ESTIMATION OF THE NUMBER OF DIFFERENT SEQUENCES IN MYOSIN HEAVY CHAIN mRNA PREPARED BY DIFFERENT METHODS

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1. Introduction

The mRNA for the myosin heavy chain sediments at 26 S on sucrose gradients [1–4] and has est. mol. wt 2×10^6 [5]. We have calculated [4] that if the mRNA contained only one sequence of this molecular weight and it was hybridized to a cDNA copy of itself under conditions of mRNA excess the theoretical $R_0t_{1/2}$* would be 2.7×10^{-3}. However, widely different estimates of $R_0t_{1/2}$ have been obtained in practice [2,4,6,7] suggesting there may be a considerable variation in the complexity (the number of different mRNA sequences) of the myosin heavy chain mRNA preparations used.

Myosin heavy chain mRNA was purified [2] from the large polysomes of 14 day embryonic chick skeletal muscle which sediment to near the bottom of a sucrose gradient. Hybridization kinetics suggested that this mRNA was of high complexity ($R_0t_{1/2}$ 5.3 in 50% formamide, 0.4 M NaCl, 0.05 M Hepes (pH 6.8), 0.001 M EDTA, 0.1% SDS at 52°C). However, we showed [4] that these large polysomes synthesised a number of proteins in the 100 000–200 000 mol. wt range which may partly account for the high complexity of the mRNA. It was further demonstrated [4] that more precise fractionation of the large polysomes gave a size-class which synthesized almost exclusively the myosin heavy chain and the mRNA purified from this size class was of low complexity containing only two different sequences ($R_0t_{1/2}$ 6.3×10^{-3} in 0.24 M equimolar Na phosphate (pH ~6.8), 0.001 M EDTA at 70°C).

Total cytoplasmic mRNA of embryonic calf muscle was fractionated on sucrose gradients and the mRNA sedimenting at 26 S was taken and assumed to be myosin heavy chain mRNA [6]. When a cDNA copy of this was hybridized to 26 S RNA from myoblast cultures the $R_0t_{1/2}$ of the reaction was >10 (0.3 M NaCl, 0.03 M Na citrate, 0.05 M EDTA, 0.1% SDS, 65°C). The interpretation of this result is difficult because the cDNA was not hybridized to its own template. Similarly, when 26 S poly(A) containing RNA was isolated from the 80–120 S (myosin mRNP) region of a sucrose gradient fractionation of polysomes and ribosomes derived from 12 day embryonic chick leg, the cDNA back-hybridized with a $R_0t_{1/2}$ of 2.0 ([7]; hybridization conditions as in [4]).

Here, a comparison was made of the sequence complexity, frequency distribution and translation capacities of two preparations of chick myosin heavy chain mRNA, one (MHC mRNA) purified from a size class of large polysomes as in [4] and the other (26 S mRNA) obtained by sucrose gradient fractionation of total cytoplasmic mRNA. The kinetics of hybridization carried out under identical conditions indicated that MHC mRNA contained two different sequences only, while 26 S mRNA also contained two sequences in high concentration (27% of the total) but in addition ~700 sequences in lower concentration. Cross hybridization experiments indicated that the two sequences present in abundance were the same in both preparations. On translation MHC mRNA yielded almost exclusively a polypeptide chain with a molecular weight (200 000) identical to the myosin heavy chain. In contrast, 26 S mRNA contained the templates for the synthesis of a number of proteins in the 100 000–230 000 mol. wt range and only ~36% of

* $R_0t_{1/2}$ is the product of the initial RNA concentration and time at which the reaction is half completed (mol.s.l^{-1})

the protein synthesized was myosin heavy chain. The results therefore suggest that the high complexity of 26 S mRNA was due to the presence of a large number of different sequences coding for other proteins and not to a large number of different myosin heavy chain mRNAs present as fewer copies.

2. Experimental

MHC mRNA was prepared from a size class of polysomes from 14 day chick embryo leg skeletal muscle as in [4].

26 S mRNA was prepared as follows: Leg skeletal muscle from 14 day chick embryos was homogenized in an equal volume of ice-cold 0.25 M KCl, 0.01 M $MgCl_2$, 0.01 Tris–HCl (pH 7.4) containing 50 μg/ml dextran sulphate. The homogenate was centrifuged at 10 000 × *g* for 10 min to remove unbroken tissue, nuclei and mitochondria. Total RNA was extracted from the supernatant (cytoplasmic fraction) as in [8,9]. Poly(A) containing RNA was separated by two passages through an oligo(dT) cellulose column (Collaborative Res., grade T_3) by the method in [10] and fractionated by sucrose gradient centrifugation. The size distribution of the mRNA was determined by hybridizing poly([^{3}H]U) to each fraction of the gradient as in the legend to fig.1. The fractions of the gradient containing 26 S mRNA were pooled as indicated in fig.1a and the RNA was precipitated with ethanol several times. The 26 S mRNA was dissolved in distilled H_2O and kept at −70°C.

mRNA concentrations were estimated by hybridizing poly([^{3}H]U) of known specific activity to the mRNA [11]. This gives an estimate of the amount of poly(A) which, in turn, provides a value for the mRNA concentration assuming that the poly(A) sequence represented 3% of the MHC mRNA molecule [5].

cDNA was prepared as in [4] using 2–6 μg MHC mRNA or 26 S mRNA. All cDNA preparations were sized on alkaline sucrose gradients. Aliquots of cDNA were made 0.2 M NaOH, 0.5% SDS, layered on a 16 ml 5–20% sucrose gradient in the same buffer and centrifuged for 41 h at 24 000 rev./min in the Beckman SW 27 rotor at 24°C. Unlabelled, sonicated mouse DNA of known size was used as a marker. Using both mRNA preparations the cDNA had an average size of ~530 nucleotides (6.5 S).

Hybridization was carried out at 70°C in 0.24 M PEB (equimolar sodium phosphate buffer containing 1 mM EDTA) and the amount of cDNA in the hybrid was measured by resistance to nuclease S1 as in [12].

3. Results and discussion

3.1. *Preparation of 26 S mRNA – comparison with* S *values of MHC mRNA*

Poly(A) containing mRNA was prepared by oligo-(dT) cellulose chromatography of total cytoplasmic RNA extracted from 14 day embryonic chick leg

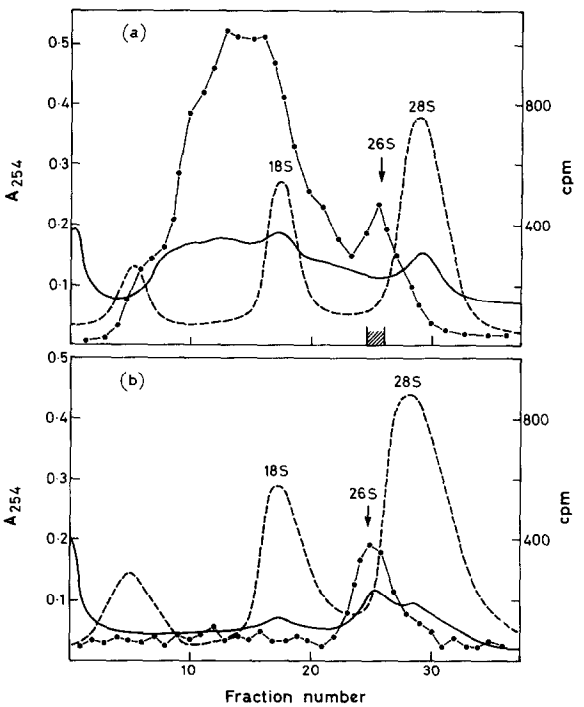

Fig.1. Fractionation of mRNA by sucrose gradient centrifugation and analysis of size distribution by hybridization with poly([^{3}H]U). Poly(A)containing RNA prepared as in section 2 was loaded onto a 16 ml 15–30% sucrose gradient in 0.02 M sodium acetate, 0.5% SDS, 0.005 M EDTA, 0.04 M Tris–HCl (pH 7.8) and centrifuged for 16 h at 25 000 rev./min in the Beckman SW27 rotor at 22°C. The A_{254} was recorded continuously using an Isco ultraviolet absorbance monitor during fractionation of the gradients. Aliquots (200 μl) from each gradient fraction were hybridized directly with poly([^{3}H]U) (spec. act. 2.5 × 10^5 cpm/μg) in poly(U) excess [10]. The samples were precipitated with trichloroacetic acid, collected on nitrocellulose filters and radioactivity determined. (a) Total cytoplasmic poly(A) containing RNA; (b) MHC mRNA. (———) absorbancy of poly(A) containing RNA; (•———•) poly([^{3}H]U) hybridization; (– – –) absorbancy of ribosomal RNA run on parallel gradients.

skeletal muscle and fractionated by centrifugation on a sucrose gradient. The size distribution of the mRNA was determined by hybridizing poly([^{3}H]U) to each fraction of the gradient (fig.1a). The total mRNA had a heterodisperse size distribution ranging from 4–28 S with a discrete shoulder at 26 S. The fractions of the gradient containing the 26 S mRNA were pooled (as indicated in fig.1a) and the mRNA used for in vitro protein synthesis, as a template to prepare cDNA and in hybridization experiments.

The size distribution of MHC mRNA was also determined on an identical sucrose gradient. A single peak of poly(A) containing RNA which hybridized with poly([^{3}H]U) sedimented at 26 S (fig.1b).

3.2. *Determination of complexity of mRNA*

To compare the sequence complexity of 26 S mRNA and MHC mRNA, cDNA copies of each were prepared and hybridized back to a vast excess of the respective template.

When MHC mRNA was hybridized to cDNA, a single sharp transition was obtained characteristic of a homogeneous mRNA population (fig.2) which had an observed $R_0t_{1/2}$ of 6.3×10^{-3} ($\log R_0t$ −2.2). The observed $R_0t_{1/2}$ was approximately double the theoretically expected value (2.7×10^{-3}) suggesting that there were two different sequences in the MHC mRNA preparation. Under identical conditions rabbit globin cDNA and globin mRNA hybridized with a $R_0t_{1/2}$ of 5.3×10^{-4} ($\log R_0t$ −3.27).

In contrast, the curve representing the hybridization of 26 S cDNA to 26 S mRNA extended over ~5 log units (fig.2) indicating a heterogeneous mRNA population. The presence of two transitions in the curve indicated two abundance classes in which different mRNAs were present in different concentrations. The first transition included 27% of the cDNA and had an observed $R_0t_{1/2}$ of 2.4×10^{-2} ($\log R_0t$ −1.6). The

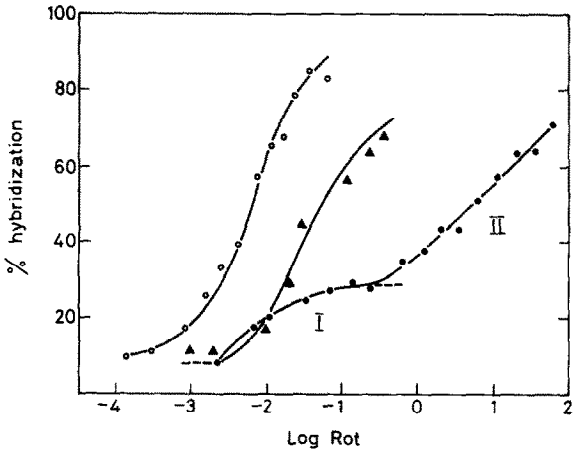

Fig.2. Hybridization of MHC mRNA (o———o) and 26 S mRNA (•———•) to their respective cDNAs. 26 S mRNA was also crosshybridized to the cDNA of MHC mRNA (▲———▲).

second transition included 44% of the cDNA and had an observed $R_0t_{1/2}$ of 4.47 ($\log R_0t$ + 0.65).

The number of different sequences present in these transitions was calculated as in [12]. The $R_0t_{1/2}$ values were first corrected to the values which would be obtained if the RNA responsible for any transition was present on its own (table 1). The corrected values of $R_0t_{1/2}$ for each transition were then divided by the theoretical $R_0t_{1/2}$ 2.7×10^{-3} to determine the number of sequences which were represented in each transition. Table 1 shows that the RNA represented in the first transition consisted of 2 sequences present in high concentration in the 26 S mRNA population, while the RNA represented in the second transition consisted of ~700 sequences present at much lower concentrations.

The first transition had a corrected $R_0t_{1/2}$ of 6.5×10^{-3} which was very close to the $R_0t_{1/2}$ observed

Table 1
Analysis of 26 S mRNA–cDNA hybridization reaction

Transition	Fraction of hybridizable cDNA	Observed $R_0t_{1/2}$	Corrected $R_0t_{1/2}$[a]	Number of sequences[b]
I	0.27	2.4×10^{-2}	6.5×10^{-3}	2.4
II	0.44	4.47	1.97	729

[a] Corrected $R_0t_{1/2}$ = Observed $R_0t_{1/2}$ × fraction of hybridizable cDNA
[b] The number of sequences of av. mol. wt 2×10^6 were obtained by dividing the corrected $R_0t_{1/2}$ value by the theoretically expected $R_0t_{1/2}$ of 2.7×10^{-3} [4]

for the MHC mRNA–cDNA hybridization reaction suggesting that the first transition represented the hybridization of MHC mRNA sequences. That this was the case was confirmed when it was shown that the cDNA of MHC mRNA crosshybridized with the abundant class mRNA sequences of the 26 S mRNA (fig.2) with an observed $R_0t_{1/2}$ of 3.02×10^{-2} (log R_0t −1.52), reasonably close to the expected value of 2.4×10^{-2} ($\log R_0t$ −1.6). Thus crosshybridization indicated that the abundant mRNA sequences were the same in both mRNA preparations. Dividing the $R_0t_{1/2}$ of the MHC mRNA–cDNA hybridization reaction (6.3×10^{-3}) by the $R_0t_{1/2}$ of the crosshybridization reaction (3.02×10^{-2}) gives the amount of MHC mRNA present in the 26 S mRNA preparation expressed as a fraction: 0.21 or 21%. This value is in reasonable agreement with the estimate of 27% obtained from the hybridization kinetics of 26 S mRNA to its own cDNA (fig.2).

3.3. *Translation in the wheat germ cell-free system*

Preparations of 26 S mRNA and MHC mRNA were translated in the wheat germ system. Translation was in the presence of 150 mM KCl since elevated KCl concentration favours the complete translation of high molecular weight mRNAs [4]. 26 S mRNA stimulated incorporation of [^{3}H]leucine into trichloroacetic acid-precipitable material to the same extent as MHC mRNA. The total translation products were analyzed by electrophoresis on SDS–polyacrylamide gels (fig.3). Over 90% of the radioactive protein synthesized by MHC mRNA co-electrophoresed with the marker myosin heavy chain (fig.3a).

In contrast, only ~35% of the radioactive protein synthesized by 26 S mRNA co-electrophoresed with the marker (fig.3b). The other proteins synthesized by 26 S mRNA were in the 100 000–230 000 mol. wt range (fig.3b). The 200 000 mol. wt polypeptide synthesized by MHC mRNA had properties identical to the myosin heavy chain [4].

There was reasonable agreement between the amount of myosin heavy chain synthesized in vitro (35%) and the proportion of the mRNA attributed to myosin heavy chain mRNA sequences (27%). It seems that the high complexity of 26 S mRNA was due to the presence of many sequences coding for other proteins and not due to the presence of a large number of myosin heavy chain mRNA sequences.

The results of this investigation in conjunction with [4] suggest that there are two preponderant MHC

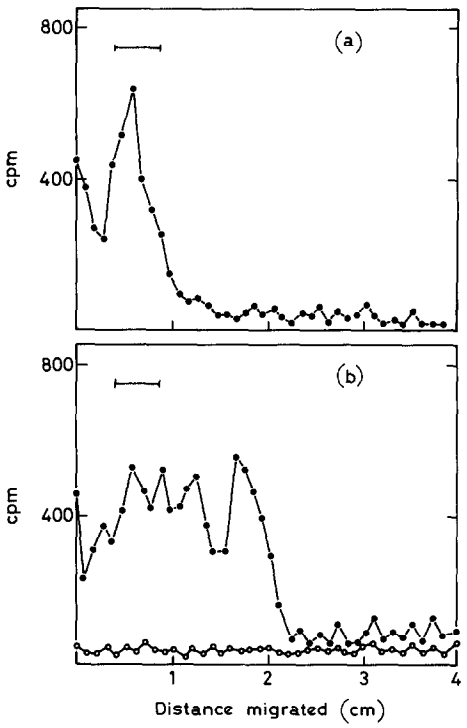

Fig.3. Direct analysis on SDS–polyacrylamide gels of the translation products of mRNA. 0.9 μg of each mRNA preparation was translated in the wheat germ cell-free system [12] as in [4]. The incubation mixture contained 25 μM unlabelled amino acids excluding leucine and 5 μCi [^{3}H]leucine (50 Ci/mmol). For direct analysis of translation products by electrophoresis the incubation mixture was added to 100 μl 12 M urea, 0.05 M sodium phosphate (pH 7.0), 1.5% SDS, 1.5% β-mercaptoethanol containing 50 μg chicken skeletal myosin as a marker and electrophoresed on SDS–polyacrylamide gels and stained as in [13]. Gels were sectioned into 1 mm slices and each slice was dissolved by incubation in 0.5 ml 30% H_2O_2 at 50°C for 16 h. After adding 10 ml Aquasol scintillation medium the radioactivity was determined in a Packard liquid scintillation counter. The position and width of the myosin heavy chain marker run on the same gel is indicated by the horizontal bar. (a) MHC mRNA; (b) 26 S mRNA – incubation mixture with (•———•) and without mRNA (o———o).

mRNA sequences present in 14 day embryonic chick leg skeletal muscle. Rat muscle myosin heavy chain mRNA has been prepared from 26 S mRNA by two rounds of sucrose density gradient centrifugation followed by fractionation on an agarose/polyacrylamide gel [15]. When the RNA was used to prepare cDNA and the cDNA was hybridized back to the template the $R_0t_{1/2}$ was $\sim 5 \times 10^{-3}$ indicating a nucleotide sequence complexity very similar to chicken MHC mRNA.

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