Changes in cytoplasmic terminal transferase activities during chick embryo skeletal muscle development

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1. INTRODUCTION

Ribonucleotidyl transferases are a group of primer-dependent terminal transferases, of nuclear and cytoplasmic origin, that have been known to exist for many years [1–6]. The best known perhaps are the cytoplasmic tRNA nucleotidyl transferases, shown some time ago to be responsible for the addition of the 3′-terminal trinucleotide CCA to tRNA [7,8]. Poly(A)polymerase activities have also been described and shown to play a role in the polyadenylation of newly synthesized pre-mRNA [9–11].

We have been concerned with a terminal transferase activity in the ribosome fraction of maturing avian erythrocytes responsible for the synthesis of polymers of oligo(U) [12]. We have shown the product of this enzyme activity to be present in the cytoplasm of intact cells [13], but the enzyme is not related to cytoplasmic tRNA nucleotidyl transferase [14]. This lends considerable support to the idea that this terminal transferase is not an artefact of isolation or assay. What then is the function of this enzyme? We have previously provided correlative evidence [12] suggesting that such a ribosome-associated terminal transferase may play a role in the synthesis or control of synthesis of globin, during the development of the immature avian erythrocyte. In order to investigate a possible translational control function more directly we have continued these studies using developing embryonic chick skeletal muscle.

The development of mononucleated myoblasts to form nonproliferating myotubes has been extensively studied in terms of the synthesis of muscle-specific proteins and the translational regulation of this synthesis [15,16]. It represents therefore a suitable system for the analysis of primer-dependent terminal transferases and their possible role in regulating differential mRNA availability and translation.

This paper reports the presence of cytoplasmic terminal transferases in developing chick embryo skeletal muscle and the changes in the activities of these polysome- and 80 S-associated terminal transferases during myoblast development in vivo.

2. MATERIALS AND METHODS

The sources of many of the chemicals used have been mentioned previously [12,13,17]. Ribonuclease-free sucrose was purchased from Bethesda Research Laboratories; unlabelled uridine and ribonucleotide monophosphates from Miles Laboratories. Acrylamide and bis-acrylamide (electrophoresis grade) were purchased from Bio-Rad and Instagel from Packard Instruments.

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2.1. *Preparation of skeletal muscle subcellular fractions*

Post-mitochondrial supernatant fractions were prepared from 12-day through to 14-day old chick embryo leg muscle as in [18], except that the leg muscle was dissected out and homogenized in an equal volume of 0.15 M KCl, 10 mM $MgCl_2$, 20 mM Tris–HCl (pH 7.4), 5 mM 2-mercaptoethanol. Total ribosomes were prepared by centrifugation of the clear post-mitochondrial supernatant through 36% (w/w) sucrose in the same buffer (160500 × *g* for 90 min, Beckman 60 Ti rotor). (The term 'total ribosomes' refers specifically to a pellet obtained from a post-mitochondrial supernatant that contains both polyribosomes and single ribosomes or monosomes.) Pellets were suspended in 50 mM Tris–HCl (pH 7.4), 1.0 mM dithiothreitol, 0.25 M sucrose (TSD buffer). For the analysis of terminal transferase activity during embryonic development, polysome and 80 S particles were isolated by centrifugation of post-mitochondrial supernatants through 15–30% sucrose gradients as in [19]. The collected pellets were suspended in TSD buffer and all fractions when not required were stored under liquid nitrogen.

2.2. *Assay of terminal ribonucleotidyl transferase activity*

Subcellular preparations were assayed for terminal transferase activity as in [12] except that, unless otherwise stated, no added RNA was included in the incubation mixture. In addition, [^{3}H]-ribonucleotides were present at a spec. act. of 1200 μCi/μmol (final conc. 8.2 μM). For preparative isolation of newly-synthesized radioactively-labelled RNA, the 0.25 ml incubation mix was scaled up 10-times; incubations were carried out for 60 min at 37°C and RNA extracted with phenol as in [20], except that the final aqueous phase, prior to the addition of ethanol, was made 0.2 M with respect to Tris–HCl (pH 7.4).

2.3. *Polyacrylamide gel electrophoresis*

Washed and drained RNA pellets were dissolved in a solution containing 10 mM Tris–HCl (pH 7.4), 0.5% (w/w) SDS and 10% (v/v) glycerol and applied to 2.6% polyacrylamide disc gels prepared as in [21]. Electrophoresis was carried out at 5 mA/gel for 90 min. Gels were scanned at 254 nm using a Unicam SP1700 UV spectrophotometer equipped with gel scanner.

3. RESULTS

Embryonic chick skeletal muscle contains a cytoplasmic terminal transferase activity catalyzing the incorporation of UTP and CTP into trichloroacetic acid-precipitable material. Table 1 illustrates the relative incorporation of the 4 [^{3}H]triphosphates, in the presence of unlabelled ribonucleotides. This terminal transferase,

Table 1

The incorporation of [^{3}H]ribonucleotides by a cytoplasmic ribonucleotidyltransferase

Ribonucleotides present				Incorp. (dpm)	Rel. act. (%)
[^{3}H]UTP	CTP	ATP	GTP	33726	100
UTP	[^{3}H]CTP	ATP	GTP	45195	134
UTP	CTP	[^{3}H]ATP	GTP	762	2
UTP	CTP	ATP	[^{3}H]GTP	1581	5

Polysomes (0.27 mg) were included in each reaction mixture, prepared as described in section 2, from day 12 embryonic skeletal muscle. [^{3}H]ribonucleotides were used at a spec. act. of 1200 μCi/μmol and a final concentration of 8.2 μM; unlabelled ribonucleotides were present at a concentration of 0.16 mM. No additional RNA was added. A zero time of incorporation of 740 dpm has been subtracted to obtain the values presented

recoverable in the total ribosome fraction, was identical (with respect to cation requirements, stimulation by exogenously added RNA and incorporation of individual [^{3}H]ribonucleotides) to a similar enzyme activity described by us [12], found in maturing avian erythrocytes. To determine whether the incorporation of UTP and CTP was due to hetero- or homopolymer synthesis, we conducted a series of nearest neighbour experiments using [α-^{32}P]ribonucleotides. Table 2 shows, using [α-^{32}P]UTP, the recovery of most of the incorporated radioactivity as UMP, indicating oligo(U)homopolymer synthesis. The recovery of low amounts of radioactivity as CMP, AMP and GMP suggests either a small amount of heteropolymer synthesis or, more likely, heterogeneity in the terminal primer nucleotide. Incorporation of [α-^{32}P]CTP, by a total ribosome preparation, into RNA, demonstrated the recovery, after alkaline hydrolysis, of most of the radioactivity as CMP, suggestive of oligo(C)homopolymer synthesis. Further, thermal denaturation studies assaying the incorporation of [^{3}H]UTP and [^{3}H]CTP, indicated two separate terminal transferases present in this total ribosome preparation from skeletal muscle (fig.1). The incorporation of [^{3}H]UTP is catalyzed by a heat-labile enzyme whereas [^{3}H]CTP incorporation continues even after pre-incubation for 20 min at 53°C. These findings are similar to data in [14] and

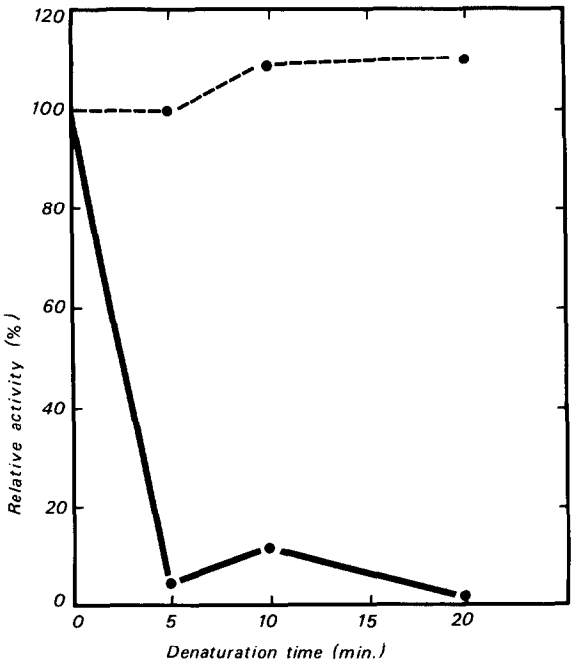

Fig.1. Rates of heat inactivation of cytoplasmic terminal transferases in embryonic chick skeletal muscle. Total ribosomes prepared from either day 12 or day 15 embryonic muscle were incubated at 53°C for up to 20 min. During the incubation, aliquots were removed and assayed for incorporation of either [^{3}H]UTP or [^{3}H]CTP into trichloroacetic acid-precipitable material. The results are expressed as a percentage activity, relative to untreated total ribosome preparations: (●---●) [^{3}H]CTP incorporation; (●——●) [^{3}H]UTP incorporation.

Table 2

Distribution of radioactivity in ribonucleotides obtained after alkaline hydrolysis of RNA isolated from total ribosomes incubated with [α-^{32}P]UTP or [α-^{32}P]CTP

[α-^{32}P]Ribonucleotide	Recovered radioactivity (% of total)			
	CMP	AMP	GMP	UMP
CTP	80	14	5	1
UTP	11	10	10	69

Total ribosomes were incubated with either of the [α-^{32}P]ribonucleotides (final concentration of each of 4 μM; 2 Ci/mmol), in the presence of the other 3 unlabelled ribonucleotides (0.16 mM each). RNA was prepared by phenol extraction (section 2). Alkaline hydrolysis and the separation and recovery of hydrolysis products was done as previously described

further strengthen the suggestion that the incorporation of [^{3}H]UTP by embryonic skeletal muscle is mediated by a terminal transferase similar to the activity found in maturing avian erythrocytes.

Analysis of the hydrolysis products obtained after alkali treatment of RNA extracted from total ribosomes incubated with [^{3}H]UTP, revealed a UMP/uridine ratio (non-terminal/terminal incorporation of UTP) of about 2 (table 3). Further, polyacrylamide gel electrophoresis of total RNA extracted from total ribosomes previously incubated with [^{3}H]UTP showed a single peak of radioactivity co-incident with the 3.8 S region of the gel (fig.2). The product of this terminal transferase therefore is a short oligo(U)homopolymer attached to a low-M_r RNA primer.

Table 3

Distribution of radioactivity in ribonucleosides and ribonucleotides after alkaline hydrolysis of newly-synthesized labelled total RNA

Hydrolysis products	Radioactivity (%)	Nucleotide / Nucleoside
UMP	69	2.23
Uridine	31	
GMP, AMP, CMP	0	

Electrophoretic analysis and the determination of radioactive distribution were carried out as in [13]. The recovery of radioactivity from the thin-layer plates was usually 90–95%

In order to begin to investigate possible functions for such a cytoplasmic terminal transferase in modulating protein synthesis at the level of translation, as our previous studies in maturing erythrocytes had suggested [12], we separated 80 S particles (containing ribosomes and presumptive myosin messenger ribonucleoprotein particles, mRNPs) from polysomes, on sucrose gradients, derived from skeletal muscle, obtained from day 12 through to day 15 embryonic chicks. It has been shown [22] that skeletal muscle undergoes fusion

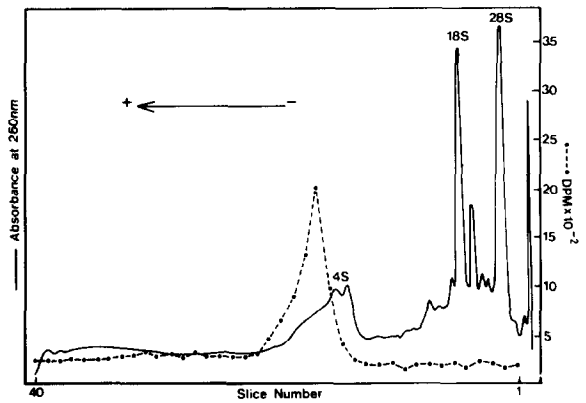

Fig.2. SDS–polyacrylamide gel electrophoresis of newly-synthesized radioactively-labelled total RNA. Electrophoresis of unfractionated radioactively-labelled total RNA on 2.6% polyacrylamide gels was done as in section 2. 1.4 A_{260} units (7500 dpm) were applied to each gel and the samples electrophoresed for 90 min at 5 mA/gel. Gels were scanned, without staining, at 260 nm and sliced. The slices were dried, oxidized in a Packard model 306 sample oxidizer and counted.

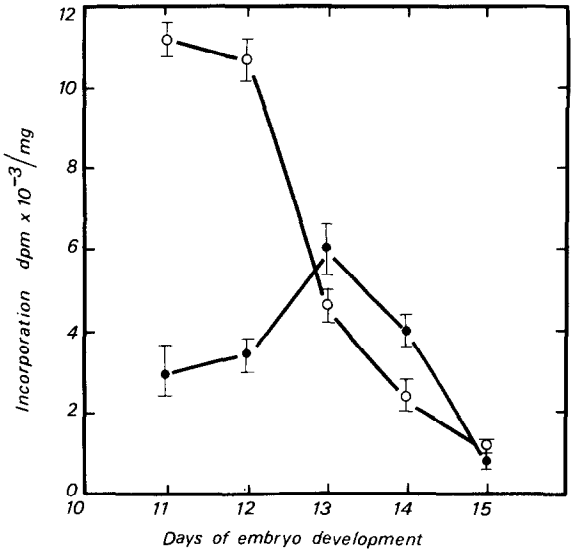

Fig.3. Ribonucleotidyl transferase activities in 80 S and polysomal fractions from day 12 to day 15 in embryonic chick skeletal muscle development. 80 S and polysome fractions were assayed for UTP–RNA uridilyltransferase activity as described in section 2. Results are expressed as incorporation of [^{3}H]UTP, into trichloroacetic acid-precipitable material/mg polysomal or 80 S material. A_{260}/A_{280} ratios were measured and shown to be constant in all 80 S and polysome preparations, obtained from developing chick embryonic muscle. Further, the yields of total ribonucleotidyl transferase activities present in the post-mitochondrial supernatant and subsequently recovered in the 80 S and polysome fractions, were shown to remain constant during embryonic muscle development. (○—○) 80 S fraction; (●—●) polysome fraction.

to form multinucleated myotubes during this period of skeletal muscle development. The 80 S and polysome fractions were assayed for terminal transferase activity ([^{3}H]UTP incorporation) and the data are summarized in fig.3. Both fractions contain a terminal transferase activity catalyzing the incorporation of [^{3}H]UTP into trichloroacetic acid-precipitable material. Whilst there is an overall decrease in the polysome-associated ribonucleotidyl transferase activity during skeletal muscle development, a marked 10-fold decrease in the 80 S-associated activity occurs during the same period. As the recovery of overall ribonucleotidyl transferase activities from post-mitochondrial supernatants remains constant, this decrease in polysome-associated and particularly 80 S-

associated enzyme activities during embryonic skeletal muscle development clearly represents a decrease in specific enzyme activity and not selective loss of enzymatic moieties from polysomes or 80 S RNP particles as a consequence of isolation.

4. DISCUSSION

A cytoplasmic UTP–RNA uridilyltransferase (EC 2.7.7) [6] in chick embryonic skeletal muscle has been described. It catalyzes the primer-dependent synthesis of short oligo(U)homopolymers. The primer has been identified as low-M_r RNA (less than 4 S). Small nuclear and cytoplasmic RNAs have been the subject of considerable investigation in recent years [23–27]. Some of these RNAs (tcRNA and iRNA) have been implicated as being involved in mediating the availability of translationally active mRNA. Many of them are also rich in oligo(U) sequences. The present data allow us to speculate that if such low-M_r RNAs are indeed involved in translational control, that they in turn may be subject to modification by a cytoplasmic terminal transferase. This hypothesis is further supported by the presence of a UTP–RNA uridilyltransferase in the 80 S fraction of embryonic skeletal muscle (fig.3). This 80 S preparation contains both monosomes (single ribosomes) and myosin mRNPs [28]. We separated these two components on 40% (w/w) metrizamide gradients and found in confirmation of earlier, but as yet unpublished, observations (Bester, A.J. and Durrheim, G.), that the 80 S mRNP particle disappeared during embryonic skeletal muscle development, to the extent that by day 16, no detectable RNP particles could be recovered by metrizamide centrifugation. Whilst we were not successful in recovering terminal transferase activity in pre-fusion 80 S mRNP particles after metrizamide centrifugation, newly-synthesized oligo(U)-homopolymer could be recovered co-incident with 80 S RNP particles in metrizamide gradients. This strongly suggests that a terminal transferase in the 80 S fraction is specifically associated with the 80 S RNP particle during skeletal muscle development. The relationship between the modulation in polysome-associated ribonucleotidyl transferase and the decrease in 80 S-associated activity is not at all clear. Further work would be necessary to substantiate a more detailed role that this enzyme might have during the mobilization of translationally active myosin mRNA from RNP particle to polysome, a mechanism suggested to occur during myoblast fusion [23].

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