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AN ANTISENSE OLIGODEOXYNUCLEOTIDE APPROACH TO INVESTIGATE THE FUNCTION OF THE NUCLEAR-ENCODED SUBUNITS OF HUMAN CYTOCHROME *C* OXIDASE

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On treatment of human liver Hep G2 cells with thiamphenicol, intramitochondrial levels of cytochrome *c* oxidase (COX) nuclear gene products were shown to decrease in tandem with the mitochondrially-encoded subunits except, however, for subunit IV which proved to be remarkably stable. This nonspecific decrease in subunit level was utilized to assess the function of subunit VIIa-L. Thiamphenicol-pretreated cells were bathed in anti-VIIa-L oligonucleotides and the recovery rate of COX activity compared to control oligomer-untreated cells or cells with a similar concentration of a randomized oligomer. No difference in recovery rate was noted for the first two days post oligomer addition, after which a sharp decrease in recovery was noted for the anti-VIIa-L treated cells only. We conclude: (i) subunit IV is stable in the absence of other COX components, (ii) subunit VIIa-L is required for maintaining normal levels of COX activity, (iii) an antisense oligodeoxynucleotide approach for assessing the function of COX nuclear gene products is made feasible by first reducing levels of the complex and then assaying its recovery rate in the presence of transcript-specific antisense oligonucleotides.

Defects of the mitochondrial respiratory chain are increasingly becoming recognized as important causes of neuromuscular diseases (1-3). Whilst most attention has focussed on the role of mitochondrial DNA mutations in the aetiology of these diseases (4), we have maintained an interest in those autosomal disorders which may be caused by defective respiratory chain polypeptides encoded by the nucleus . In particular, deficiencies of cytochrome *c* oxidase (COX) a commonly affected complex, have been associated with a variety of muscular and neurodegenerative disorders. Purified preparations of mammalian COX invariably include three mitochondrially-encoded components normally associated with ten nuclear gene products, a subset of which are present as tissue specific isoforms (5-7). The analogous bacterial *aa3*-type terminal oxidases are comprised solely of three subunits homologous to the mitochondrially-encoded gene products (8). As the bacterial polypeptides contain the same redox components, translocate protons and function in a similar manner to their eukaryote counterparts, what function do these nuclear gene products play in the activity and homeostasis of the eukaryote enzyme complex ? It is apparent that when attempting to correlate any given human COX

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Abbreviations: **EGTA**: ethylene glycol -O,O'-bis(aminoethyl)-N,N,N,'N' tetraacetic acid, **PMSF**: phenylmethylsulphonyl fluoride, **PBS**: phosphate-buffered saline, **DTT**: 1,4 -Dithio-DL-threitol, **SDS**: sodium dodecylsulphate, **BSA**: bovine serum albumin, **TAP**: thiamphenicol.

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deficiency with a nuclear gene lesion we, and members of many other research groups, have been frustrated by a lack of knowledge concerning the function of these nuclear encoded COX polypeptides. In *Saccharomyces cerevisiae*, gene ablation techniques have demonstrated that at least five of their nine associated polypeptides are required for assembly of the enzyme complex (9, 10). In the absence of an assembled enzyme, it is impossible to assess any more subtle regulatory role these subunits may perform *in vivo*. There has been only one report of a fully-characterized missense mutation of a yeast nuclear COX gene (11). This temperature-sensitive mutation, of subunit IV (Vb in mammals) revealed a requirement for this subunit in both COX assembly and activity . To date, however, no such studies have been undertaken in the higher eukaryote.

One method of addressing the function of mammalian COX subunits is to down regulate individual polypeptides using antisense oligodeoxynucleotides. To facilitate this approach, it is imperative to overcome several problems. First, it is likely that high concentrations (1-20μM) of oligonucleotides may be required to be effective. This would prove prohibitively expensive for repeated analysis of large volumes of cells. Consequently, we have established a novel COX microassay allowing measurement of activity in microwells of less than 20,000 cells (12). Second, the half life of COX in hepatocytes has been estimated at 5.7 days (13). Initial experiments with phosphorothioate oligonucleotide analogues confirmed that these oligomers were grossly degraded within this time period (L.A.B, unpublished observation). One potential way of circumventing this problem is by reducing the level of many mitochondrial proteins and enzyme activities before the addition of the antisense oligonucleotide, and then assessing the efficacy of antisense downregulation by comparing the recovery rates of COX activity in control and oligomer-untreated cells. In the following paper, we detail such a regimen which involves pretreatment of human liver Hep G2 cells with the mitochondrial synthesis inhibitor thiamphenicol (TAP), and present our results obtained with antisense oligonucleotides directed against the transcript encoding human COX subunit VIIa-Liver (VIIa-L).

METHODS

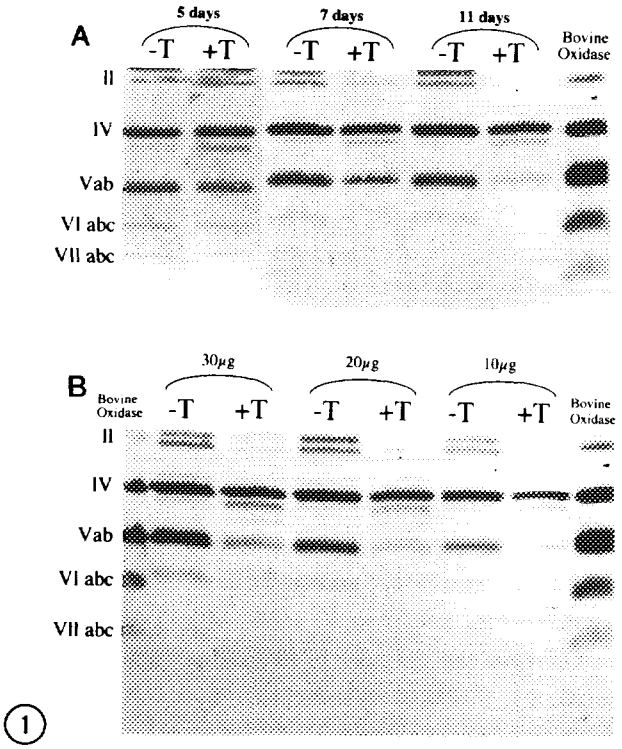
Miscellaneous: All manipulations were performed on human Hep G2 liver cells, grown as previously described (12). Whole cell COX microassays, based on the oxidation of diaminobenzidine, were also detailed in (12). Thiamphenicol (Sigma), was dissolved in 100% ethanol and the concentration calculated spectrophotometrically (E_{mM} 0.64 @ 273nm) to ensure that exactly 50μg/ml final concentration was used in all experiments. An equal volume of ethanol was added to untreated control cells.
Mitochondrial preparation: All manipulations were at 4°C. Cells were removed from flasks using 1mM EDTA and washed twice in homogenisation buffer (0.6M mannitol, 10mM Tris-HCl pH 7.4, 1mM EGTA, 0.1% BSA, 1mM PMSF), resuspended in homogenisation buffer to 50 x 10^6 cells/ml and hand homogenised (15 strokes) in a glass:teflon tissue grinder. An equal volume of buffer was added, and after centrifugation at 600g 10mins, the supernatant was decanted and the pellet rehomogenised as before. The two supernatants were combined and a further 600g clearing spin was performed. The supernatant was removed and spun at 15000g 10mins and the resultant mitochondrial pellet was washed in buffer minus BSA before final resuspension in a minimal volume of wash buffer. Mitochondrial protein concentration was estimated using the Bradford method and aliquots were flash frozen in liquid nitrogen.
Oligodeoxynucleotide synthesis and incubation: The following fully sulphurized oligonucleotides were synthesized on an Applied Biosystems Model 380A oligonucleotide synthesizer using tetraethylthiuram disulphide as the sulphurizing reagent:

Oli-1, 5'-AGATTCCGCAGCATCTTGGCTGTTAC-3; Oli-2 5'-CCGCAGCATCTTGGCT-3'; Random, 5'-CTCGCGTGCTAGTCAGC-3'. Oli-1 and Oli-2, complementary to the mRNA sequence of subunit VIIa-L, are centered over the initiation codon (14). 'Random' oligomer contains the same base composition as Oli-2 but with a randomized sequence. To assess efficacy of antisense down regulation, cells were grown in TAP for 9 days (as outlined above) before counting and seeding in 96-well plates at 8.5 x 10^4 cells/well After a further 2 days in a 200µl aliquot of medium supplemented with TAP, cells were washed and resuspended in EMEM to a final volume of 80µl. Oligomer was added in PBS to TAP-free cells, and an equal volume of PBS was added to control cells. To minimize oligomer degradation, foetal calf serum was heat inactivated (30min @ 56°C) and added to a final 10% v;v 5 hours post oligonucleotide addition. Western blotting: Mitochondrial protein (6-100µg) isolated from cells after various times of incubation in TAP, were solubilized in dissociation buffer (4M urea, 5% SDS, 50mM DTT final concentrations) and separated, together with molecular weight standards and purified bovine oxidase, by electrophoresis through a 19% polyacrylamide;0.1% SDS gel (1120 V.hrs). Individual COX subunits were visualized after transfer of the protein to Immobilon filters (Pharmacia)(15) using either anti-holoenzyme or subunit specific polyclonal antibodies. Amplification of the signal was achieved using a biotinylated secondary antibody and streptavidin-alkaline phosphatase conjugate, affording visualisation of the antigen by the standard colour reaction. Relative amounts of each subunit were calculated by video densitometry (Ultra-Violet Products Ltd. Package SW2000) on several repeats of differential protein loadings.

RESULTS

Susceptibility of COX subunit levels and activity to thiamphenicol treatment- The first step of our antisense strategy required evidence that nuclear COX gene products decreased in cells inhibited for mitochondrial protein translation. Consequently, human liver Hep G2 cells were exposed to sublethal doses of TAP, the methylsulphonyl analogue of chloramphenicol, and mitochondria were isolated from these cells at numerous time points during an eleven day treatment with the inhibitor. Protein aliquots were separated by denaturing gel electrophoresis before being subjected to Western analysis. To account for inconsistencies in protein transfer and membrane binding, several different amounts of protein were loaded for each time point. These blots were probed with holoenzyme antibody (Fig 1) or with monospecific antisera to COX subunits MtII, IV, VIa, VIbc and VIIa and the amounts of individual subunits present at the indicated time points were calculated by video densitometry (Fig 2). Activity of COX was also compared during the time course and is presented as a percentage of the untreated control cells in figure 2. In the presence of 50µg/ml TAP, COX activity drops to less than 20% of control untreated cells within five days. Concomitantly, levels of subunits MtII and COX VIIa-L also drop within this period. Intriguingly, steady state levels of COX subunit IV remained high throughout the eleven day period. These results show that by inhibiting mitochondrial protein synthesis, levels of mitochondrial gene products decrease and the majority of the nuclear-encoded COX polypeptides are unstable in their absence.

Subunit VIIa-L antisense oligodeoxynucleotides decrease the recovery rate of COX activity in thiamphenicol-pretreated cells- Thiamphenicol pretreated cells recover full COX activity within five days (Z.M. C-L, unpublished observation) making it possible to compare recovery rates on addition of anti-COX oligonucleotides. To verify this approach, two anti-COX subunit VIIa-L oligodeoxynucleotides were synthesized (qv **materials and methods**). Both phosphorothioate oligomers were targetted to the AUG start codon of the VIIa-L transcript. Hep G2 cells were grown in TAP for nine days, before transfer to microtitre plates. Forty-eight hours



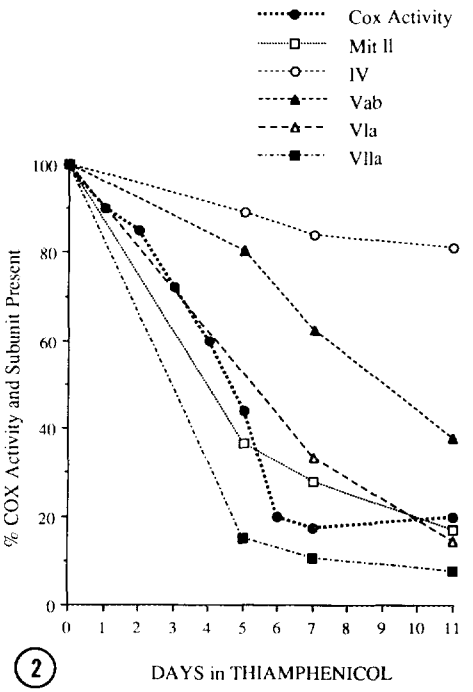


Figure 1. Effect of thiamphenicol on the intramitochondrial levels of COX subunits. A; Mitochondrial protein (20μg) was isolated from cells after the indicated incubation period in the presence (+T) or absence (-T) of TAP. Protein was separated by gel electrophoresis and COX subunits identified with anti-COX holoenzyme antibodies. B; To establish the fidelity of quantitation using Western analysis, differential amounts of protein were analysed after each incubation period (The figure shows protein isolated after eleven days incubation).

Figure 2. Quantitation of COX activity and subunit levels after thiamphenicol treatment. Cells were incubated in TAP for various time periods and COX subunits/activities were estimated as detailed in **materials and methods**. Activities and polypeptide levels are represented as a percentage of the levels estimated in identical amounts of mitochondrial protein isolated from control-untreated cells.

later, TAP was removed and fresh media supplemented with one of the anti-VIIa-L oligomers to 0.1, 1 or 10μM final concentration. Cells were bathed in the oligonucleotides for four days before COX activity was assayed (Table 1). At the highest concentration, both oligonucleotides induced a decrease in COX recovery rate in comparison to the oligomer-untreated cells. To confirm this result, and to establish whether the decrease in recovery was specifically due to the anti-VIIa-L oligomers or a nonspecific effect induced by the addition of high concentrations of oligonucleotides, the experiment was repeated with a control oligomer. Large scale (10μmol) syntheses of anti-VIIa-L oli-2 and an oligomer identical in base composition but differing in nucleotide sequence (random; qv **materials and methods**) were undertaken. Cells were pretreated as before, and oligonucleotide added to 10μM final concentration. Figure 3 shows a comparison of the recovery rates of COX activity in control and oligomer-treated cells measured over four days. As noted before, COX activity was clearly lower than control after a four day incubation with anti-subunit VIIa-L oli-2, although the recovery rate of COX for both sets of

TABLE 1: Recovery rates of cytochrome *c* oxidase activity in cells treated with antisense oligonucleotides directed against subunit VIIa-L. Cells were pretreated with thiamphenicol before addition of the oligomers and incubated for 4 days in the absence of thiamphenicol. COX activity was assayed in control and oligomer-treated cells and is expressed as a percentage of the activity in oligomer-untreated cells[a].

	ANTI-VIIa-L OLIGONUCLEOTIDE	
(μM)	OLI- 1 %[a] ± SD	OLI- 2 %[a] ± SD
10	69 ± 4	49 ± 11
1	78 ± 12	71 ± 2
0.1	102 ± 4	78 ± 9

oligomer-treated cells was identical to the untreated controls for the first two days. COX activity in cells treated with the control oligomer were, however, similar to the untreated cells throughout the duration of the experiment. Each time point represents the mean of five independently-treated aliquots of cells. We therefore conclude that oli-2 is likely to be acting by specifically inhibiting COX activity by down regulating levels of subunit VIIa-L.

DISCUSSION

The five isolated complexes responsible for coupling oxidative phosphorylation in the mammalian mitochondrion comprise more than 70 polypeptides, of which only thirteen are

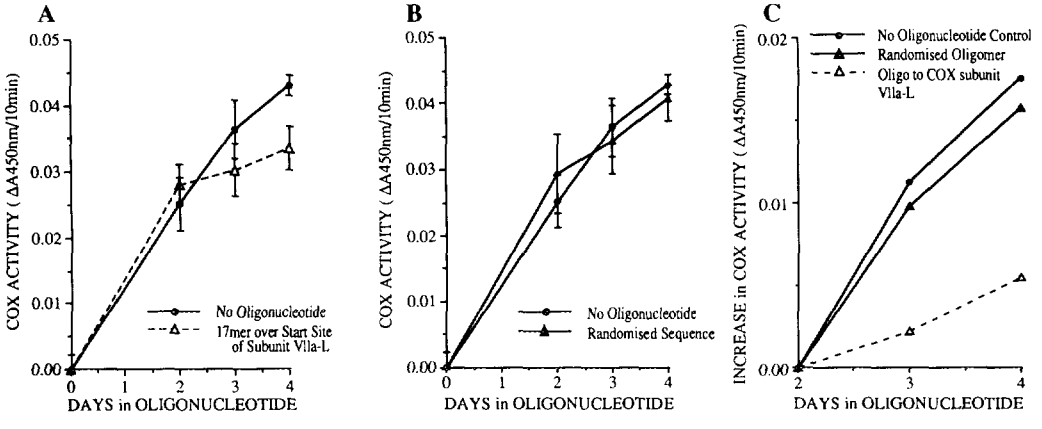


Figure 3. Effect of antisense oligonucleotides on the COX recovery rate of thiamphenicol-treated cells. Cells were pretreated with inhibitor and exposed to 10μM of either anti-COX subunit VIIa-L Oli-2 (panel A) or a 'random' oligomer (panel B; qv **materials and methods**). Panel C highlights the difference in COX recovery rate between cells exposed to either oligomer-untreated or 'random' oligomer and anti-COX VIIa-L oligomer treated cells. COX activity of whole cells estimated by the DAB-linked microassay () is represented as a change in absorbance at 450nm over a 10min period.

encoded by the mtDNA. The functions of the vast majority of the nuclear gene products are unknown. To elucidate the function of a subset of these polypeptides, namely the nuclear-encoded COX subunits, we have established an antisense oligonucleotide rationale. To facilitate this approach we have had to overcome the problem of the extensive half-life of COX. The method of choice was to nonspecifically reduce the levels of target subunits by pretreatment with TAP before addition of the antisense oligomer, and then to assess the oligomer's effect on the recovery rate of COX activity. It has been known for many years that TAP acts as an effective mitochondrial protein synthesis inhibitor and is preferred to the naturally occurring inhibitor chloramphenicol due to its resilience against hepatic detoxification (16). Furthermore, there have been several reports of a concomitant decrease in mitochondrial COX activity (17, 18). We are unaware, however, of any analysis of the temporal changes in COX subunit levels in cells treated with TAP. Addition of TAP to the culture medium dramatically reduced the levels of most nuclear COX gene products analysed. There was, however, little reduction of COX subunit IV. The intriguing observation that COX IV is stable in the absence of other partner polypeptides is in accord with several published reports (20,21).

Other work, however, appears to contradict these findings. Tetracycline derivatives have also been used to inhibit mitochondrial protein synthesis, both in cultured cells and *in vivo*. In contrast to our observation, intravenous injection of rats with oxytetracycline was reported to produce a generalized decrease of all COX subunits when liver mitochondrial protein was analyzed (21). Under these conditions, however, COX activity was inhibited by only 50% and no direct subunit quantitation was documented. A study to assess the efficacy of immuno-flow cytometric quantitation of COX subunits as an indicator of COX deficiency also showed decreased levels of all COX polypeptides, including COX IV, in human leukaemia cells treated with doxycycline (22). The legitimacy of this approach was established by comparing levels of subunit IV in solubilized mitochondria from doxycycline-treated cells, immunoprecipitated with holoenzyme antibody and quantitated after separation by gel electrophoresis. If the holoenzyme was unable to immunoprecipitate uncomplexed subunit IV, the demonstrated reduced levels of this polypeptide would have been artificially low. Consequently, we feel it is necessary to stress the hazards of using COX subunit IV monospecific antibodies (or holoenzyme antibody whose major epitope recognition sites lies within subunit IV) to preclude COX deficiency in patients potentially suffering from mitochondrial cytopathies.

The possibility of assessing the function of nuclear COX gene products by assaying the affect of antisense oligonucleotides on the recovery rate of COX activity, was tested by antisense oligomers directed against subunit VIIa-L. Efficient inhibition of recovery rate was noted in each of several independent experiments. The inhibition was also shown to be sequence dependent, as COX activity in cells treated with identical concentrations of the VIIa-L random oligomer recovered at a similar rate to the control. A substantial time lag of inhibition for the anti-VIIa-L, however, was repeatedly noted. Recovery rates were identical for the oligomer-treated and control cells during the first two days, after which the rate was dramatically lowered in the anti-VIIa-L cells. Western analysis had confirmed the substantial decrease in mitochondrial COX VIIa-L and the extramitochondrial amounts were negligible after TAP treatment. Thus, *if the decrease in recovery rate is indeed due to successful antisense inhibition,*

why is the inhibition not more immediate ? One explanation is that the nucleus was able to 'sense' the mitochondrial stress caused by mitochondrial protein synthesis inhibition, and responded by expressing a protein which functions to protect transcripts whose products are mitochondrially destined. Protection of mRNA by formation of ribonucleoproteins is a well documented phenomenon which has also previously been reported to impair binding of antisense RNA (23) and strong evidence for similar interactions mediating the tissue-specific expression of COX isoforms has also recently been reported (24). We have recently calculated COX and control transcript half lives in cells pretreated with TAP and have demonstrated a stabilizing effect of the inhibitor on COX transcripts (Z.M.C-L and R.N.L, manuscript in preparation) . Although the COX transcript steady state levels were similar for control and treated cells, the half lives were increased by greater than ten fold for COX 4 and eight fold for COX 7a-L. No difference was noted for β-actin, tubulin or several mitochondrially-encoded transcripts. We are currently attempting to identify an RNA-binding protein activated or induced by TAP.

In conclusion, we have highlighted the stability of COX subunit IV in the absence of other partner COX proteins and established an inhibitory effect of anti-COX subunit VIIa-L on enzyme activity in cultured cells. We believe this antisense protocol may prove invaluable for confirming which of the nuclear-encoded COX subunits are required *in vivo* for assembly of a fully functional COX complex and consequently may aid in localizing the molecular defect associated with some autosomally inherited mitochondrial cytopathies.

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