the subcellular location of the sites of protein synthesis within the muscle fibre.

However, surprisingly little attention has been given to the distribution of ribosomes within the muscle fibre, although basophilic staining of the A-bands (myosin filaments) in muscle sections has been attributed to the presence of RNA (Clavert *et al.*, 1949). Previous studies of striated muscle using electron microscopy have consistently shown that ribosomes are present in the subsarcolemmal sarcoplasm, especially the paranuclear cones (Padykula & Gauthier, 1970; Galavazi, 1971; Galavazi & Szirmai, 1971; Gauthier & Dunn, 1973; Gauthier & Schaeffer, 1975). In addition, certain investigations (Larson *et al.*, 1969; Galavazi, 1971; Galavazi & Szirmai, 1971) have suggested that ribosomes are also present in the intermyofibrillary cytoplasm, possibly associated with the contractile apparatus. The various reports do not agree, however, on the precise location; thus the presumptive myofibrillar ribosomes have been reported associated with either the filaments of the I bands (Galavazi, 1971; Galavazi & Szirmai, 1971) or the A bands (Larson *et al.*, 1969), whereas in some cases they have been reported not to be present at all (Gauthier & Schaeffer, 1974). These discrepancies may be related to the problems of distinguishing ribosomes from glycogen granules in muscle sections examined by electron microscopy (Galavazi, 1971).

The present work uses an alternative approach to study the localization of ribosomes in striated muscle, particularly to investigate the putative association of ribosomal material with the myofibrils. Polyclonal antibodies raised against the large ribosomal subunit permitted immunohistochemical localization with a light-microscope and, combined with microdensitometry (Campbell *et al.*, 1986), it was possible to quantify both sub-sarcolemmal and myofibrillar staining. These techniques have been used to investigate changes in ribosome location in muscle as a function of age.

MATERIALS AND METHODS

Materials

Biotinylated anti-rabbit IgG and streptavidin–biotinylated horseradish peroxidase complex were purchased from Amersham International, Amersham, Bucks., U.K. All other chemicals were of reagent grade and purchased from either Sigma Chemical Co or British Drug Houses, both of Poole, Dorset, U.K.

Ribosome-subunit preparation

Ribosomes were prepared from livers of male Hooded Lister rats (Rowett strain) by the method of Metlas *et al.* (1973). Subunits were subsequently separated on linear 10–30% (w/v) sucrose density gradients after pretreatment with 0.1 mM-puromycin for 15 min at 37 °C (Leader *et al.*, 1976). Gradients were monitored by the A_{260}, and the two fractions containing 60 S and 40 S subunits collected. Each subunit fraction was centrifuged (175000 *g* for 16 h at 4 °C), and the pellets were then rinsed with 10 mM-Tris/HCl, pH 7.6, and stored at −20 °C until required.

Antibody production

Ribosome-subunit pellets were resuspended in phosphate-buffered saline (10 mM-sodium phosphate buffer, pH 7.4, containing 0.9% NaCl; PBS) to a final concentration of 4 mg of protein/ml. Before immunization each subunit suspension was mixed with an equal volume of Freund's adjuvant, either complete (injection 1) or incomplete (subsequent injections). New Zealand White rabbits (approx. 4 kg in weight) were

Abbreviation used: PBS, phosphate-buffered saline.
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immunized with 1 mg of protein on days 0, 14, 28 and 42; antigen was injected subcutaneously at six sites on the upper back. On day 50, approx. 30 ml of blood (per rabbit) was collected from a peripheral ear vein. The IgG fraction was separated from the serum by $(NH_4)_2SO_4$ precipitation and then dialysed to remove salt and lipoproteins (Harboe & Ingild, 1973). Antibodies were stored at −20 °C in 0.5 ml batches.

Antibody characterization

Antibody specificity was tested by enzyme-linked immunosorbent assay (e.l.i.s.a.), carried out in 96-well Immunol 1 plates (Dynatech Labs, Billinghurst, Sussex, U.K.). Wells were coated with antigen (200 μl of 1 μg/ml in PBS) overnight at room temperature, washed three times with PBS containing 0.05% Tween 20 (PBS/Tween), then incubated (1 h at 37 °C) successively with the primary antibody, biotinylated anti-rabbit antibody and streptavidin-biotinylated horseradish peroxidase, with washing between each incubation with three changes of PBS/Tween. All dilutions were 1:1000 in PBS/Tween. Peroxidase activity was then revealed by using the chromogen *o*-phenylenediamine (8 mg, dissolved in 20 ml of 0.05 M-Na_2HPO_4/0.025 M-citric acid plus 20 μl of H_2O_2) as substrate with a reaction time of 10 min. The reaction was stopped with 3 M-H_2SO_4 and the A_{492} measured.

Further characterization involved immunoblotting after protein separation on polyacrylamide gels by using either a one-dimensional SDS system (Laemmli, 1970) or two-dimensional urea-based gels (Lastick & McConkey, 1976). Proteins were transferred on to nitrocellulose paper by using a semi-dry multigel blotter (Kyhse-Anderson, 1984) for SDS gels and a Trans-blot cell (Bio-Rad) for the two-dimensional gels (Towbin *et al.*, 1979), and non-specific binding sites were blocked by subsequent incubation (16 h) of the paper with 3% (w/v) dried milk powder (Cadbury's Marvel)/5% (v/v) horse serum in PBS. The nitrocellulose paper was then incubated with the anti-ribosomal antibody (diluted 1:100) for 2 h, followed by successive 1 h incubations with biotinylated anti-rabbit and streptavidin-biotinylated horseradish peroxidase (diluted 1:200). Papers were washed between each incubation with PBS (three of four changes). Peroxidase activity was detected with chloronaphthol as substrate (60 mg dissolved in 20 ml of methanol, plus 100 ml of PBS and 40 μl of H_2O_2). Immunoblots were scanned with a Shimadzu CS-930 densitometer in the reflectance mode.

Immunocytochemistry

Rat striated muscles (psoas, plantaris) were carefully dissected, pinned on to cork boards to maintain fibre length and orientation, and surrounded by OCT embedding medium (Agar Aids, Stansted, Essex, U.K.). Muscles were then frozen in isopentane, which had been precooled in liquid N_2 (Dubowitz & Brooke, 1973). Cryostat sections (5 μm) were transferred to glass slides and fixed with acetone for 10 min. Sections were then treated with 3% (v/v) H_2O_2 in methanol for 30 min to remove endogenous peroxidase activity, and then with normal donkey serum (1:100 dilution, 30 min) to block non-specific binding. Sections were then incubated with the anti-ribosomal antibody (diluted 1:200 with PBS; 16 h at 4 °C), washed with PBS (three or four changes) and incubated successively with biotinylated anti-rabbit IgG and streptavidin-biotinylated horseradish peroxidase, both diluted 1:1000 with PBS. Peroxidase activity was revealed by incubation for 15 min at room temperature with diaminobenzidine as substrate (6 mg dissolved in 10 ml of PBS and 10 μl of H_2O_2). After stopping the reaction by washing in PBS, sections were mounted in Aquamount and viewed using a Zeiss (Axioplan) microscope. Pictures were taken using Kodak Pan Plus-X film for the fluorescence and Panatomic X for the peroxidase slides. Parallel controls, using an IgG fraction from normal preimmune rabbit serum at the same dilutions as the anti-ribosomal antibody, were conducted for all immunological investigations.

Microdensitometry

Peroxidase-stained sections were examined on a Vickers M85 microdensitometer, and absorbance readings were taken at 470 nm with a ×100 oil-immersion lens and a size-1 circular mask, 1.5 μm effective diameter. Absorbance readings were at 15 μm intervals along the length of the fibres, measuring both the staining in the region of the myofibrils (10 measurements) and that in the subsarcolemmal sarcoplasm (15 measurements, 8 along one side and 7 along the other). Six readings were taken on tissue-free areas as slide blanks. Five fibres were analysed per section and six sections per muscle, and the values from one muscle were then averaged. Serial sections were stained with preimmune serum, and absorbance values for similar fibre areas were subtracted from the values obtained with the specific serum.

A QUIPS image-processing work-station (Torch Computers, Cambridge, U.K.) operating with VCS image-processing software (Vision Dynamics, Hemel Hempstead, Herts., U.K.) was used to compare phase-contrast and peroxidase staining of the same section. Sections were photographed with Ektachrome 50 DX slide film, and between exposures the microscope stage was not moved; only the light source was altered to give phase-contrast or bright-field illumination. Transparencies were mounted on a photographic micrometer stage with three planes of movement, and illuminated with a photographic light-box. Images were then captured with a Hitachi KP140 CCD camera (resolution 768 by 575 pixels, at 256 grey levels). Image registration was accomplished by aligning the lower left corner of each image with lines displayed in the overlay plane of the frame store (Fig. 6).

RESULTS

When antibody and antigen concentrations were varied over a wide range, e.l.i.s.a. assays showed the optimal conditions to be an antibody dilution of 1:1000 and an antigen coating concentration of 5 μg/ml (Fig. 1). Under these conditions the antibody raised against the 60 S ribosomal subunit exhibited weak cross-reactivity with the 40 S subunit and no cross-reaction with a mitochondrial fraction (Fig. 2). The weak reaction against 40 S subunits was due to contamination with 60 S material, as shown by the weaker reaction against proteins of similar electrophoretic mobility on immunoblot analysis (Fig. 3). As expected,

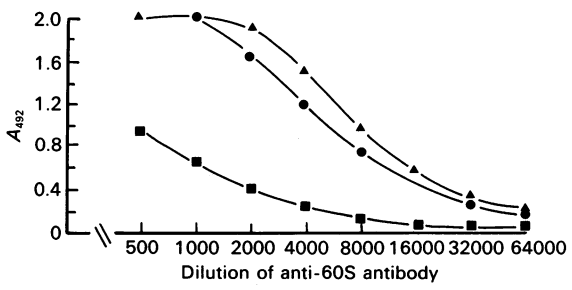

Fig. 1. Determination of the anti-(60 S subunit) antibody titre by e.l.i.s.a.

Microtitre plates were coated overnight with 60 S subunits at protein concentrations of 0.5 μg/ml (■), 5 μg/ml (●) or 10 μg/ml (▲), before incubation with serial dilutions of antibodies raised against 60 S subunits (dilutions from 1:500 to 1:64000).

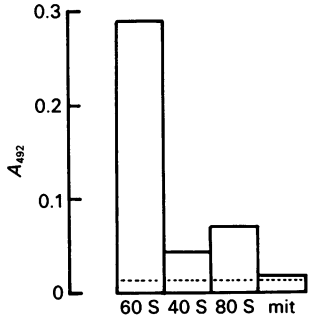

Fig. 2. Specificity of anti-(60 S subunit) antibody studied by e.l.i.s.a.

Microtitre plates were coated with either ribosomal (60 S, 40 S or 80 S) or mitochondrial (mit) material at a concentration of 5 μg of protein/ml. All samples were subsequently incubated with anti-(60 S subunit) antibody at 1:1000 dilution. The broken line shows the non-specific reaction obtained with pre-immune serum at the same dilution.

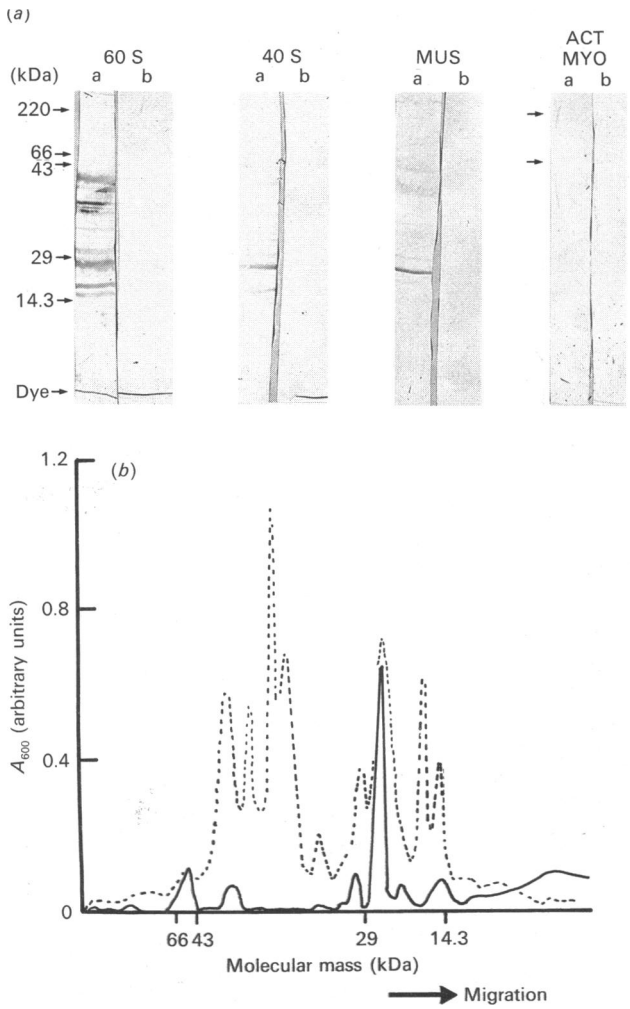

Fig. 3. Specificity of anti-(60 S subunit) antibody studied by immunoblot analysis

(*a*) Proteins from purified 60 S and 40 S ribosomal subunits (40 μg), total muscle homogenate (MUS; 120 μg) and actin and myosin (ACT, MYO; 40 μg) were separated by SDS/PAGE and transferred to nitrocellulose paper and incubated with anti-(60 S subunit) antibodies (a) or pre-immune serum (b) both diluted 1:100. Migration of actin and myosin is indicated by arrows, as is the position of the dye front. Migration of standard molecular-mass markers is shown at the left of the Figure. (*b*) The proteins labelled in 60 S (---) and muscle samples (——) were compared by densitometric scans of the appropriate lanes.

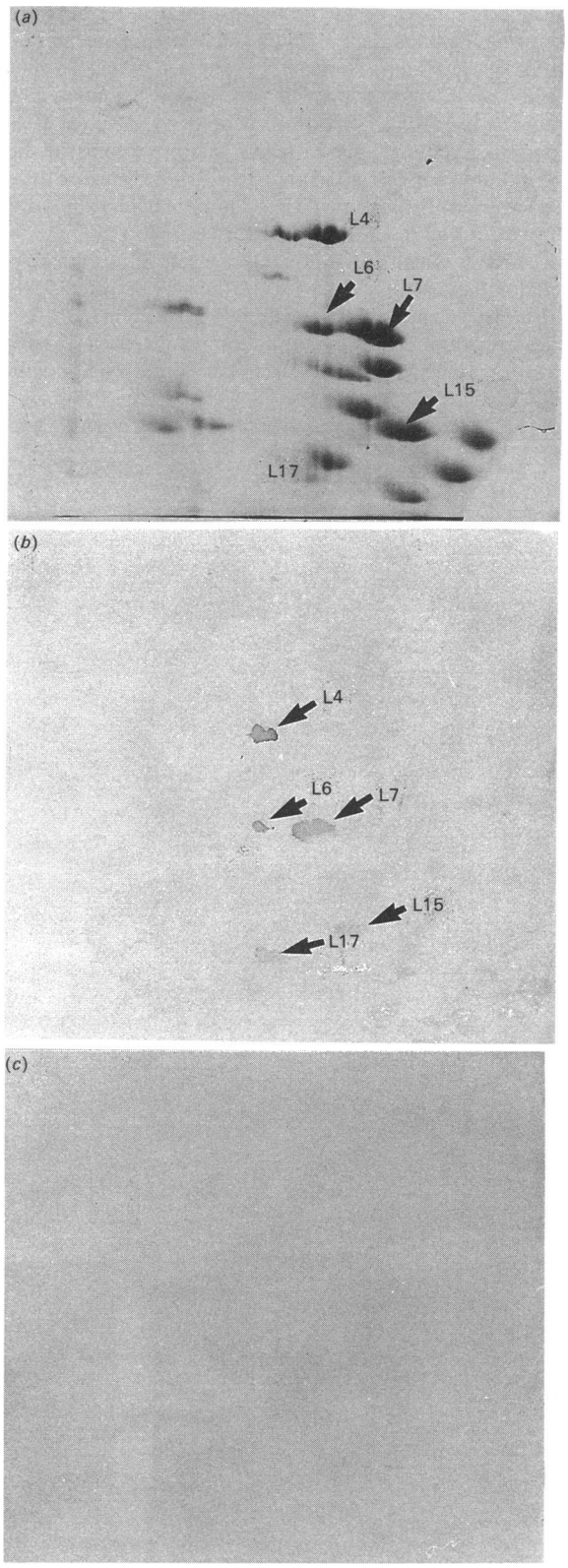

Fig. 4. Immunoblot analysis after two-dimensional gel-electrophoresis

Proteins were purified from 60 S ribosomal subunits (Sherton & Wool, 1972) and separated by two-dimensional electrophoresis in urea gels (Lastick & McConkey, 1976). Gels were either stained with Coomassie Brilliant Blue to reveal protein composition (*a*) or transferred to nitrocellulose for immunoblotting with the anti-(60 S subunit) antibody (*b*) or preimmune serum (*c*).

the antibody showed reactivity against total 80 S ribosomes (Fig. 2), but this is not as high as might have been expected, and this could have been due to some 60 S proteins to which the antibody is specific being inaccessible when the 80 S ribosome is complete. Immunoblotting of SDS/polyacrylamide gels showed that the antibody reacted strongly with five major proteins in the large (60 S) subunit and less strongly with another four or five; when tested against complete muscle homogenates the anti-(60 S subunit) antibody recognized one major component and five other proteins which corresponded in electrophoretic mobility to the major bands observed with purified 60 S subunits (Fig. 3). Importantly, no additional staining was evident. On the basis, therefore, of the e.l.i.s.a. and immunoblotting results the antibody preparation specifically recognizes ribosomal components, and there was no evidence of cross-reaction with other muscle proteins. The antibody did not recognize actin or myosin (Fig. 3). Immunoblots of two-dimensional urea gels again showed the antibody preparation to recognize five major ribosomal proteins (Fig. 4). Comparison against a parallel gel stained with Coomassie Brilliant Blue allowed identification of the proteins recognized, namely L4, L6, L7, L15 and L17 (nomenclature of Lastick & McConkey, 1976; corresponding to L4, L7, L6, L15 and L17 in the nomenclature of McConkey *et al.*, 1979). These identifications were supported by the fact that the known molecular masses (40, 30, 25, 28 and 23 kDa) of these proteins compared closely with estimates, from migration in SDS/polyacrylamide gels, of the molecular masses of the five major antigenic species (40 and 30 kDa and three in the range

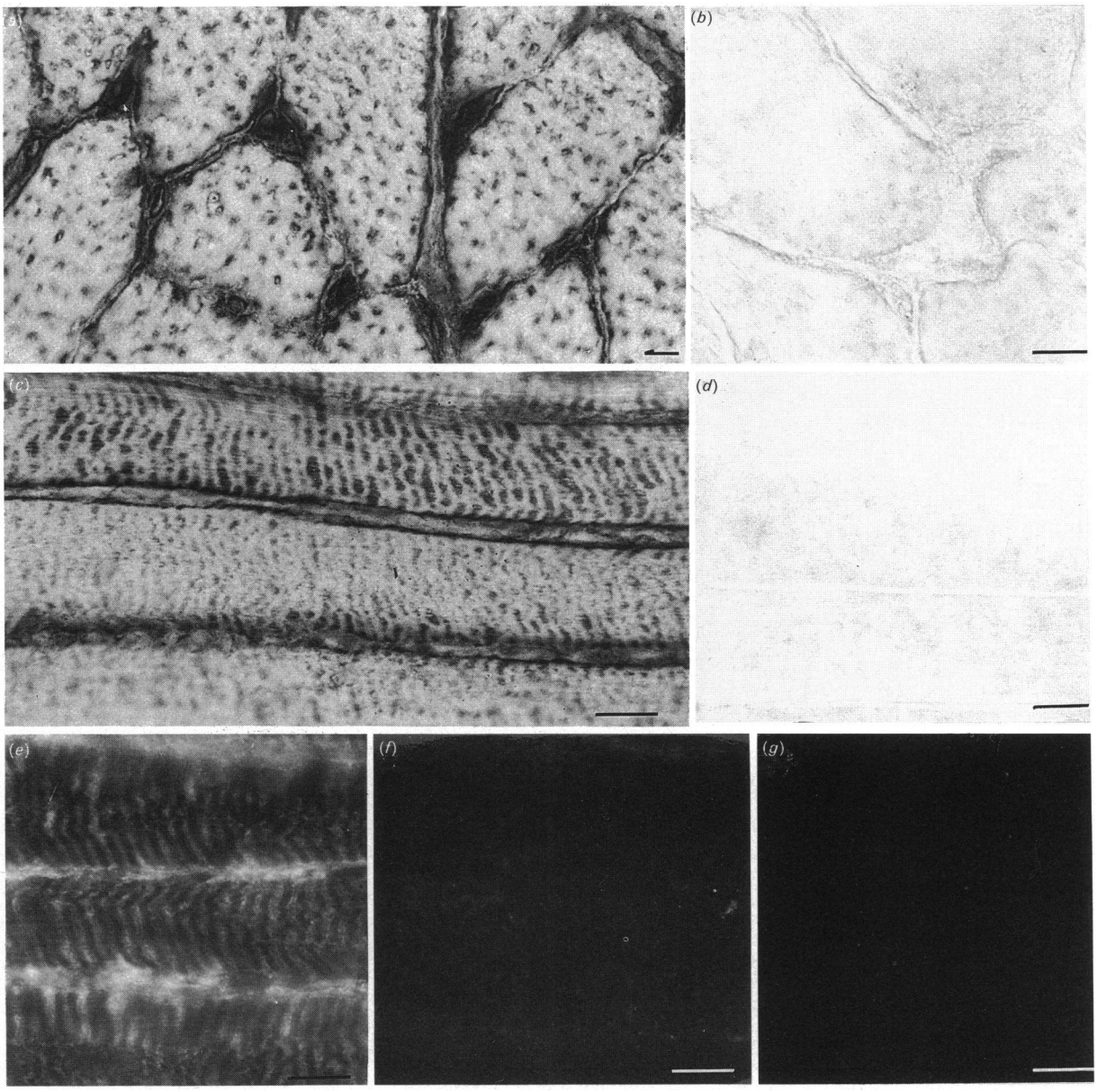

Fig. 5. Immunohistochemical localization of ribosomes in sections of skeletal muscle

Immunofluorescence with anti-(60 S subunit) antibodies (*e*, *f*) or antibodies from pre-immune serum (*g*) of longitudinal sections. Immunoperoxidase staining of transverse (*a*, *b*) and longitudinal (*c*, *d*) sections with anti-(60 S subunit) and pre-immune (*b*, *d*) antibodies. Immunofluorescence with anti-(60 S subunit) antibodies of muscle sections without (*e*) or with (*f*) treatment with RNA-ase A (type III-A, from bovine pancreas, 50 μg/ml) for 1–2 h at 37 °C before fixing. Bars represent 10 μm.

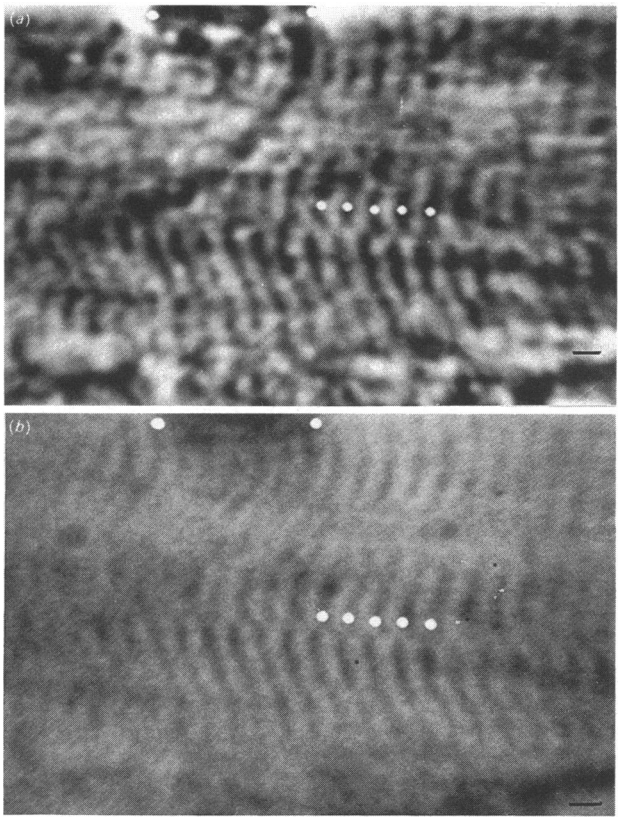

Fig. 6. Image analysis: phase contrast (*a*) and immunoperoxidase (*b*) images of the same field of a longitudinal muscle section stained with anti-(60 S subunit) antibody

White dots were placed on the banding visible in the immunoperoxidase image stored in the computer and then superimposed on the phase-contrast image. Peroxidase staining corresponded to the A-bands. Bars represent 10 μm.

20–28 kDa). The antigenicity of these proteins is consistent with their reported presence near the surface of the subunit, as judged by trypsin accessibility (Marion & Marion, 1987).

Immunocytochemistry using the anti-(60 S subunit) antibody produced reproducible staining patterns on sections of skeletal muscle (Fig. 5). Identical patterns were observed with both immunoperoxidase and immunofluorescence methods, so excluding the possibility that the patterns were partly due to artefacts of autofluorescence or endogenous peroxidase activity. Strong staining was observed in the interstitial cells between fibres and in the subsarcolemmal sarcoplasm (Figs. 5*a* and 5*c*). In addition there was staining in the myofibrillar regions of the myofibres (Figs. 5*a* and 5*c*). Preimmune serum gave a comparatively very low level of non-specific staining (Figs. 5*b* and 5*d*). The specific staining, both subsarcolemmal and myofibrillar, was more diffuse and decreased in intensity by preincubation of sections with RNAase (Fig. 5*f*).

The myofibrillar staining showed a distinctive banding pattern when viewed in longitudinal sections (Fig. 5*c*). Comparison of the banding pattern with a phase-contrast image of the same section showed that the peroxidase staining occurred over the A-bands of the myofibrils. This is illustrated by computer-based image analysis of peroxidase-stained and phase-contrast pictures of the same section in Fig. 6. The location of the peroxidase banding within fibres was marked by dots, stored in the computer memory and then overlaid on the phase-contrast image of the same section; when the two images were aligned, the white dots, corresponding to peroxidase staining, were found to coincide with the A-bands. When transverse muscle sections were labelled with the anti-ribosomal antibody, the myofibrillar staining was evident (Fig. 5*a*) as spots and circles within the fibres; the size of the circles (approx. 1.1 μm) is compatible with the staining being due to ribosomes located around or in the myofibrils (diameter approx. 1 μm). Staining was not observed around all myofibrils within a given fibre, and this is consistent with the periodic staining observed in longitudinal sections, since transverse sections would have been cut through the A band region in only a proportion of myofibrils. Taken together, the results from both longitudinal and transverse sections suggest that, in addition to being present in the perinuclear and subsarcolemmal sarcoplasm, ribosomes are also present in the myofibrillar cytoplasm in a regular fashion.

Table 1. Microdensitometry of immunoperoxidase anti-ribosomal staining of fibres from psoas muscle

Values shown are mean absorbances (470 nm) corrected for non-specific staining and are given as means ± S.E.M. There were six animals in each group. Groups were compared by a two-tailed Student's *t* test: $*P < 0.02$, $**P < 0.005$ for significant difference from 14 day old animals.

Age	Myofibrillar	Subsarcolemmal	Myofibrillar / Subsarcolemmal
14 days	0.412 ± 0.155	1.57 ± 0.37	0.257 ± 0.063
51 days	0.258 ± 0.051	2.74 ± 0.52*	0.095 ± 0.018**

The peroxidase staining of longitudinal sections was quantified by microdensitometry, by using the same basic procedures as Campbell *et al.* (1986) used with glucose oxidase-conjugated antibodies. By using a 1.5 μm mask, absorbance readings of both the subsarcolemmal staining and intermyofibrillar staining were taken. The mean coefficients of variation between fibres within a single section were 22% ± 9 (S.D.) for the myofibrillar staining and 17% ± 7 for the subsarcolemmal, and the mean coefficient of variation between sections within a muscle was 22% ± 5 (myofibrillar) and 17% ± 4 (subsarcolemmal).

With these methods the distribution of ribosomal material in muscle fibres was investigated as a function of age. Psoas muscle was dissected from male rats aged either 14 or 51 days and ribosome distribution studied by immunoperoxidase labelling. The results (Table 1) showed differences in ribosome location between the two groups of animals. The mean myofibrillar staining decreased slightly with age, but this was not statistically significant; however, the subsarcolemmal staining significantly increased by 74% between 14 and 51 days. The ratio of myofibrillar to subsarcolemmal staining declined significantly with age, with a 63% fall between 14 and 51 days of age.

DISCUSSION

On the basis of immunological reactivity, the present results show that muscle ribosomes are found not only in the subsarcolemmal and perinuclear cytoplasm but also in association with the myofibrillar apparatus. Immunostaining of longitudinal sections through the muscle fibres showed a distinct banding pattern in the region of the myofibrils. Comparison of banding patterns obtained with anti-ribosomal antibodies and with phase-contrast microscopy suggest that the ribosomes are associated

with the myosin-containing A-bands. This is consistent with several early observations suggesting that there is an interaction between myosin and ribosomes: histochemical studies found basophilic material, which could be removed by pre-treatment with RNAase and was presumed therefore to be RNA, associated with the A-bands in muscle sections (Clavert *et al.*, 1949). Furthermore, RNA was found associated with myofibrils (Zak *et al.*, 1967) and with isolated muscle myosin (Perry, 1952, 1960; Heywood *et al.*, 1968); in the latter case the RNA had a base composition similar to that of rRNA (Mihalyi *et al.*, 1957). Electron microscopy of muscle sections has also provided some evidence for ribosomes associated with myosin (Larson *et al.*, 1969), although the results have been contradictory. The reasons for the lack of consistent results from electron microscopy are not clear, but may be related to either the difficulty in distinguishing ribosomes from glycogen granules (Galavazi, 1971) or the large amount of electron-dense myofibrillar material.

Taken with the observations from RNA staining and electron microscopy, the present immunohistochemical results provide a body of evidence that ribosomes are associated with myosin-rich regions of the myofibre. Immunostaining of longitudinal sections showed a periodic staining along the myofibrils, whereas results from transverse sections suggested that ribosomal material was present either in the intermyofibrillary spaces in rings around the myofibrils or within the myofibrils. Together, these two patterns suggest that in the intact fibre ribosomes are arranged in a periodic, regular fashion around the A-bands (myosin), probably within the intermyofibrillar space. The location of ribosomes in close proximity to the myofibrils may be of considerable significance, especially if such ribosomes are engaged in the synthesis of myofibrillar proteins such as actin and myosin. It would be logical for these proteins to be synthesized close their site of incorporation into the myofibrils.

It is clearly important to know if the association of ribosomes with the myofibrils is constant, or if it changes with physiological state of the muscle. This question has been addressed by carrying out microdensitometric measurements of the immunostaining in order to quantify the staining distribution within the muscle fibres. In the first instance we have investigated muscles from rats of different ages, and the results demonstrate that as the animal ages the distribution of ribosomal material in the muscle changes. It was observed that older animals tended to have less ribosomal material associated with the myofibrils, but significantly more ribosomes in the sarcolemmal cytoplasm; thus the proportion of ribosomes associated with the myofibrils declined considerably (37%). These changes occur over an age span during which there is an increase in both fibre size and the amount of myofibrillar material. The decrease in myofibrillar staining therefore represents a decrease in ribosomes per unit of myofibrillar material, i.e. effectively a dilution of myofibrillar ribosomes. The increase in subsarcolemmal staining probably reflects the increase in total muscle RNA (mostly ribosomal) over this period. Between 14 and 51 days of age there is a considerable decrease in the overall rate of protein synthesis (Lewis *et al.*, 1984), and the relative proportion of myofibrillar to sarcoplasmic protein synthesis is decreased as growth rate falls (Waterlow *et al.* 1978). Thus at a period of rapid growth and high rates of protein, particularly myofibrillar protein, synthesis (14 days) the proportion of myofibrillar ribosomes is high, whereas at a time of lower rates of protein synthesis and muscle growth (50 days) the proportion is decreased. We hypothesize therefore that the myofibrillar ribosomes are engaged in synthesis of myosin and other myofibrillar proteins, and the ribosome redistribution as the animal ages is associated with lower rates of myosin synthesis.

The fact that changes in ribosome distribution occur with age at a time when protein synthesis is altered suggests firstly that the distribution of ribosomes between different cell compartments in muscle is of physiological significance and secondly that this distribution may be modulated by physiological factors. In recent years it has become apparent that in cultured cells ribosomes actively engaged in protein synthesis (polysomes) are found associated with the structural filaments (cytoskeleton) of cultured cells (Nielsen *et al.*, 1983; Hesketh & Pryme, 1988). Effects of salt and cytochalasin B on polysome distribution suggest that these cytoskeletal-bound polysomes are associated with the actin/myosin microfilaments (Ramaekers *et al.*, 1983; Hesketh & Pryme, 1988). It may be that the ribosomes that we have observed in association with the muscle myosin represent a highly specialized case of 'cytoskeletal-bound' polysomes.

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