The rat cytochrome *c* oxidase subunit IV gene family: tissue-specific and hormonal differences in subunit IV and cytochrome *c* mRNA expression

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ABSTRACT

We have isolated three members of the rat cytochrome *c* oxidase subunit IV gene family: one functional gene and two processed pseudogenes. The pseudogenes appear to represent the only other closely related sequences in this family. The functional gene encodes an isoform which is expressed in all tissues examined and has features characteristic of 'housekeeping' genes. These include multiple transcription start sites mapped to within an approximately 50 bp region and a GC-rich promoter lacking typical CCAAT or TATAA sequences. Although the subunit IV gene is expressed at its highest levels in cardiac and skeletal muscle, consistent with the high energy demand in those tissues, its expression differs from that of cytochrome *c* in several respects. 1) Subunit IV mRNA abundance in various tissues is relatively uniform when compared to the highly variable levels of cytochrome *c* mRNAs. 2) Unlike cytochrome *c*, subunit IV mRNA is expressed at a surprisingly high level in testis. 3) While cytochrome *c* mRNA levels in liver are increased markedly in response to thyroid hormone treatment, subunit IV mRNA is not significantly affected. Differences in the expression of these two nuclear-encoded respiratory genes are consistent with differences in regulatory elements within their promoters. Therefore, the regulation of nuclear-encoded respiratory genes in response to tissue demands for cellular energy may not be satisfactorily explained by a set of universal regulators common to all such genes.

INTRODUCTION

The five membrane-associated, multi-subunit complexes and two soluble carriers which make up the mammalian mitochondrial oxidative phosphorylation system are the products of both the nuclear and mitochondrial genomes (for review see (1). It is likely that variations in respiratory capacity among various cell types can be attributed, at least in part, to the coordinate regulation of the two genetic systems and indeed evidence for such coordinate regulation at the level of gene expression has been obtained (2). However, given that the function of many of the nuclear-encoded subunits of the respiratory complexes remains obscure, the necessity for the maintenance of identical subunit stoichiometries in all cell types and under all physiological conditions is unproven. The identification of tissue-specific subunits of several of the respiratory complexes (3-5) argues instead that unique combinations of components are required for some situations.

Using the rat cytochrome *c* promoter as a model system we have begun to identify regulators of nuclear gene activity which are involved in the control of nuclear respiratory gene expression and in the coordination of nuclear and mitochondrial gene expression (6-8). We now seek to extend this analysis by examining the structure and expression of other nuclear genes encoding components of the oxidative phosphorylation system. Here we present the initial findings concerning the expression of the rat cytochrome *c* oxidase subunit IV gene. There are several reasons for characterizing this gene. First, cytochrome *c* is the electron donor in the cytochrome oxidase reaction which is thought to be rate-limiting in mitochondrial respiration (for review see 9). It is thus reasonable to postulate coordinate expression of genes encoding these components. Second, NRF-1, a novel *trans*-activator of respiratory gene expression in mammalian cells, was previously found to interact with both cytochrome *c* and cytochrome *c* oxidase subunit VIc promoters suggesting a mechanism for coordinate regulation which may apply to other oxidase subunits (8). Finally, mammalian subunit IV is homologous to yeast subunit V (10,11) and the major isoforms of subunit V and cytochrome *c* are known to be coordinately expressed through a common activator of respiratory gene expression in this organism (12).

In the present study we find features in the cytochrome *c* oxidase subunit IV gene in common with as well as distinct from the cytochrome *c* gene which are reflected in unique patterns of regulation. These findings suggest variations in the structure and expression of nuclear respiratory genes which do not reflect a simple pattern of coordinate gene activity but rather more complex and flexible patterns allowing differential expression of individual subunits.

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MATERIALS AND METHODS

Isolation and sequencing of cDNA and genomic clones

Rat testis cDNA and liver genomic libraries were obtained from Clontech, Inc. The cDNA library was screened with a human cytochrome *c* oxidase subunit IV cDNA clone (13, a kind gift of Dr. L. Grossman) as previously described (14). A rat cDNA clone thus obtained was used to screen as described previously (14) a rat genomic library constructed from *Hae*III partial digestion products of Sprague-Dawley rat liver genomic DNA cloned into βCharon 4A. Positive isolates were subcloned into M13 vectors for sequencing by the dideoxy chain-termination method (15). With the exception of a 77 bp region of the pseudogene clone RCO4-2, all sequences reported have been determined completely on both strands.

RNA preparation and analysis

Total RNA was prepared from Sprague-Dawley rat tissues by LiCl precipitation as previously described (16). For primer extension, a synthetic oligonucleotide 5′-CCACTGCTGCCC-AGGCGACC-3′ complementary to nucleotides +66 to +85 was labeled with T4 polynucleotide kinase in the presence of γ-^{32}P ATP and annealed to RNA by heating to 70°C and slow cooling in 100 mM KCl. Complementary DNA was synthesized with 25 units AMV reverse transcriptase (Promega) in 50 mM Tris-HCl, pH 8.3, 10 mM $MgCl_2$, 10 mM dithiothreitol, 0.5 mM each deoxynucleotide triphosphate, 50 mM KCl, 0.5 units/μl RNAsin (Promega), 100μg/ml actinomycin D for 60 minutes at 42°. Extension products were separated on polyacrylamide-urea gels with a dideoxy sequencing ladder generated using the same primer. For S1 nuclease mapping, uniformly labeled, single stranded probes were generated by the method of Burke (17) using the same primer and an M13 template spanning nucleotides −183 to +226 of the RCO4-1 sequence. Hybridization to RNA and S1 nuclease digestion were carried out as previously described (18). RNAs were separated on agarose-formaldehyde gels, transferred to nitrocellulose, and hybridized with nick-translated probes as previously described (18). Prior to rehybridization with a second probe, blots were stripped by washing in 0.1×SSC for 15−30 minutes at 90°C. Hybridization signals were quantitated by scanning autoradiograms in a Zeineh laser densitometer equipped with a Hewlett-Packard reporting integrator. For cytochrome *c*, the results presented represent the sum of the three peaks resulting from the three distinct cytochrome *c* mRNAs (18). Treatment of thyroidectomized rats with thyroid hormone (3,5,3′-triiodo-L-thyronine) has been previously described (19). Briefly, induction of mRNAs was measured by Northern blotting as described above following administration of 5 daily intraperitoneal injections of thyroid hormone (30 μg thyroid hormone/ 100g body weight) or over a time course following a single intravenous tail vein injection (100 μg thyroid hormone/ 100g body weight).

RESULTS

Sequences and genomic organization of rat cytochrome *c* oxidase subunit IV loci

We initially isolated a cDNA clone from a rat testis library using the human cytochrome *c* oxidase subunit IV cDNA (13). This was in turn used to screen approximately 700,000 plaques of a Sprague-Dawley rat genomic library. The 29 positive clones obtained from this screening were found by means of restriction mapping to represent three different loci. Clones representing each of these were further mapped and the regions hybridizing to the cDNA probe sequenced. One of these, RC04-1, encodes a gene of 6234 nucleotides containing five exons (88, 74, 168, 132, and 234 nucleotides) interrupted by four introns (968, 3304, 391, and 876 nucleotides). RCO4-1 appears to be the functional locus because its exon sequences perfectly match the rat testis cDNA clone which encodes a protein having 78 and 88 percent sequence identity respectively with the known human liver (13) and bovine heart (20) proteins.

The other two loci, RCO4-2 and RCO4-3, have all of the structural features of processed pseudogenes. Like the numerous cytochrome *c* pseudogenes in mammalian systems (21,22), they both lack introns, have the remnant of a poly-A tract in a location corresponding exactly to the polyadenylation site identified in the cDNA and are flanked by direct repeats of the genomic integration site. The 5′ direct repeat in each pseudogene corresponds to an initiation site mapping to approximately +25 relative to the furthest upstream transcription start site (Figure 2) indicating that both loci were derived from nearly identical mRNAs. Although RCO4-2 has relatively few nucleotide differences with the RCO4-1 coding region, a four base-pair deletion and a G to A transition combine to create an in-frame translation termination codon (TGA). Thus, if expressed at all, RCO4-2 would give rise to a truncated and likely nonfunctional protein. The RCO4-3 pseudogene has numerous substitutions, insertions and deletions. A deletion of the G nucleotide in the translation start codon destroys the subunit IV reading frame. The RCO4-2 and RCO4-3 isolates are therefore most likely nonfunctional pseudogenes. The sequences of RCO4-1, 2, and 3 have been submitted to the EMBL database under accession numbers X54081, X54082 and X54083, respectively.

To determine the extent to which the three cloned loci account

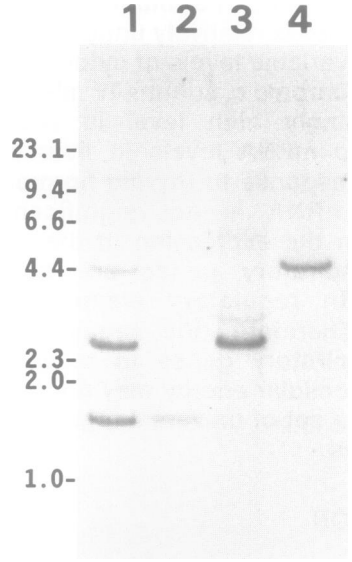

Figure 1. Correspondence of cloned subunit IV loci to specific sequences in rat genomic DNA. Individual lanes of a genomic blot each containing 10 μg rat genomic DNA digested with *Pst*I were separately hybridized with a 235 bp *Sau*3AI fragment of the subunit IV cDNA including sequences corresponding to exons 3 and 4 (lane 1); a 225 bp *Msp*I-*Bgl*II fragment from the intron of the RCO4-1 gene (lane 2); an approximately 250 bp *Pst*I-*Asp*718 fragment from the 5′-flanking region of the RCO4-2 pseudogene (lane 3); a 276 bp *Sph*I-*Xba*I fragment from the 3′-flanking region of the RCO4-3 pseudogene (lane 4). The mobilities of DNA size markers derived from *Hin*dIII digested βDNA (in kb) are indicated.

for the cytochrome *c* oxidase subunit IV gene family, we used a radiolabeled DNA fragment containing cDNA sequences corresponding to exons 3 and 4 of the functional gene to detect subunit IV-related sequences in restriction enzyme *Pst*I digested rat genomic DNA (Figure 1). This probe and digest combination was expected to generate a single hybridizing band corresponding to each locus based on mapping and sequencing of the three categories of clones. In fact, three prominent bands are detected:

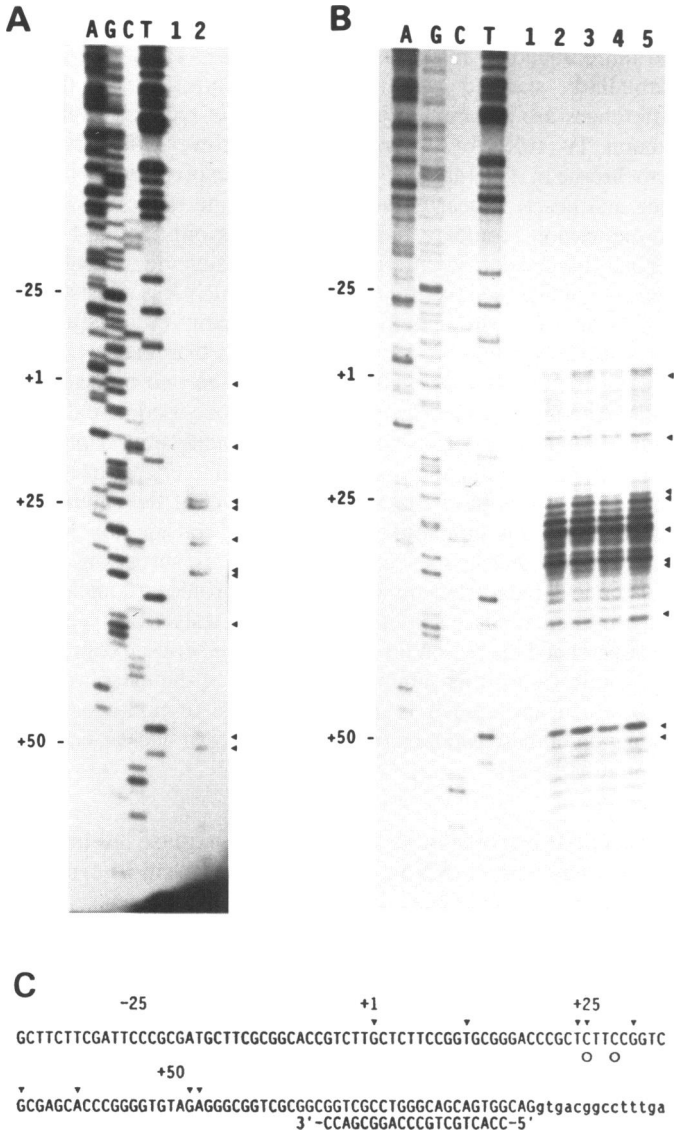

Figure 2. Transcription start site mapping. Panel A shows extension products of a ^{32}P-labeled primer hybridized with 20 μg total RNA from HeLa cells (lane 1) or rat heart (lane 2). A sequencing ladder produced by Klenow enzyme extension of the same primer with a cognate M13 template is also shown (lanes A,G,C,T). Note that the sequence is the complement of the gene sequence as written in panel C. Panel B shows products protected from S1 nuclease digestion following hybridization of a uniformly labeled, single stranded probe to 20 μg tRNA (lane 1) or 20 μg rat heart total RNA (lanes 2–5). Hybrids were digested with 1000 units S1 nuclease for 15 minutes (lanes 1,2), 30 minutes (lane 3), 45 minutes (lane 4) or 60 minutes (lane 5). The same sequencing ladder as in panel A is shown. The filled triangles indicate the positions of transcription start sites determined by primer extension (as in panel A). Panel C shows the DNA sequence spanning the transcription start sites indicated by filled triangles above the sequence. The first intron is in lower case letters and the oligonucleotide sequence used for primer extension shown below. Open circles below indicate the positions of the RCO4-2 and RCO4-3 pseudogene 5'-ends.

one fairly weak band at 4.4 kb and two stronger bands at 2.5 and 1.4 kb (lane 1). These correspond, respectively, to the expected sizes for the RCO4-3, RCO4-2 and RCO4-1 loci. To verify this we also prepared and hybridized locus-specific probes from each clone: a fragment from the third intron of RCO4-1 (lane 2) and fragments from the 5′ and 3′ flanking regions of RCO4-2 (lane 3) and RCO4-3 (lane 4), respectively. Hybridization of these probes to separate lanes of the same gel using the same *Pst*I digested rat genomic DNA detects bands which comigrate with those detected by the exon 3/4 probe as anticipated. The RCO4-2-specific probe also detects several additional bands. However, since this probe contains no subunit IV sequences, these are most likely the result of an unrelated multiple-copy sequence fortuitously present in this probe. We note that there are two additional weakly hybridizing bands of about 12 and 2.3 kb visible upon prolonged exposure of blots hybridized at low stringency with the exon probe. These may be sequences distantly related to the subunit IV sequence or the result of spurious cross-hybridization with unrelated sequences. In either case these results suggest that we have cloned all of the closely related members of the subunit IV gene family.

Transcription start-site mapping

The rat testis cDNA clone which was originally isolated included 47 bp of first-exon sequences. To determine the complete length of the 5′ end of the messenger RNA we used a 20 bp synthetic oligonucleotide complementary to nucleotides +66 to +85 for primer extension (Figure 2A) and to generate an uniformly-labeled, single-stranded probe for S1 nuclease protection (Figure 2B). Both of these assays detect the same heterogeneous collection of start sites spanning a region of approximately 50 bp with a cluster of strong start sites within 12 bp. No change in the pattern of protected products is detected over an extended time course of treatment with S1 nuclease (Figure 2B, lanes 2–5). Prolonged exposures of the gels are required to detect some of the weaker start sites using the primer extension technique but these correspond in all cases to sites detected more readily with the S1 nuclease protection method (Figure 2A, lane 3). We note that these results differ from those of Yamada, et al. (23), who, using only primer extension, identify a single strong start site corresponding to position +45 of our sequence and 11 bp downstream of the major cluster of the sites which we find. We find only a minor site near this position using both S1 nuclease protection and primer extension. Furthermore their own published cDNA sequence as well as our unpublished sequence include nucleotides 5′ to their proposed major start site indicating that at least some transcripts must start farther upstream. Finally, the 5′ direct repeats which in the cytochrome *c* system are usually found at or near locations corresponding to transcription start sites (21,22), are found near the positions of major start sites in both the RCO4 pseudogenes. No sequences resembling the consensus for TATA box or CCAAT recognition sequences are found in the several hundred nucleotides upstream of the start sites. In this respect and in the very high proportion of G and C nucleotides in the regions surrounding the start sites this promoter resembles those of the so-called housekeeping genes (24). It is therefore not surprising to find multiple start sites which is another characteristic typical of these genes.

Comparison of cytochrome oxidase subunit IV and cytochrome *c* mRNA expression in rat tissues

We have previously reported that the level of expression of the cytochrome *c* gene varies widely among rat tissues and generally

reflects their oxidative capacities (16,18). To determine whether the expression of the subunit IV mRNA follows the same pattern of cell-specific regulation, equal amounts of total RNA from adult rat brain, heart, kidney, liver, lung, skeletal muscle and testis were separated on an agarose gel, transferred to nitrocellulose and hybridized with the subunit IV cDNA. Each tissue contains an mRNA in the expected size range of about 0.9 kb which is consistent with the length of the cDNA allowing for a 150−200 nucleotide poly A tail (Figure 3A). The blot was subsequently stripped and then rehybridized with a cytochrome *c* coding region probe (Figure 3B). Using this probe, each tissue contains the expected pattern of three cytochrome *c* mRNAs (1.4, 1.1, and 0.7 kb) which have previously been shown to differ in the lengths of their 3′-untranslated regions because of alternative polyadenylation sites (18).

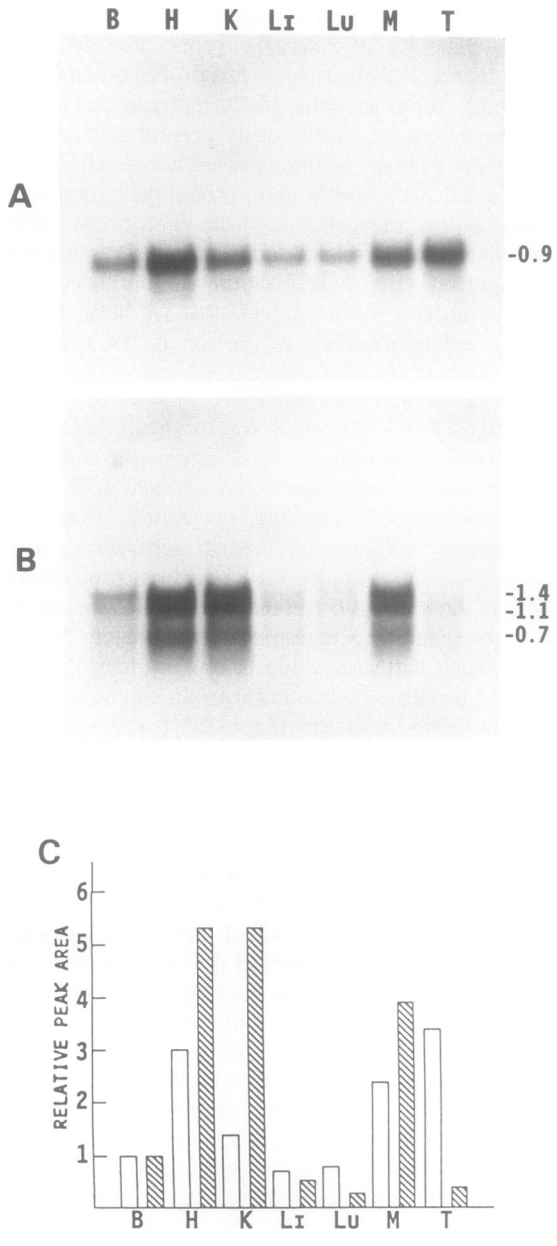

Figure 3. Expression of the subunit IV and cytochrome *c* mRNAs in rat tissues. Total RNA (50 μg) from rat brain (B), heart (H), kidney (K), liver (Li), lung (Lu), thigh muscle (M), and testis (T) was separated on an agarose-formaldehyde gel, transferred to nitrocellulose, and hybridized with a nick-translated fragment containing the rat subunit IV cDNA (panel A). The blot was subsequently stripped and rehybridized with a rat cytochrome *c* coding region probe (panel B). Positions of the 0.9 kb subunit IV message and the 1.4, 1.1 and 0.7 kb cytochrome *c* mRNAs are indicated. Autoradiograms of the two blots were scanned by laser densitometry, and the relative areas of the resulting peaks for cytochrome *c* (hatched bars) and subunit IV (open bars) mRNAs are shown in panel C. For each blot the peak area resulting from hybridization to RNA in brain is arbitrarily taken as equal to 1.0.

Estimation of the relative levels of expression of the two genes by densitometric scanning of the blot (Figure 3C) demonstrates that although both subunit IV and cytochrome *c* messenger RNAs are more abundant in the tissues with higher energy demand, particularly cardiac and skeletal muscle, several notable differences are apparent. First, the range of expression of the subunit IV mRNA is considerably narrower than that of cytochrome *c*. The range of cytochrome *c* expression (compare lung and heart) is nearly 19-fold whereas the range of subunit IV expression (compare lung and testis) is only about 4-fold. Second, tissue-specific differences in expression are evident. For example, while the level of cytochrome *c* mRNA is consistently similar in rat heart and kidney, the subunit IV message is approximately 2-fold less abundant in kidney than in heart. Most striking is the difference in expression of the two messages in testis. Although the cytochrome *c* coding probe used here detects both the somatic and testis-specific cytochrome *c* transcripts (16) their combined population is barely detectable except in longer exposures of this blot. By contrast, the level of the subunit IV mRNA in testis is similar to that in heart and considerably higher than in the other tissues examined. This is surprising since biochemical measurements of both cytochrome *c* content and cytochrome *c* oxidase activity are lowest in testis (25). We have not ascertained the reason for this discordance between expression of the mRNA for this subunit and activity of the cytochrome *c* oxidase. In any case it is a clear example of non-coordinate regulation of these two members of the family of nuclear-encoded respiratory genes.

Differential expression of cytochrome *c* oxidase subunit IV and cytochrome *c*mRNAs in response to thyroid hormones

The effects of thyroid hormone on respiratory function have been well documented (26,27), and we have demonstrated that cytochrome *c* transcription is highly sensitive to the thyroid status of experimental animals (19). We therefore wished to determine whether cytochrome *c* oxidase subunit IV gene expression is similarly regulated. Messenger RNAs prepared from the livers of thyroidectomized rats following injections of saline (−) or saline plus thyroid hormone (+) were detected with a subunit IV cDNA probe (Figure 4A) and the same blot subsequently stripped and rehybridized with a cytochrome *c* coding region probe (Figure 4B). In agreement with our previous findings (19), cytochrome *c* mRNA levels are increased approximately 6-fold by thyroid hormone based on densitometric scanning of the blot shown (Figure 4C). By contrast the subunit IV message level is increased only about two-fold, within the range of the general increase in mRNA transcription which results from thyroid hormone treatment (28). A similar result is obtained by comparing mRNA levels from thyroidectomized animals sacrificed at various times following a single intravenous administration of thyroid hormone. Between 0.5 and 24 hours after injection the cytochrome *c* oxidase subunit IV mRNA level increases about 1.5 fold whereas the cytochrome *c* mRNA level increases nearly 6-fold. While we cannot rule out from these experiments that

there is a modest level of specific stimulation of subunit IV gene activity by thyroid hormone, it is clear that the magnitude of the increase in cytochrome *c* mRNA accumulation is significantly greater.

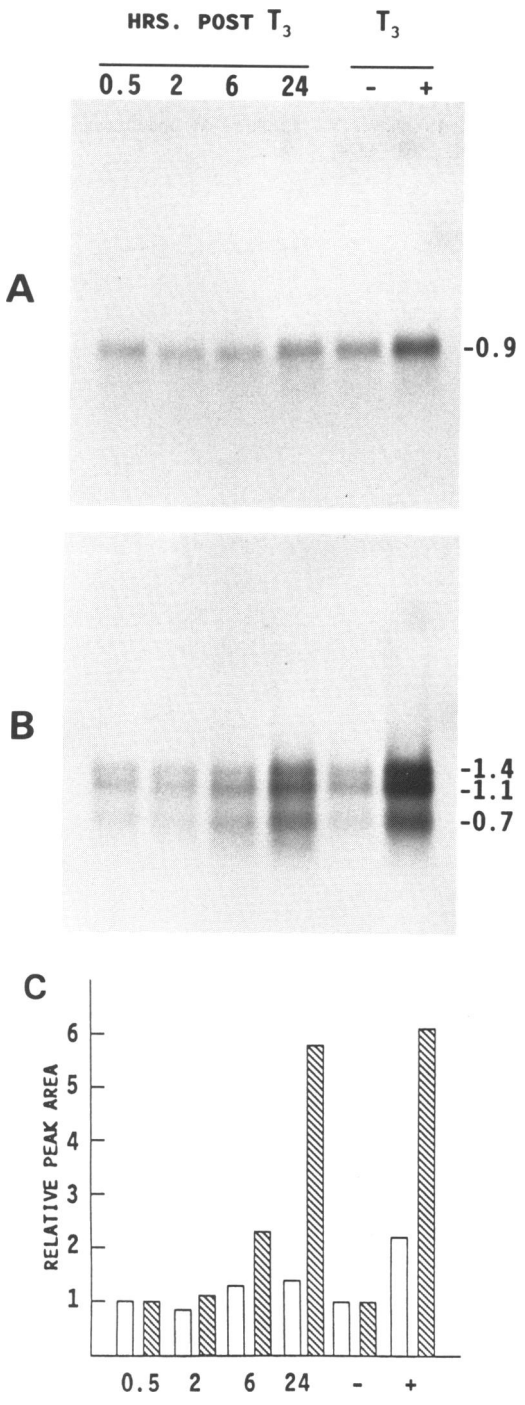

Figure 4. Response of subunit IV and cytochrome *c* mRNA expression to thyroid hormone. RNA was prepared from the livers of rats 0.5, 2, 6, and 24 hrs after a single injection of thyroid hormone, and from the livers of rats given 5 daily injections of a buffered saline solution (−) or buffered saline plus thyroid hormone (+). RNA was separated on an agarose-formaldehyde gel, transferred to nitrocellulose and hybridized with a subunit IV probe (panel A). The same blot was stripped and rehybridized with a cytochrome *c* coding region probe (panel B). Relative peak areas resulting from densitometric scanning of the autoradiograms is shown for cytochrome *c* (hatched bars) and subunit IV (open bars) mRNAs in panel C. For each probe, the peak area for the 0.5 hr and saline control samples (−) is taken as equal to 1.0.

DISCUSSION

The results presented here suggest that the cytochrome *c* oxidase subunit IV gene family consists of a single functional gene and two processed pseudogenes. In addition to the rat testis cDNA used in the isolation of these genomic loci we isolated a cDNA encoding an identical subunit IV protein from a rat kidney cDNA library (J.V.V. and R.C.S. unpublished). Clones with the same sequence have also been isolated from adult rat brain and liver (29) and rat fetal liver (30). These results are in keeping with the isolation of a single bovine functional gene (31) and with biochemical studies indicating that bovine heart and liver proteins are identical (32). Thus it seems likely that there is only a single isoform of subunit IV in contrast to other cytochrome *c* oxidase subunits (3,5), an ATP/ADP translocase subunit (4), and cytochrome *c* (16) for which tissue-specific genes have been identified.

Our interest in the coordination of nuclear respiratory gene expression led us to compare the expression of the subunit IV gene with that of the well-characterized cytochrome *c* gene. Given the significance of thyroid hormone in regulating respiratory function (26,27) and cytochrome *c* expression (19) it might be expected that other nuclear respiratory genes would be similarly responsive. We find, however, that subunit IV expression is not significantly affected by thyroid hormone status. A similar finding has been reported for cytochrome *c* oxidase subunit Va expression which is unchanged by thyroid hormone treatment (33). It is clear from these results that the physiological effectors such as thyroid hormone need not regulate, at least at the level of mRNA expression, all respiratory chain genes in order to significantly affect respiratory function.

In its ubiquitous and perhaps constitutive expression, the subunit IV gene may be described as a 'housekeeping' gene, and indeed we find that its structure is similar to a number of other well-characterized genes of this category (24). The region upstream of the transcription start sites as well as the first exon and 5'proximal region of the first intron are extremely rich in G and C nucleotides and there are a number of sequences with similarities to the core recognition sequence GGGCGG for the transcription factor Sp1 (34). We have established that at least one of these Sp1 sites contributes to promoter function (J.V.V. and R.C.S., unpublished). In its G-C content and likely Sp1 recognition sites the promoter region is similar to that of the cytochrome *c* gene which possesses a potent intron promoter element consisting of tandem Sp1 binding sites (7).

Outside of this similarity, however, we find little in common between the cytochrome *c* and subunit IV promoters. The subunit IV promoter region lacks sequences resembling the consensus for TATA or CCAAT elements. The absence of a CCAAT box is noteworthy in that the cytochrome *c* promoter is dependent on a *cis*-acting element containing evolutionarily conserved CCAAT homologies (6,7). This contrasts with the yeast system where the coordinate regulation of genes encoding the major isoforms of yeast COXV (mammalian COXIV) and cytochrome *c* is thought to occur through HAP2, a subunit of the yeast counterpart to mammalian CCAAT box transcription factor CP1 (12,35). We have also been unable to detect by either sequence searches or *in vitro* DNA binding assays any recognition site for transcription factor NRF-1. This factor binds to the promoter of the cytochrome *c* gene as well as a number of other nuclear-encoded respiratory genes including the cytochrome oxidase subunit VIc gene (8). It is of particular interest to us that the human cytochrome c_1 gene (36), like the subunit IV gene,

apparently lacks a functional NRF-1 binding site and is also unresponsive to thyroid hormone (27). We presume that it is differences in regulatory sequences such as these that lead to differences in tissue-specific and hormone-regulated patterns of expression among nuclear-encoded respiratory genes. Thus, while subsets of respiratory chain genes share common activators such as NRF-1, it cannot be assumed that all the genes which contribute to mitochondrial respiratory function are coordinately controlled by one or a few global regulators.

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