
Gene for OTC: characterisation and linkage to Duchenne muscular dystrophy

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SUMMARY

Cloned coding sequences for rat and human ornithine transcarbamylase (OTC) were obtained by screening a rat and a human cDNA library respectively with a synthetic oligonucleotide corresponding to 27 bases of the rat sequence. These clones, 1100 bp long for the rat clone and 1300 bp for the human, contain approximately 80% of the human OTC coding sequence. The OTC mRNA length determined by Northern blot analysis is 1700bp. The human OTC sequence was shown to be localised Xp_{11.4}-Xp₁₁ using somatic cell hybrids. There is a frequent RFLP revealed with the restriction enzyme MspI. OTC is located more closely to the Duchenne muscular dystrophy mutation than previously reported markers such as RC8 and L1.28, and therefore should prove useful in carrier detection and haplotype analysis of families carrying the mutation causing the disease.

INTRODUCTION

Ornithine transcarbamylase (OTC) (EC 2.1.3.3) is a mitochondrial enzyme of the urea cycle found in the liver, which catalyses the condensation of carbamylphosphate with ornithine to form citrulline (1). This enzyme has several important biological and clinical features. The structural gene is localised on the X chromosome (2,3) and is thus a good candidate for study using molecular approaches to X inactivation. The enzyme is synthesised in the cytoplasm as a larger precursor and is then transported to the mitochondria with apparently coincident processing to its mature form (4,5). It is consequently a good model for studies of compartmentation and post-translation modification. A partial sequence of the rat cDNA (6), and more recently the complete sequence of the human cDNA, has been published, shedding light on the aminoterminal peptide involved in the up-take and processing of the enzyme (7). The precise mechanism of transport remains to be elucidated. OTC is therefore a useful model for the study of the biogenesis and turnover of cytosolic synthesised mitochondrial proteins.

We have previously reported that the mutations causing the X-linked diseases Duchenne muscular dystrophy (DMD) and Becker muscular dystrophy (BMD) are located in the region Xp21, linked to and between two random cloned human DNA sequences RC8 and L1.28 (8,9). Since the coding sequence for OTC is

located at this region of the human X chromosome, it may be of value in linkage mapping and carrier detection for DMD, BMD and other X-linked diseases which map to Xp21 and surrounding regions.

We describe the isolation of two clones obtained by screening a rat and a human cDNA library. We have characterised these clones, identified the mature mRNA seen in the cytoplasm and compared the cDNA restriction maps with the human sequence recently published (7). We demonstrate the potential of this probe, which is closer to DMD than previously reported DNA markers, for the study of the Duchenne mutation.

MATERIALS AND METHODS

Chemicals were of the highest purity available and were from Merck, Sigma or Boehringer-Mannheim. Restriction enzymes were from Genofit (Hinf I, Alu I) and Biolab (Pst I, Hind III, Bgl II, BamHI, Pvu II Kpn, EcoRV). Agarose was from Biorad. Royal XO Mat AR films were from Eastman Kodak. BA 85 nitrocellulose filters were from Schleicher and Schuell. Radioactivity was measured in a liquid scintillation counter (LKB 1211 Rackbeta). Horizontal electrophoresis were performed in a Biorad apparatus.

RNA PURIFICATION

Total cellular RNAs were prepared by ethanol precipitation in guanidinium hydrochloride as described (10,11). Polyadenylated RNAs were isolated by chromatography on oligodT cellulose (12).

CHEMICAL SYNTHESIS AND PURIFICATION OF THE OLIGONUCLEOTIDE

The oligonucleotide 27-bases long was synthesised on a solid support by Pasteur Institute. Purification was performed by polyacrylamide (20%) gel electrophoresis in the presence of 8M urea 8m. The sample was diluted in the following buffer: 0.8% formamide (v/v)/ 0.005M EDTA/0.02% bromophenol blue/0.05% xylene cyanol, at the rate of 1 µl of buffer for 1 OD of oligonucleotide. Electrophoresis was run at 40 mA (1100 volts) until xylene cyanol had reached the middle of the gel. The gel was then analysed on a fluorescent plate under UV 254 nm and the upper band was recovered. The product was taken out of the gel by diffusion in 0.1M triethylammonium at 37°C overnight. The supernatant, evaporated and rediluted in the minimum of 0.05M triethylammonium, was completely purified on a Sephadex G10 column. Elution was performed with the same buffer and the first peak recovered.

The oligonucleotide was labelled at the 5' end by transfer of ³²P from (³²P)ATP using T4 polynucleotide kinase as described previously (13).

SCREENING OF THE cDNA LIBRARY

The rat liver cDNA library was kindly supplied by M.P. Simon et al (14)

and the human liver library was a kind gift from Derek Woods (15). The prehybridisation was performed for 2 hours at 55°C in 6 X SSC/0.5% SDS/5 X Denhardtts/carrier DNA (100 µg/ml). Hybridisation was carried out overnight in the same buffer containing 3 X 10⁴ cpm/ml (10 ml/filter) of the ³²P-labelled oligonucleotide. The filter was then washed in 6 X SSC at 0°C 10 min and in 6 X SSC at 37°C for 10 min each four times. They were then washed in alcohol, dried and exposed for 6 hours at -80°C to Kodak X OM at AR film with intensifying screens.

Recombinant plasmids were amplified with chloramphenicol (17). Clear lysates were prepared and plasmid DNA purified by centrifugation in CsCl/ethidium bromide density gradient (18).

NORTHERN BLOT HYBRIDISATION ANALYSIS

RNAs were separated by electrophoresis in 1.5% agarose gels in 10mM phosphate buffer pH 7.4, 1.1M formaldehyde. RNAs (2 to 10 µg) were heated 10 min at 65°C in 50% formamide, 10mM phosphate, pH 8, 2.2M formaldehyde/1mM EDTA. They were then loaded in 1/5 X v of 0.025% bromophenol blue/25% glycerol/25mM EDTA. After migration RNAs are transferred directly to nitrocellulose in 20 X SSC and hybridised with nick-translated OTC DNA insert. Prehybridisation was for 4 hours at 42° in 50% formamide, 5 X SSC, 5 X Denhardtts, 50mM Na-phosphate pH 6.8, 1% glycine, 500µg/ml carrier DNA. Hybridisation was in 50% formamide, 5 X SSC, 1 X Denhardtts, 20mM Na-phosphate, pH 8.8, carrier DNA 100 µg/ml. Washing solution was with 2 X SSC at room temperature.

HYBRID LINES

Somatic cell hybrid lines were a kind gift of Professor H-H Ropers and have been described in detail elsewhere (28). Hybrid Sin176 was constructed from a fibroblast line obtained from a skin biopsy of a female deleted in the region Xp_{11.4}-Xp_{21.3} (24).

RESTRICTION MAPS OF THE cDNA CLONES

Plasmid DNA were first diluted in 10 mM Tris-HCl buffer, pH 8.3/0.1 mM EDTA to a final concentration of 0.2 µg/ml. We used the simplified buffers described by Maniatis et al (18) for the digestion with 2 units of each enzyme/µg of DNA, for 2 hours incubation. Restriction fragments were analysed either by 1% (w/v) agarose, 5 or 7.5% (w/v) polyacrylamide gel electrophoresis in 50mM Tris-borate, pH 8.3/EDTA 1 mM. Polyacrylamide gel was used instead of agarose when the fragments to be analysed were smaller than 400 bp.

SOUTHERN BLOT ANALYSIS OF DUCHENNE MUSCULAR DYSTROPHY FAMILIES

Blood samples were collected and analysed as previously described (8).

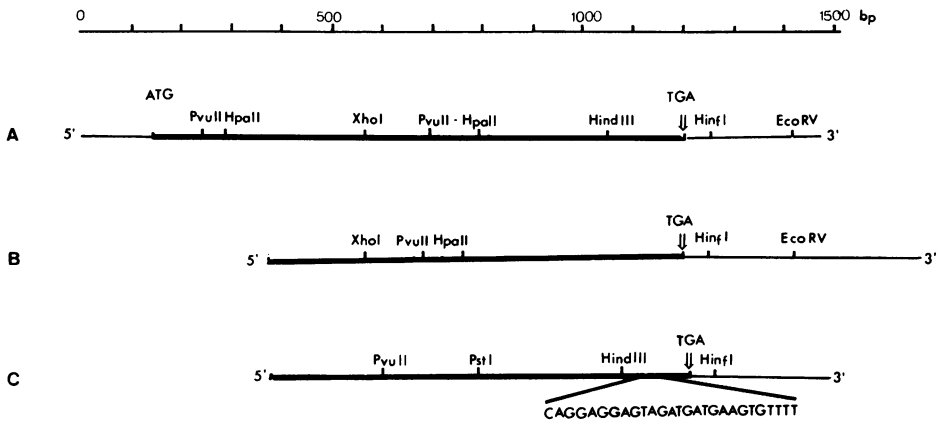


Figure 1 Partial restriction map analysis of the human and rat OTC clones. Comparison with the restriction map of the human clone published by Horwich *et al* (6). The inserts are represented by thin lines, the coding sequence by thick lines.
 A - Human OTC clone according to Horwich *et al* (6)
 B - Human OTC clone
 C - Rat OTC clone
 The sequence of the synthetic oligonucleotide used for the screening is positioned on the rat clone C.

PATIENTS

The diagnosis of DMD was established on the basis of clinical evaluation (proximal limb weakness and atrophy, weakness of the back muscles and neck flexors, enlarged calves, tight heelcords) highly evaluated serum CK level and muscle biopsy demonstrating dystrophic pathologic alterations.

RESULTS

Human and liver libraries were screened as described in methods with an end-labelled oligonucleotide consisting of 27 bases deduced from the published sequence (6,7). The partial restriction map analysis of the resulting clones is shown in figure 1 and compared with the restriction map of the human clone published by Horwich *et al* (7). Our human clone is identical except for one Hind III which is not present in our human OTC clone. The cDNA contains 80% of the total coding region of this gene and demonstrates that there is a fairly long stretch of non-coding sequence at the 3'-end. Confirmation that this sequence does code for ornithine transcarbamylase was obtained by the antenatal diagnosis of OTC deficiency in a patient lacking the enzyme and known to possess a deletion at this locus (32).

The size of OTC mRNA was determined by Northern blot analysis. As shown in figure 2, the rat probe hybridised with an mRNA species of 1700bp which

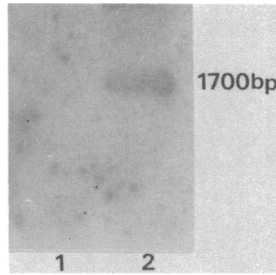


Figure 2 Northern blot analysis of OTC mRNA. RNA (20µg) was transferred from 1% (w/v) agarose/formaldehyde gel onto nitrocellulose filter as described in Materials and Methods. The RNA was probed with nick-translated rat OTC cDNA. Lane 1 RNA from rat kidney. Lane 2 polyA⁺ RNA from rat liver. Sizes were obtained from HindIII digest of DNA. Autoradiography was for 24 hours.

appears to be expressed predominantly in the liver. The human cDNA probe showed a similar result. Long exposures indicate a 1700bp species expressed in kidney. Verification that this mRNA species encodes the OTC gene was

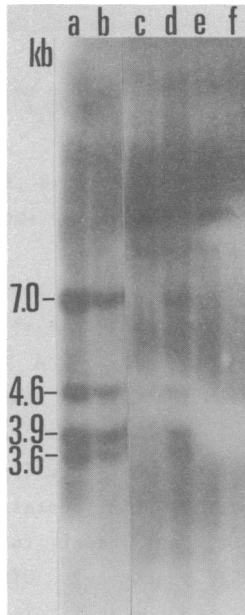


Figure 3 Localisation of the OTC gene. BglIII digest of (a) female (46,XX) DNA; (b) male (46,XY) DNA; (c) (d) and (e) hybrid cell lines with the following human X chromosome segments: (c) Xp11.0-Xqter; (d) Xp11.4-Xqter; (e) Xp11.4-Xqter but deleted Xp11.4-Xp21.3 (f) Chinese hamster parent of hybrid lines.

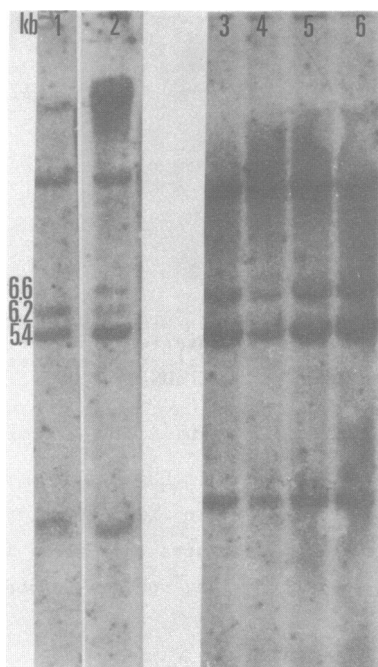


Figure 4 Southern blot of random females and a male after restriction enzyme digest with MspI. Lane 1 male, lanes 2-6 females.

obtained by analysing polyA⁺ mRNA enriched for OTC coding sequences by polysome-immunoprecipitation. Only the 1700bp species was observed (data not shown).

Ornithine transcarbamylase deficiency is known to be an X-linked disorder (21). The X-specificity of the human clone is shown in figure 3 together with its localisation to the region Xp_{11.4}-Xp₂₁. A BglII digest was performed since this allowed the bands from the Chinese hamster parent DNA of the hybrid to be resolved from the human bands. All of the bands dose with the X chromosome. Thus no autosomal pseudogenes are apparent at this stringency. Bands are present in the hybrid cell line containing Xp_{11.4}-Xq_{ter} as its only human X chromosome counterpart but absent in the cell line containing Xp_{11.0}-Xq_{ter} (lanes c and d respectively). OTC does not give a signal with the hybrid Sin 176 which is deleted for Xp_{11.4}-Xp_{21.3} (lane e). A signal was observed, as expected, with a hybrid containing Xp₂₁-Xq_{ter} (results not shown). Thus OTC must lie in the proximal area of the Sin 176 X chromosome deletion, in the region Xp_{11.4}. Hybridisation is seen with Chinese hamster

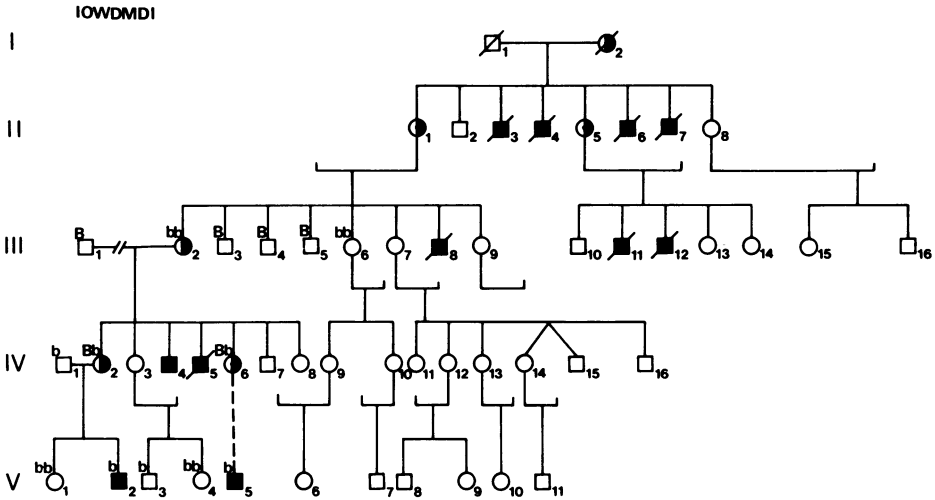


Figure 5 DMD family segregating for the OTC polymorphism. (○, normal female; □, normal male; ●, carrier female; ■, affected male). Alleles A and a refer to the OTC locus and are seen as bands at 6.6kb and 5.8kb respectively in a Southern blot. (Dotted line between IV-6 and V-5 indicates uncertain paternity).

DNA demonstrating conservation of this sequence. The OTC sequence does cross-hybridise with many non-X-linked bands at low stringency (3 X SSC). The relationship of these sequences to the functional gene is unknown at present.

The DMD locus has been identified in the region Xp₂₁ by linkage to flanking DNA markers at ~15cM on either side (8). One of these markers, laboratory acronym L1.28, is localised at Xp_{11.4} proximal to OTC (24). OTC

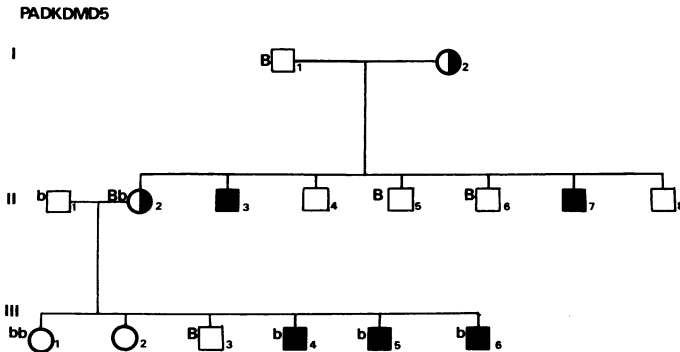


Figure 6 DMD family segregating for the OTC polymorphism. (Nomenclature as in figure 5).

TABLE 1 Linkage data for Duchenne muscular dystrophy and OTC

| $\theta =$ | 0.05 | 0.1 | 0.15 | 0.2 | 0.25 | 0.3 | 0.35 | 0.4 | 0.45 |
|------------|--------|--------|--------|--------|--------|--------|--------|--------|--------|
| PADK DMD5 | +1.650 | +1.486 | +1.314 | +1.134 | +0.947 | +0.755 | +0.560 | +0.366 | +0.178 |
| IOW DMD1 | +0.938 | +0.811 | +0.738 | +0.629 | +0.516 | +0.400 | +0.286 | +0.178 | +0.081 |
| TOTAL | +2.588 | +2.327 | +2.052 | +1.764 | +1.463 | +1.155 | +0.846 | +0.544 | +0.259 |

Results are expressed as lod scores (log of the odds) for different values of recombination fraction (θ).

should therefore provide a good closer marker for DMD. We searched for restriction fragment polymorphisms with this DNA probe using a panel of random females and one male. Figure 4 shows the MspI polymorphism detected in such a panel. One allele is seen as a 6.6kb band and the rarer allele gives a band at 6.2kb in a Southern blot. Thirty per cent of the females studied were heterozygous at this locus.

Figures 5 and 6 show the segregation of this polymorphism in two DMD families. In family IOWDMD1 no crossovers are observed and the OTC allele b is inherited with DMD in all other individuals in the pedigree. In family PADKDMD5, the OTC locus also cosegregates with DMD in all meioses. The lod scores for linkage between DMD and OTC are given in Table 1.

DISCUSSION

The partial nucleotide sequence of the rat OTC recently published (6) has allowed the synthesis of a 27 base oligonucleotide corresponding to part of the 3' coding region. With this probe, a rat and a human cDNA library were screened. Although prepared using data for the rat gene, the probe successfully identified an OTC clone in the human cDNA library. The clone was confirmed as coding for OTC because it was absent in a patient deficient for the enzyme (32). The human OTC clone obtained has 80% of the coding sequence and has a restriction map very similar to that reported by Horwich et al (7). Nevertheless, it diverges in the Hind III site. The rat clone is a little more divergent but differences such as the Pst I site can be explained by a conservative mutation (leu CTC giving LEU CTG in the codon 147) (7).

We found a 3' untranslated region of 500 bp as also described previously (7,22). Examples of such mRNA with even longer 3' non-coding region have been published for L-pyruvate kinase (16) interferon (17) and crystallin (18). The significance and the physiological role of these regions are not clear. In some of them alternative polyadenylation sites and corresponding alternative stop transcription points are found resulting in several mRNA species (20) but

this does not seem to be the case for OTC (6).

In this study we found a 1700bp mRNA species hybridising with the OTC probe on a Northern blot. The 1700bp band appears to be liver specific and is in good agreement with previously reported data (7,19). This band was also the only species observed in polyA⁺ RNA obtained after polysome-immunoprecipitation with OTC antibodies. It is interesting to note that the AATAAA sequence, which is supposed to play a role in polyadenylation, is not present in the rat sequence but is present in the human one.

The hybrid cell lines described here localise OTC to the region Xp_{11.4} on the human X chromosome. A signal was observed in the cell hybrids containing Xp_{11.4}-Xq_{ter}, in a hybrid containing Xp_{21.1}-Xq_{ter}, and also in the hybrid deleted for the region Xp_{11.4}-Xp_{21.3}. The result is at variance with that reported by Lindgren (33) who localised OTC within Xp₂₁. This discrepancy cannot be simply explained by a rearrangement of the Xp_{11.4}-Xq_{ter} hybrid during growth since the localisation of OTC in Xp₂₁ would represent a gain of X chromosomal material. Alternatively, it may be that the chromosome aberration in the patient from which this hybrid was originally made is more complex and possibly a rearrangement preceded the translocation event. If this is a highly mutable region, then inversions and deletions might be more common than elsewhere in the genome. For example, we have reported two cases of deletions for Xp_{11.4}-Xp_{21.3} in females (24,32) and Lindgren *et al* (33) have reported another. Whether this is related to the high mutation rate observed for Duchenne muscular dystrophy should be resolved through the ordering of more closely linked probes.

At present, the study of the DMD mutation by linkage has progressed due to the isolation of several cloned DNAs located at Xp₂₁ (8,28,30,31). Carrier detection can only be carried out reliably in families informative for at least one probe on each side of the DMD mutation. Even the most informative RFLP will only be useful in approximately one half of the families, so it is essential to find more sequences with frequent RFLPs which are linked to DMD. OTC is one such probe, as it is informative in approximately one third of the DMD families, and gives an RFLP which is easy to analyse experimentally. In a random panel of twenty Caucasian females, six were found to be heterozygous at this locus. Since OTC maps to the region Xp_{11.4}-Xp₂₁, it should be very useful not only for genetic mapping of the short arm of the human X chromosome, but also for the carrier status determination of females at risk for DMD.

In the family PADKMD5 in figure 6, the DMD mutation appears to

cosegregate with the b allele at the OTC locus and the B allele segregates with the DMD normal allele. If this is the case, no recombinants are apparent in seven meioses (II-2, II-5, II-6, III-3, III-4, III-5, and III-6). Female III-1 has inherited b from her carrier mother and b from her father, indicating that, unless there has been a crossover, III-1 is a carrier. This family was uninformative for RCE, L1.28, and 754 and could not previously be offered any information on carrier status. Similarly, in family IOWDMD1, allele b is segregating with the DMD mutation. V-1 has inherited the b allele from both parents and therefore has a high risk of being a carrier.

The two families presented here suggest that OTC is closely linked to the DMD locus and more extensive studies suggest that it is linked at approximately 10cM (Davies et al, in preparation). OTC, used in conjunction with other distal markers, will be an important additional locus to those previously reported (8,29) for the genetic counselling of families.

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