Changes in mRNA length accompany translational regulation of the somatic and testis-specific cytochrome c genes during spermatogenesis in the mouse

LAURA E. HAKE, ACACIA A. ALCIVAR and NORMAN B. HECHT*

Department of Biology, Tufts University, Medford, MA 02155, USA

*To whom reprint requests should be addressed

Summary

The mouse testis contains two isotypes of cytochrome c, which differ in 14 of 104 amino acids: cytochrome c§ is present in all somatic tissues and cytochrome cT is testis specific. The regulation of cytochrome c§ and cytochrome cT gene expression during spermatogenesis was examined by Northern blot analysis using specific cDNA probes. Total RNA was isolated from adult tissues, enriched germinal cell populations and polysomal gradients of total testis and isolated germinal cells. Three cytochrome c§ mRNAs were detected averaging 1.3 kb, 1.1 kb and 0.7 kb in all tissues examined; an additional 1.7 kb mRNA was observed in testis. Isolated germinal cells through prepuberal pachytene spermatocytes contained only the three smaller mRNAs; the 1.7 kb mRNA was enriched in round spermatids. All three smaller cytochrome c§ mRNAs were present on polysomes; the 1.7 kb mRNA was non-polysomal. Cytochrome cT mRNA of 0.6–0.9 kb was detected in testis; mRNA levels were low in early spermatogonia and peaked in prepuberal pachytene spermatocytes. In adult pachytene spermatocytes, a subset of the cytochrome cT mRNAs, 0.7–0.9 kb, was present on polysomes; a shortened size class, 0.6–0.75 kb, was non-polysomal. A distinct, primarily non-polysomal, cytochrome cT 0.7 kb mRNA was present in round spermatids. These results indicate that (1) both cytochrome c§ and cytochrome cT mRNAs are present in early meiotic cells, (2) a 1.7 kb cytochrome c§ mRNA is post-meiotically expressed and non-polysomal and (3) cytochrome c§ and cytochrome cT mRNAs are each developmentally and translationally regulated during spermatogenesis.

Key words: mRNA, translational regulation, cytochrome c, testis-specific gene, spermatogenesis, mouse.

Introduction

Cytochrome c is a nuclear encoded mitochondrial protein that functions in the transport of electrons in the mitochondrial intermembrane space (Petitgrew and Moore, 1987). The regulation of cytochrome c gene expression has been most extensively studied in Saccharomyces cerevisiae. Transcriptional control of cytochrome c expression appears to be mediated by interaction of cis- and trans-acting factors in the flanking region of the gene in response to physiological stimuli such as heme, glucose and oxygen (Prezant et al. 1987; Cerdan and Zitomer, 1988). In mammalian systems, a similar analysis of rat somatic cytochrome c gene regulation has identified necessary cis-acting promoter elements (Evans and Scarpulla, 1989). Transcriptional induction of cytochrome c in mammals has been observed only in response to thyroid hormone, which is known to increase metabolic activity (Scarpulla et al. 1986). Post-transcriptional regulation of cytochrome c has been demonstrated in the Trypanosoma brucei life cycle (Torri and Hajduk, 1988) and in the increased half-life of cytochrome c mRNA in response to thyroid hormone in rat (Scarpulla et al. 1986).

Isozymic variants for several nuclear encoded mitochondrial proteins exist in several different organisms and tissues (reviewed in Kadenbach et al. 1987). Little is known of the physiological significance of these isoforms or how they are differentially regulated to provide the appropriate amount of each gene product to the mitochondrion. The best characterized example in eucaryotes is the regulation of two cytochrome c genes in yeast. In this case, the differential activation of these two genes appears to arise from the interaction of identical trans factors with variant cis-acting elements (Prezant et al. 1987; Cerdan and Zitomer, 1988). In mammalian systems, this issue has not been addressed.

The mammalian testis contains two isoforms of cytochrome c which have distinct distributions in spermatogenic cells. In the mouse, the somatic form of cytochrome c, cytochrome c§, has been localized by immunofluorescence to interstitial cells, Sertoli cells...
and early germ cells of the adult mouse testis (Goldberg et al. 1977). The testis-specific cytochrome c, cytochrome cTS (Hennig 1975), has been localized in all the later, more differentiated germ cell stages beginning with mid-pachytenic spermatocytes (Goldberg et al. 1977).

The existence of these isofoms provides a unique opportunity to study the coordinate regulation of two genes that code for proteins that are presumed to be functionally similar, yet are present in different testicular compartments. Through cell separation techniques, it has been possible to examine gene expression in each of the three phases of spermatogenesis: spermatogonial renewal/proliferation, meiosis and spermiogenesis (Bellvé, 1979). These analyses have led to the identification of unique modes of gene regulation (reviewed in Hecht, 1986, 1990).

The primary goal of this investigation is to examine the regulation of the two cytochrome c genes during mouse spermatogenesis. In this paper, we utilize cDNA clones encoding the somatic and testis-specific forms of cytochrome c to characterize cytochrome c gene expression in mouse tissues and throughout mouse spermatogenesis. We also examine the translational status of cytochrome c mRNAs in various spermatogenic developmental stages. We conclude that translational regulation of both genes occurs during spermatogenesis and is accompanied by changes in mRNA length.

Materials and methods

Animals and chemicals

CD-I mice were obtained from Charles River Laboratory, Inc. (Wilmington, MA). Laboratory reagents were obtained from the sources described previously (Alcivar et al. 1989). Additional reagents were obtained from the following sources: magnesium chloride, sucrose and Heps from Sigma (St Louis, MO): oligo d(T)12-18 from Collaborative Research Inc. (Bedford, MA).

Isolation and identification of cytochrome cS and cT cDNA clones

The cytochrome cS cDNA was isolated by plaque hybridization (Maniatis et al. 1982) with a 1 kb rat genomic cytochrome cS clone (Scarpulla et al. 1981) from a lambda gt-10 adult CD-1 mouse testis cDNA library generously provided by Dr K. Kleene. A 679 base pair lambda gt-10 insert from a positively hybridizing clone was subcloned into the EcoKl site of the M13 mpl8 cloning vector. Nucleotide sequencing was performed by the Sanger dideoxy chain termination method (Sanger et al. 1977) following the methodology described in the BRL M13 Cloning and Sequencing Manual.

A cDNA coding for cytochrome cT was obtained by screening the same library with the cytochrome cS cDNA under conditions of reduced stringency (the same conditions as described below for Northern blot hybridization, with the second wash temperature at 50°C). 24 positive clones were obtained after screening 20000 plagues. Inserts from positive clones that hybridized with less intensity after washing at 65°C when compared to control cytochrome cS blots were subcloned into the EcoRI site of the M13 mpl8 sequencing vector. In order to obtain the sequence of the second strand and a probe suitable for Northern blot analysis, the poly(A)+ tail of one of the two positive clones was removed by unidirectional digestion with exonuclease III (BRL, Inc.) as described previously (Kim et al. 1989).

Purification of total RNA

Total RNA was purified from adult mouse tissues (whole blood, brain, epididymis, heart, intestine, kidney, liver, lung, seminal vesicle, spleen) and the testes of prepuberal mice at days 6, 8, 12, 13, 14, 15, 16, 18, 20, 22, 30 and 45 after birth by methods described previously (Alcivar et al. 1989).

Hybridization and labelling conditions

Northern blot analysis was performed as described previously (Alcivar et al. 1989) with the following modifications: RNA was electrophoresed in 1.5 or 2% agarose/formaldehyde gels and the gels were subsequently denatured in 50 mM NaOH: 10 mM NaCl for 1 h at 25°C and neutralized in 0.1 M Tris pH 7.5 before blotting. Probes containing full-length cytochrome cS and cytochrome cT cDNAs were used in Northern blot analysis and no cross hybridization was observed because of the relatively low nucleotide similarity between the cytochrome cS and cytochrome cT genes (approx. 75%). The lacZ dehydrogenase probe was a random prime labeled insert from pLDH-R8 obtained from Dr Yun-fai Lau (Wu et al. 1987). For screening of libraries, the plaque lifts on nitrocellulose filters were processed as described by Maniatis et al. (1982). Prehybridization of the filters for screening procedures and Northern blots was performed by incubation at 65°C in a prehybridization solution containing 0.40 M phosphate buffer, pH6.5, 5×Denhardt's solution (1×Denhardt's=0.02% bovine serum albumin, 0.02% polyvinyl-pyrrolidone, 0.02% Ficoll), 1% SDS and 50–100 μg single-stranded salmon sperm DNA for 2–4 h (Alcivar et al. 1989). Hybridization was performed in fresh prehybridization buffer with either nick-translated or random-prime labeled probes (Random Primers DNA Labelling System, BRL, Bethesda, MD.) with specific activities ranging from 1–10×106 cts min⁻¹ μg⁻¹ for 12–16 h at 65°C. Following hybridization, filters were washed two times for 15 min each at 24°C in 0.25 M phosphate buffer with 0.1% SDS and then two times for 30 min each at 65°C in 0.4 M phosphate buffer with 0.1% SDS. Filters were exposed to X-Omat AR Kodak film (Eastman Kodak, Rochester, NY) with an intensifying screen for 2–8 days.

To determine the integrity of the RNA used in Northern blots, control hybridizations were performed with a probe for 18s ribosomal RNA obtained from Dr N. Arnheim.

Purification of spermatogenic cells

Isolated populations of spermatogenic cells from the testes of days 8, 17 and adult CD-1 mice were obtained by the gravity sedimentation procedure (Romrell et al. 1976; Meistrich, 1977; Bellvé et al. 1977). The cell number and the purity of the fractions used in Northern blot analysis are as follows: type A spermatogonia – 4.6–7×10⁶ cells, 76–90% pure; type B spermatogonia – 1.4–8×10⁶ cells, 78–95% pure; preleptotene spermatocytes – 2.7–3.2×10⁶ cells, 85% pure; leptotene/zygote spermatocytes – 3.5–5.9×10⁶ cells, 80–90% pure; preleptotene pachytene spermatocytes – 3.1–4.3×10⁶ cells, 75–88% pure; pachytene spermatocytes – 7–28×10⁶ cells, 80–90% pure; round spermatids – 6.7–28×10⁶ cells, 88–95% pure; elongating spermatids – 8.2–10×10⁶ cells, 40% pure; residual body/cytoplasm – 5.5–8×10⁶ cells, 77–92% pure. RNA was purified from the isolated cells by the guanidium isothiocyanate/CsCl procedure as previously described (Alcivar et al. 1989).
Total testis polysomal gradients

Polysomal gradients were established essentially as described by Kleene et al. (1984) with minor modifications. Testes from three mice were decapsulated and homogenized at 4°C with 10 strokes of a motor driven teflon homogenizer in 3 ml of MgCl₂ buffer (20 mM Hepes pH 7.6, 100 mM NaCl and 1.5 mM MgCl₂). Control fractions were isolated by replacing the MgCl₂ in the resuspension solution with 10 mM EDTA, to release the polysomes (Penman et al. 1968). Post-mitochondrial supernatants, obtained after centrifugation at 5000 revs min⁻¹ for 10 min at 4°C, were layered over a 33 ml 10-35% sucrose gradient with a 2 ml 60% sucrose cushion. The gradients were centrifuged in a Beckman SW28 rotor for 3 h at 28000 revs min⁻¹ at 4°C. When the rotor decelerated to 10000 revs min⁻¹, the brake was turned off and the rotor coasted to a stop. Six fractions from each gradient were collected with an ISCO fraction collector and the absorbance at 250 nm was measured. RNA was isolated using one of the following methods: (1) one extraction with 1:1 phenol:chloroform mixture followed by three extractions with chloroform and EtOH precipitation, or (2) collection of the gradient fractions directly into 2.5 volumes of EtOH containing 0.3 M sodium acetate. The precipitate was recovered by centrifugation at 10000 revs min⁻¹ for 30 min, resuspended in 3.5 ml of guanidine isothiocyanate buffer and RNA was isolated as previously described (Alcivar et al. 1989). 1% of the RNA from each gradient fraction was analyzed by Northern blots (Alcivar et al. 1989).

Polysomal gradients of purified spermatogenic cells

Pellets of germinal cells isolated as described above were resuspended in 1 ml MgCl₂ buffer. For the pachytene spermatocyte Mg²⁺ gradients, the cell number was 1.2-1.4 x 10⁸; n = 3. The round spermatid Mg²⁺ gradients were performed with 8-9.7 x 10⁶ cells; n = 3. One-quarter to one-third of the cells used in the Mg²⁺ gradients were used in control gradients in the presence of EDTA. The cell suspensions were homogenized in the presence of 0.5% Nonidet P-40 with 5 strokes of a hand-driven Dounce homogenizer. The post-mitochondrial supernatant, obtained after centrifugation at 10000 revs min⁻¹ for 5 min, was then layered on a 10.5 ml 10-35% sucrose gradient with a 2 ml 60% sucrose cushion. The gradient was centrifuged for 2 h at 35000 revs min⁻¹ in a Beckman SW41 rotor and 2 ml fractions were collected. RNA was isolated as described above. Fifty percent of the RNA from each gradient fraction was analyzed by Northern blot (Alcivar et al. 1989).

RNAse H analysis

500 picomoles of oligo dT were hybridized to 15 μg RNA from total testis polysomal gradient fractions and from isolated adult spermatogenic cells. The oligo dT-poly A tail hybrids formed were digested from the mRNAs by using 1 unit of RNAse H/50/60 reaction (Sherman et al. 1984). After digestion, the resulting RNAs were analyzed by Northern blot as described above.

Results

Characterization of cDNAs

Somatic and testis-specific cytochrome c cDNA clones were identified by comparison of their nucleotide sequences with the published sequence for the mouse somatic genomic clone (Wu and Limbach, 1985) and the DNA sequences for a rat genomic cytochrome c⁷ and a mouse cytochrome c⁷ cDNA (Virbasius and Scarpulla, 1988). Comparisons of the protein and nucleic acid sequences for mouse cytochrome c⁷ and cytochrome c⁷ as well as a comparison of the rat cytochrome c⁷ gene with the mouse cytochrome c⁷ cDNA have been published (Hennig, 1975; Virbasius and Scarpulla, 1988). Two clones were identified as the testis-specific cytochrome c. Sequence comparison of our mouse cytochrome c⁷ cDNA with the cDNA isolated by Virbasius and Scarpulla (Fig. 1) revealed that our cDNA clone begins at nucleotide 63 of their sequence and an adenine is substituted for a cytosine at base 75 of their cytochrome c⁷ cDNA. The sequence of the remainder of the 3' untranslated region, the entire coding region and half of the 3' untranslated region in cytochrome c⁷ cDNAs from both laboratories are identical. However, beginning at base 502 in our cDNA, the 3' untranslated regions of the two cDNAs differ substantially. Comparison of the region of discrepancy to the rat genomic testis-specific cytochrome c⁷ reveals that our cytochrome c⁷ cDNA is very similar to the rat 3' UT whereas the cytochrome c⁷ cDNA sequence reported by Virbasius and Scarpulla (1988) is highly divergent in this region. The clones isolated in

Fig. 1. Nucleotide sequence of mouse cytochrome c⁷. The sequence is numbered beginning with the first base of clone pmrcc.2. The rest of the sequence is identical for both cDNA clones (pmrcc.1 and pmrcc.2). 5' and 3' untranslated regions are in lower case and the coding region is in capital letters. The ATG translation start is underlined. The putative poly(A) + addition sequence is overlined and underlined. The poly(A) + tail of 90 nucleotides is denoted by Aₙ. The sequence corresponding to the 3' untranslated region of a cDNA clone isolated by Virbasius and Scarpulla (1988) numbered according to their published sequence is shown below our sequence beginning at the translation stop codon at base 441.
this study also contained poly(A)+ addition sequences and portions of poly(A)+ tails, suggesting that they represent copies of functional cytochrome cT mRNAs.

**Tissue distribution of cytochrome cS and cT transcripts**

To examine the expression of cytochrome cS and cytochrome cT transcripts in somatic tissues, Northern blot analysis was performed on RNA samples obtained from various tissues from adult mice utilizing the cytochrome cS and cytochrome cT cDNAs as probes (Fig. 2). In Fig. 2A three cytochrome cS mRNAs of approximately 0.5–0.7, 1.1 and 1.3 kb are detected in most of the tissues examined; an additional transcript of 1.7 kb is detected only in the testis upon longer exposure of the gel. The quantity of cytochrome cS transcript in the different tissues is highly variable, with the highest mRNA level observed in heart and the lowest in whole blood and lung. These variations may reflect differences in the respiratory capacities of the various tissues. The cytochrome cT cDNA hybridizes to a heterogeneous 0.6–0.9 kb transcript in testis (Fig. 2B). This transcript was not detected in any of the other tissues examined, even after longer exposure of the filter. The same Northern blot, when hybridized with a cDNA probe for the 1.2 kb mitochondrial encoded cytochrome b mRNA, reveals that these mitochondrially encoded mitochondrial proteins have the same pattern of expression as the nuclear encoded somatic cytochrome c (Fig. 2C).

**Temporal expression of cytochrome c during spermatogenesis**

**Prepuberal mouse testes**

In the testes of immature mice, spermatogenesis has only proceeded up to a given point, depending on the age of the animal (Bellvé et al. 1977). We compared the temporal appearance of cytochrome cT to that of cytochrome cS in total testicular RNA from sexually immature and mature mice. Fig. 3 demonstrates that the three smaller cytochrome cS transcripts are present at roughly equivalent concentrations in testes of mice ranging from 6 to 45 days in age (Fig. 3A). The 1.7 kb somatic transcript is first detected at day 22 of testis development, corresponding to the progression of spermatogenesis to the round spermatid stage (Bellvé et al. 1977). Cytochrome cT transcripts are not detected in the testes of 6- or 8-day-old mice (Fig. 3B). The mRNA is first detected in 12-day-old mice and increases markedly...
between days 12 and 15 of prepuberal mouse testis development correlating with the appearance of pachytene spermatocytes in the development of the spermatogenic epithelium at day 13 (Bellevé et al. 1977).

**Isolated spermatogenic cells**

To examine more closely the relationship between expression of isozymes of cytochrome c, we analyzed their mRNA levels in purified populations of germ cells (Fig. 4). The three smaller cytochrome c₅ transcripts are present in type A and B spermatogonia and preleptotene spermatocytes at approximately equivalent levels. The level of cytochrome c₅ transcripts is observed to increase in leptotene/zygotene and prepuberal pachytene spermatocytes and to decrease in adult pachytene spermatocytes, round spermatids and residual bodies. A distinct, abundant 1.7 kb transcript appears in the post-meiotic spermatids.

Cytochrome c₅ transcripts (0.6–0.9 kb) are not detected in type A and type B spermatogonia (Fig. 4B). Low levels are first detected in preleptotene spermatocytes upon longer exposure of the blot (data not shown). The signal becomes stronger and more heterogeneous in leptotene/zygotene spermatocytes and this trend continues in prepuberal pachytene spermatocytes. In cells isolated from adult mice, the highest levels of mRNA (0.6–0.9 kb) are observed in pachytene spermatocytes, while a reduced and less heterogeneous 0.7 kb band is found in round spermatids. Very little cytochrome c₅ transcript is detected in residual bodies, the cytoplasmic droplets extruded from the developing spermatid.

**Translational status of cytochrome c isozymes during spermatogenesis**

**Total testis polysomal gradients**

To determine the distribution of cytochrome c transcripts on polysomes, Northern blot analysis was performed on non-polysomal and polysomal RNAs prepared from total testis polysomal gradients (Fig. 5). The three smaller cytochrome c₅ transcripts are found predominantly in the polysomal fractions (M5–M6) of the gradient (Fig. 5A) whereas the 1.7 kb cytochrome c₅ transcript is detected only in the non-polysomal fraction (M2–M3). In contrast to the localization of cytochrome c₅ mRNA on polysomes, cytochrome c₅ mRNA shows a much more diverse polysomal distribution (Fig. 5B). A 0.7–0.9 kb subset of the heterogenous in leptotene/zygotene spermatocytes and this trend continues in prepuberal pachytene spermatocytes. In cells isolated from adult mice, the highest levels of mRNA (0.6–0.9 kb) are observed in pachytene spermatocytes, while a reduced and less heterogeneous 0.7 kb band is found in round spermatids. Very little cytochrome c₅ transcript is detected in residual bodies, the cytoplasmic droplets extruded from the developing spermatid.

**Fig. 4.** Size and distribution of cytochrome c transcripts in purified populations of spermatogenic cells. 10 µg aliquots of RNA from day 8 total testis (T₈), day 17 total testis (T₁₇), adult total testis (Tₐ), type A spermatogonia (A), type B spermatogonia (B), preleptotene spermatocytes (PL), leptotene/zygotene spermatocytes (LZ), prepuberal pachytene spermatocytes (PP), pachytene spermatocytes (P), round spermatids (RS) and residual body/cytoplasts (RB) were electrophoresed in a 1.5 % agarose gel (Fig. 4A) or a 2 % agarose gel (Fig. 4B) and blotted to nitrocellulose. Autoradiograms were obtained after hybridization with probes encoding (A) cytochrome c₅, (B) cytochrome c₇, and (C) 18s rRNA. The filter in Fig. 4B was rehybridized with 18s rRNA (Fig. 4C).

**Fig. 5.** Distribution of mRNAs for cytochrome c₅ and cytochrome c₇ in polysomal gradients from adult mouse testis. Fractions M1 and E1 are the top of the gradients. Lanes M1–M6 contain RNA isolated from gradients run with Mg²⁺ and lanes E1–E6 contain RNA isolated from gradients containing EDTA in place of Mg²⁺. Total testis (T). One percent of the RNA from each fraction was electrophoresed in 1.5 % agarose gels and blotted onto nitrocellulose filters. Autoradiograms were obtained after hybridization with probes encoding (A) cytochrome c₅, (B) cytochrome c₇ and (C) lactate dehydrogenase X (rehybridization of B).
geneous cytochrome cT transcript is enriched in the polysomal fraction when compared with a 0.6–0.75 kb subset enriched in the non-polysomal fraction (Fig. 5B). The presence of two overlapping subsets of cytochrome cT transcripts has been observed in four separate polysomal gradients and is also apparent in isolated cell type polysomal gradients (Fig. 6B). All of the transcripts shift to the non-polysomal fraction of the gradients when the polyosomes are released from the mRNAs with EDTA (Fig. 5A,B). The polysomal gradients are believed to be intact since the three smaller cytochrome cS transcripts are located completely in the polysomal fraction. In addition, using identical fractions, approximately 60% of the lactate dehydrogenase -X transcripts of 1.2 and 1.3 kb, appear in the polysomal fraction in our experiments, as has been reported previously (Fujimoto et al. 1988).

**Polysomal gradients from isolated spermatogenic cells**

Two questions arise upon consideration of the preceding Northern blots: (1) is the 1.7 kb cytochrome cS transcript translated, i.e. present on polyosomes? and (2) do the polysomal distributions of the heterogeneous cytochrome cT transcripts in pachytene spermatocytes and the more homogeneous round spermatid transcripts differ? Polysomal gradients using cell homogenates from purified pachytene spermatocytes and round spermatids were run to address these issues.

Fig. 6A demonstrates that the 1.7 kb cytochrome cS transcript is abundant in the non-polysomal fraction of round spermatids. The three smaller cytochrome cS transcripts are present primarily in the non-polysomal fraction of both cell types at greatly reduced levels compared to their abundance and polysomal location in total testis polysomal gradients. The cytochrome cT transcripts have distinct distributions in the polysome gradients from isolated pachytene spermatocytes and round spermatids (Fig. 6B). The transcript distribution in pachytene spermatocyte polysomal gradients is essentially the same as that observed for the total testis polysomal gradients: The majority of the non-polysomal transcripts are 0.6–0.75 kb, while the polysomal transcripts are 0.7–0.9 kb. In round spermatids a small transcript size class of about 0.7 kb is present primarily in the non-polysomal fraction, with a minor fraction in the polysomal portion of the gradient.

**Analysis of transcript heterogeneity**

To understand the cause of the heterogeneity in transcript size of the cytochrome cS and cytochrome cT mRNAs, the sizes of their poly(A)+ tails were examined. The poly(A)+ tail was removed by RNAs H digestion of poly(A)+ tail:oligo d(T) hybrids using total RNA from total adult testis, purified populations of spermatogenic cells and polysomal gradient fractions. After digestion, the size of the RNAs were determined by Northern blot analysis. Removal of the poly(A)+ tail from testicular cytochrome cS transcripts (T-A) revealed four size classes of mRNAs: 0.45, 0.9, 1.1 and 1.5 kb (Fig. 7A), establishing that there are four distinct classes of transcripts in the tests. From the increased rate of migration, we estimate that cytochrome cS mRNAs contain approximately 200 adenylic acid residues.

Analysis of cytochrome cT transcripts lacking poly(A)+ tails in samples from isolated spermatogenic cells reveal that both the heterogeneous pachytene spermatocyte transcripts and the more homogeneous round spermatid transcripts shorten to approximately
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Fig. 7. RNA blot analysis of cytochrome c mRNAs from total testis, spermatogenic cells and polysomal gradient fractions after removal of the poly(A) tail with RNAse H. 10 μg of RNA with poly(A)+ tails intact or removed following incubation with oligo dT and RNAse H were electrophoresed in 2% agarose gels and blotted onto nitrocellulose filters. (A) Autoradiogram of a filter hybridized with a probe encoding cytochrome cs to total mRNAs from adult liver (L), adult testis after deadenylation of the mRNA (T_A) and adult testis RNA (T). Fig. 7B and C are autoradiograms of filters hybridized with cytochrome cT cDNA probes. Fig. 7B contains intact or deadenylated (denoted by −Poly(A)+) mRNAs from total adult testis (T), pachytene spermatocyte (P) and round spermatid (RS). Fig. 7C contains intact (T on the right) or deadenylated (−Poly(A)+) mRNAs from total adult testis (T) and polysome gradient fractions M2–M6.

0.5 kb (Fig. 7B). Similar results are obtained with the heterogeneously sized polysomal and non-polysomal transcripts from total testis with all mRNAs appearing to shorten to approximately 0.5 kb (Fig. 7C). We estimate from the difference in migration of the deadenylated transcripts that the poly(A)+ tails on cytochrome cT mRNAs range from 50–400 residues.

Discussion

To study cytochrome c gene expression during spermatogenesis, cDNA clones encoding the mouse somatic and testis-specific forms of cytochrome c were isolated and sequenced. Comparison of the cytochrome cS cDNA sequence with the mouse genomic sequence (Limbach and Wu, 1985) reveals 100% similarity, thus confirming the identity of this clone. The cytochrome cT cDNA coding region sequence is 93% similar to that of rat and is identical to that previously found for mouse (Virbasius and Scarpulla, 1988). The high divergence of the 3' untranslated region sequence of the mouse cytochrome cT cDNA found by Virbasius and Scarpulla (1988) from the sequence currently reported is puzzling. The high homology (86%) between our 3' untranslated region and that found in rat (Virbasius and Scarpulla, 1988) suggests that our sequence probably represents a mRNA transcript from the mouse cytochrome cT gene.

The pattern of expression of cytochrome cs, a nuclear encoded gene, in somatic tissues is similar to that of cytochrome b, a mitochondrially encoded gene. This similarity may represent an example of the coordinate regulation of respiratory chain components by heme (reviewed in Padmanaban et al. 1989). The three smaller cytochrome cS transcripts appear to be regulated in concert in all tissues examined. The size differences between the transcripts are probably due to the alternative use of three 3' polyadenylation signals located at positions 562, 723 and 980 of the genomic clone (Limbach and Wu, 1985) as is the case in the rat (Scarpulla, 1984). The only observed difference in these three transcripts has been propensity for the middle transcript to be reverse transcribed and reinserted into the genome as a pseudogene (Scarpulla, 1984).

The presence of a fourth cytochrome cS transcript of 1.7 kb in mouse testis has also been reported by Wu and Limbach (1985). In this original observation, the additional transcript was presumed to encode the testis-specific form of cytochrome c. However, subsequent studies utilizing a cytochrome cT cDNA in rat and mouse (Virbasius and Scarpulla, 1988 and this study) revealed that cytochrome cT is encoded by mRNAs of 0.6–0.9 kb. The relationship between the 1.7 kb transcript and the smaller cytochrome cS transcripts is at present unclear.

To define the 1.7 kb transcript, its expression and translational status during spermatogenesis have been examined. Analysis of cytochrome cS expression in prepuberal testis RNA and isolated spermatogenic cell types reveals that the 1.7 kb transcript is abundant in more advanced germ cell stages such as round spermatids while the three smaller transcripts are present at reduced levels. This switch in transcript size is most evident in round spermatids, a cell type where no cytochrome cS protein is detected (Goldberg et al. 1977); we were therefore interested in a closer examination of the functional status of the 1.7 kb transcript in round spermatids. Analysis of polysomal gradients from total testis and isolated round spermatids revealed that, while the three smaller cytochrome cS transcripts
are polysomal, the 1.7kb transcript is not associated with polysomes. We conclude that the post-meiotically enriched, non-polysomal 1.7kb transcript is non-functional and may be evidence for a mechanism that operates to down-regulate this gene during spermatogenesis.

Closer examination of the 1.7kb cytochrome cS transcript reveals that, although this mRNA is polyadenylated, the larger size of this transcript is not due solely to poly(A)+ tail length. The 1.7kb cytochrome cS transcript may (1) be derived from a pseudogene that is transcribed post-meiotically in the testis or (2) represent a cytochrome cS mRNA precursor which is not correctly processed into translatable cytochrome c mRNA in the later stages of spermatogenesis. Additional 3' or 5' untranslated regions with secondary structure or additional open reading frames may interfere with translation (Kozak, 1988). In the rat, testis-specific alternative splicing of the proenkephalin message produces a 1.75kb mRNA which contains a large portion of the first intron and is not loaded efficiently onto polysomes (Garrett et al. 1989). Further investigation of the identity of the 1.7kb cytochrome cS transcript is in progress.

The regulation of cytochrome cT expression during spermatogenesis was also examined. The observation that cytochrome cT mRNA is only found in the testis is in agreement with results of Virbasius and Scarpulla (1989), suggesting strongly that this gene is regulated at the level of transcription in a tissue-specific manner. There may also be coordinate regulation of cytochrome cT transcripts with mitochondrially encoded transcripts during spermiogenesis, as levels of both sets of mRNAs are high in pachytene spermatocytes, decreased in round spermatids and absent in residual bodies (Alcivar et al. 1989). Further complexity in regulation is supported by the presence of cytochrome cT mRNA in both the polysomal and non-polysomal fractions. These two subsets differ in size: non-polysomal transcripts are 0.6-0.75 kb while polysomal transcripts are 0.7-0.9 kb. The majority of the size variability in the cytochrome cT transcript appears to be due to changes in poly(A) tail length; however, it is not possible to determine if this was the only cause of the size discrepancy between polysomal and non-polysomal RNAs because of the limited resolution in the agarose gel system used.

An increase in poly(A)+ tail length has been correlated with polysomal loading of transcripts in oocyte maturation and fertilization in several organisms, most notably Xenopus (McGrew et al. 1989) and Spisula (Rosenthal and Ruderman, 1987). Recently, a short sequence element, UUUUUUAU, located 8 nucleotides upstream from the poly(A) addition sequence in a Xenopus maternal mRNA (G10), has been found to be both necessary and sufficient in combination with the poly(A) addition signal for the polyadenylation and translational recruitment of the transcript during oocyte development (McGrew et al. 1989). A sequence very similar to this, UUUUUGAU, is present in two locations in the cytochrome cT mRNA: (1) 27 nucleotides upstream from the poly(A) addition signal (at base 522 in cytochrome cT, Fig. 1) and (2) 8 nucleotides downstream from the poly(A) addition signal (at base 571). The function of this sequence in the cytochrome cT transcript during spermatogenesis requires further investigation.

In conclusion, cytochrome cS and cytochrome cT are developmentally and translationally regulated during spermatogenesis. As the quantity of the three smaller cytochrome cS transcripts declines, the amount of cytochrome cT transcripts increases and is highest when very little somatic cytochrome c transcript can be detected. The 1.7 kb cytochrome cS mRNA may be the result of a novel form of gene down-regulation through an alteration in transcript processing or may represent a transcript from a differentially expressed cytochrome cS pseudogene. The complex size distribution of cytochrome cT transcripts on polysomal gradients indicates that significant changes in poly(A) tail length and perhaps slight 3' or 5' untranslated region variations are concurrent with the translational regulation of this transcript. Further investigation of these two mechanisms of mRNA regulation will provide greater insight into both the coordination of nuclear encoded mitochondrial genes and the intricacies of gene expression during spermatogenesis.

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