Mapping of the MYCL2 processed gene to Xq22-23 and identification of an additional L MYC-related sequence in Xq27.2

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Abstract We report here the identification of a human genomic sequence from the q27.2 region of the X chromosome which shows a high homology to the L-MYC proto-oncogene. This sequence is not the MYCL2 homology, previously mapped to the long arm of the X chromosome at q22-qter by Morton et al., as we located the MYCL2-processed gene in Xq22-23, using a panel containing a combination of hybrid DNA carrying different portions of the human X chromosome. Based on computer analysis, the MYC-like sequence (MYCL3) is 98.2% identical to a portion of exon 3 of the MYCL1 gene and maps to the Xq27.2 region, between the DXS312 and DXS292 loci.

Key words: Xq27; Gene mapping; MYC homology

1. Introduction

After the completion of the physical map of the entire Xq27 cytogenetic band [1] and the definition of a continuous 12 Mb YAC contig, we started the assembly of a transcriptional map by the systematic search for transcription units. In order to detect functionally transcribed sequences, different methods were developed and applied, testing for STSs by retro-transcribed PCR (RT-PCR) on mRNAs from different tissues, hybridisation of yeast artificial chromosomes (YACs) to heterologous cDNA libraries, Zoo blot analysis for conservation of sequence fragments between divergent species, cDNA direct selection [2–4], trapping of exons [5], STS virtual mapping [6] and expressed sequence tag (EST) mapping. By combining these methods, a number of transcribed sequences have been identified providing a functional analysis of this region of the human genome [7].

Here we report that by using the cDNA direct selection method with the YAC yWXD340 from Xq27.2 and a combination of cDNA libraries, we found that a sequence related to the L-MYC proto-oncogene is located in Xq27.2, a region associated with neurological disorders [8,9]. However, in spite of its proximity to a genomic region with high homology to several ESTs, we were unable to find evidence of its expression, suggesting that the L-MYC-like element (denominated hereafter MYCL3) represents a pseudogene sequence.

2. Materials and methods

2.1. YAC genomic clones, libraries and Southern blot analysis

The YAC yWXD340 used for the cDNA selection is a genomic clone from the yWXD X3000.11 library [10]. It is located in Xq27.2 and carries the probe DXS292 [1]. Gridded filters containing YACs specific for the Xq27 chromosomal region were prepared according to reported procedures [10]. Southern blot analysis was performed as in Sambrook et al. [11]. Radiolabelling of DNA probes was performed by random priming [12] using [α-^{32}P]dCTP.

2.2. YAC DNA preparation, linker ligation and biotin DNA fragments conjugation

Chromosomal DNA preparation of the clone yWXD 340 was carried out in agarose blocks. The YAC was isolated by pulsed field gel electrophoresis (PFGE) and excised from the AB1380 host chromosomes. Five blocks containing the isolated yWXD340 YAC DNA were *Sau*3A (New England Biolabs) digested to completion, the agarose was removed by agarase treatment, as recommended by the manufacturer (Sigma-Aldridge), the DNA was phenol extracted and EtOH precipitated. The YAC DNA fragments were ligated to adapter A [4] and amplified by PCR using a biotin-conjugated oligonucleotide complementary to the same adapter sequence as a polymerisation primer [4].

2.3. cDNA synthesis

The cDNA selection protocol is basically as described in Pizzuti et al. [4]. Poly A mRNAs were isolated from different human tissues and ds cDNAs were synthesised as described in Maggi et al. [13]. The cDNAs were ligated to the adapter B and amplified by PCR using an oligonucleotide complementary to the adapter B sequence as a polymerisation primer.

2.4. YAC to cDNA fragments hybridisation

Fragments of digested YAC and cDNAs in a ratio of 1:25 were mixed, denatured and hybridised for 48 h at 65°C. The hybridisation was carried out in 5×SSPE, 5×Denhart's, 0.1% SDS and 10 μg of Cot1 human genomic DNA and 5 μg of yeast DNA were used for competition. The hybridisation reaction was completed by adding 50 μl of streptavidin-conjugated magnetic beads (Dynabeads Dynal, USA).

The bead-biotinylated YAC fragment cDNA complexes were separated magnetically and after washing in 0.5×SSC at 65°C for 15 min, the DNAs were recovered by boiling in water.

The recovered heteroduplex complexes, containing cDNA and genomic fragments, were amplified using the oligonucleotide complementary to the adapter B as a polymerisation primer and cloned into the pCRII vector (Invitrogen).

2.5. Genomic sequencing and sequence analysis

PCR products were purified from low melting point agarose gel and directly sequenced by the dideoxynucleotide chain termination method using a Perkin Elmer kit. Nucleotide sequence analyses were per-

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Abbreviations: UTR, untranslated region; PFGE, pulsed field gel electrophoresis; YAC, yeast artificial chromosome; RT-PCR, retro-transcribed polymerase chain reaction; EST, expressed sequence tag

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Table 1
Primers and PCR conditions

Primer name	Primer sequence		Primer pair	Product size (bp)	Annealing T*
3IFI	CTAGTCTGGGAAAGTCACCC	MYCL3	31FI/31RI	191	58°C
3IRI	TGGTTTTCCAGAGCCATATA				
np5834	CTCTTTCCCACCTTCCTCCTCAT	MYCL3	np5834/31RI	527	60°C
5FF	TTAAAGTTGTTCTGCAACCATGC	MYCL3	5FF/3F	2055	60°C
3F	AAGCTGGGGTGGGTAGCGG	Genomic			
3R	AGTCCCAACGGCTCTCCCC	(inverse PCR)	3F/3R	590	60°C
pvu57	CACTGCTTTTTGGACAACATGT	MYCL3	pvu57/3REV	2846	60°C
3REV	TATGGCACACAAGACTCTTTTTG	Genomic			
ILE6	AGCGAGCAGAGATTGAGCCAC	MYCL2	ILE6/ILE7	231	62°C
ILE7	CTATCCTTATGCCAGTACCACA				
MYCL2-1	ACCCCCTTCCTCCCACTCTCAATGC	MYCL2	MYCL2-1/MYCL2-2	354	60°C
MYCL2-2	TACAGTTCTCCCTTCTAAGGTGCCT				

*For all primer pairs, denaturation and extension temperatures were at 94°C and 72°C, respectively. PCR products were detected after 30 amplification cycles.

formed with the GCG software package. Homology searches were performed using FastA and BLAST programs.

2.6. Cloning by inverse PCR

The genomic regions at the 5′ and 3′ ends of the sequence homologous to the L-MYC were cloned by inverse PCR from the YAC clone yWXD340 and sequenced. For the 5′ end, Pvu II-digested DNA was diluted and ligated as in Collins and Weissman [14] with minor modifications. A PCR reaction was then performed with primers 5R and 3F according to the cycle conditions reported in Table 1. The 3′ end of the region was also obtained by inverse PCR, starting from *Sau*3A digested yWXD340 DNA. The PCR reaction was performed with primers 3F and 3R. The PCR amplification products were cloned into the pCR2.1 vector (TA cloning kit, Invitrogen).

From the nucleotide sequences generated by inverse PCR, we designed the additional primers pvu57 and 3REV (see Table 1) that allowed us to amplify the complete sequence we produced.

3. Results

3.1. Isolation of cDNAs hybridising to the Xq27.2 YAC contig

The method used is a hybridisation and PCR-based protocol for the isolation of cDNAs encoded by large genomic fragments [15]. The tissue expression pattern was initially unknown for the expressed sequence of interest. Therefore we used a combination of human cDNA libraries including liver, skeletal muscle, placenta, heart and foetal brain. To perform our experiments, we decided to work with YACs carrying evolutionary conserved sequences syntenic to mouse. We started with the YAC yWXD340 containing the locus DXS292 that is conserved in mouse X DNA [16,1]. *Sau*3A restriction fragments from YAC yWXD340 were incubated with the before mentioned cDNA libraries as described in Pizzuti et al. [4]. The cDNAs hybridising to the homologous YAC fragments were cloned and analysed for the presence of Xq27-specific transcripts. We focused on inserts ranging from 100 to 350 bp (19 clones out of 48). Two identical membranes from a sandwich blot containing the amplification products were individually hybridised to total human DNA, yeast AB1380 and pBR322 DNA, respectively, in order to select clones carrying only human non-repetitive sequences. After the double hybridisation screening, we selected five clones containing non-repetitive human DNA, that were individually hybridised to gridded filters containing YACs specific for the Xq27 chromosomal region. One strong signal and four medium/weak signals were obtained by hybridisation of the five selected cDNAs.

The strongest signal was detected using cDNA31 that recognised YAC yWXD340 and other overlapping YACs of the DXS292 contig. The other four cDNAs that gave weak signals, when analysed at the sequence level, did not show any

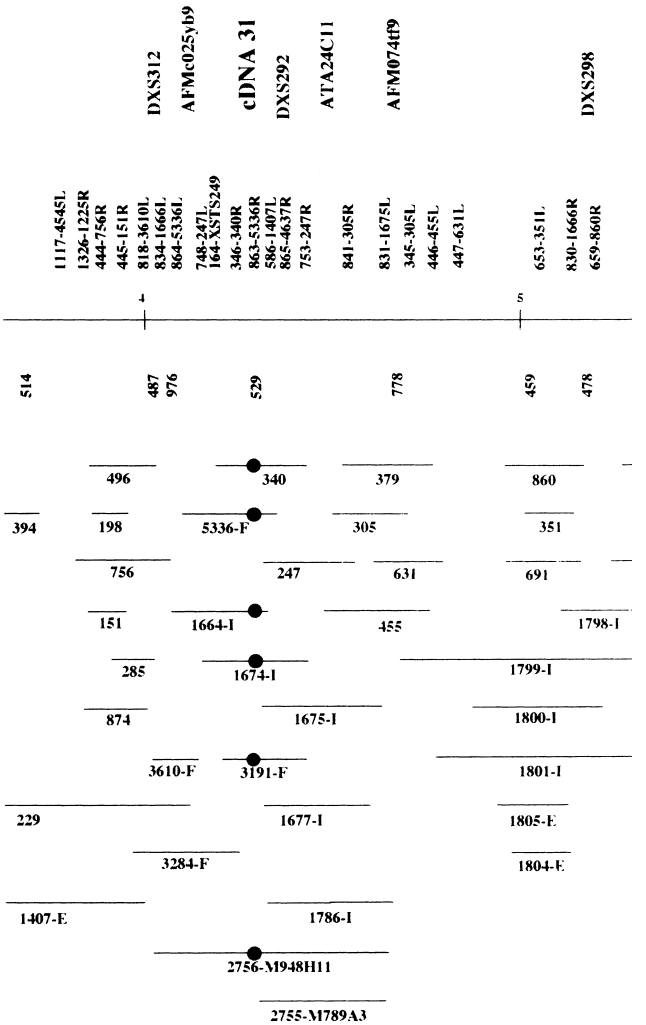

Fig. 1. YAC-based physical map of the Xq27.2 region. YACs hybridizing to cDNA31 are indicated by a black ring. The cDNA31 is located between the DXS312 and the DXS292 loci as shown, since it hybridizes to yWXD1664 that is part of the DXS292 contig, but is negative for the DXS292 probe.

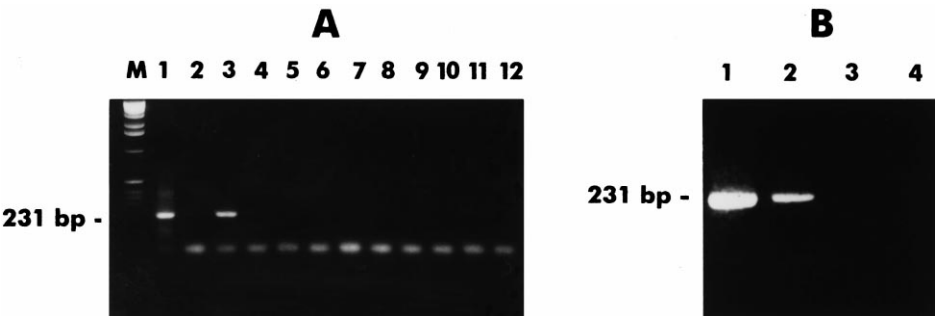

Fig. 2. A: PCR amplification using primers the IL6-IL7, specific for the MYC homologous L2. Lanes 1 and 3: total human and X only DNA. Lanes 2 and 5–12: CHO, YY, Chr7, yWXD340, 1677, 1674, 2756-M948, 1664 and 3191 DNA, respectively. Lane 4: a PCR control containing no DNA. Lane 4: no template. B: mapping of MYCL2 processed gene. PCR assay was performed using the primers IL6 and IL7 from the MYCL2 sequence and DNA from different hybrids. Lane 1: Ben3B hybrid (Xq13-21 to Xqter), lane 2: X only hybrid, lane 3: X3000.11 hybrid (Xq24-qter), lane 4: no template.

homology with known human or rodent DNA sequences and are under investigation at present.

Hybridisation of cDNA31 to the Xq27-specific YAC collection identified the following YACs: yWXD340, 3191, 1664, 1674, 5336 and 2756-M948H11, all belonging to the DXS292 YAC contig as reported in Zucchi et al. [1] and presented in Fig. 1 where it is also shown that cDNA31 is located between DXS312 and DXS292. We excluded the possibility of any artefact, rearrangement or chromosomal abnormalities attributable to the FraXA hybrid DNA [17], the source of the ×3000.11 library since cDNA31 also recognised other YACs, recovered from different collections of YAC clones as the CGM library [18], 5×cell line library [19], 'F' library [20] and CEPH library [21]. Each individual YAC positive for cDNA31 was *Eco*RI digested, blotted and hybridised with the same cDNA31 probe. A single *Eco*RI hybridisation band of approximately 4 kb was found on YAC DNA belonging to the DXS292 contig, no signal was detected in any YACs included in the filter as negative controls (data not shown).

3.2. Mapping of the MYC L2 processed gene

cDNA31 (227 bp) was analysed at the sequence level. In its 227 nucleotides (nt), we found a 98.2% identity with the 3′-untranslated region (UTR) of the MYCL1 gene exon 3, from nt 6166 to 6392.

A different MYC-related sequence, the MYC homologous L2 (MYCL2), was reported by Morton et al. [22] to be present on the long arm of the X chromosome in the q22-qter region. Considering the possibility that this MYCL2 sequence could be located in Xq27, we designed the primers IL6 and IL7 (see Table 1) in a region of the sequence (a.n. J03069) published by Morton et al. [22], where the MYCL2 gene diverges from MYCL1. Theses primers amplified a PCR fragment of the expected size (231 bp) from total human DNA and X only sorted DNA (see Fig. 2 (A, lanes 1 and 3)), as well as from Ben3B hybrid containing the Xq13-p21 to Xq-ter region (Fig. 2, B, lane 1). However, no PCR product was obtained from YAC DNA of the DXS292 contig or ×3000.11 DNA (Fig. 2, A, lanes 7–12 and B, lane 3, respectively).

Consistent with this result is that when we used a PCR probe, obtained with the primers MYCL2-1 and MYCL2-2 (derived from the exon 3 of the MYCL2 sequence, see Table 1) to hybridise an *Eco*RI Southern blot, we did not detect any bands from the ×3000.11 DNA (Fig. 3, A, lane 4) while we obtained a 6.5 kb fragment (corresponding to the MYCL2

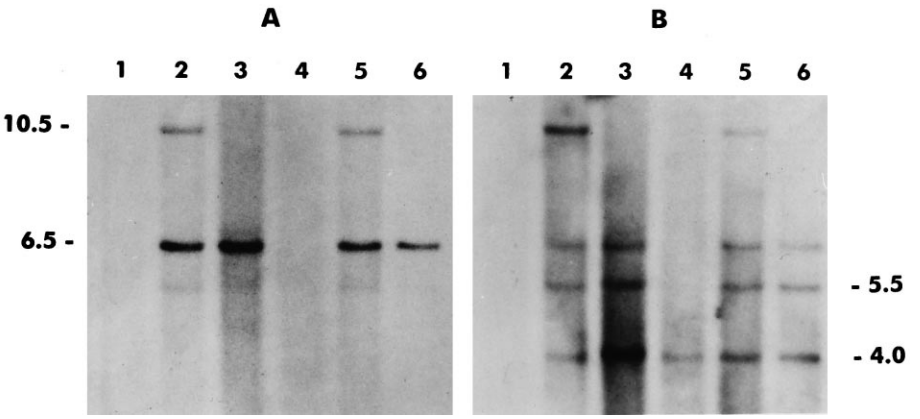

Fig. 3. Hybridization of the MYCL2-derived probe (A) and cDNA31 (B) to *Eco*RI-digested genomic DNAs. A: A probe generated by primers MYCL2-1 and MYCL2-2 from the MYCL2 gene was hybridized to the following DNA: lanes 1–6: CHO, total human, X only, ×3000.11, total human and 5×DNA, respectively. The ×3000.11 DNA (lane 4) does not hybridize to the MYCL2-derived probe. B: Hybridization of the cDNA31 to the same blot. Total human (lanes 2 and 5), 5×DNA (lane 6), X only DNA (lane 3), ×3000.11 DNA (lane 4). For both figures no hybridization signal was detected for CHO DNA (lane 1). Samples were loaded with the purpose of having the same amount of XDNA.

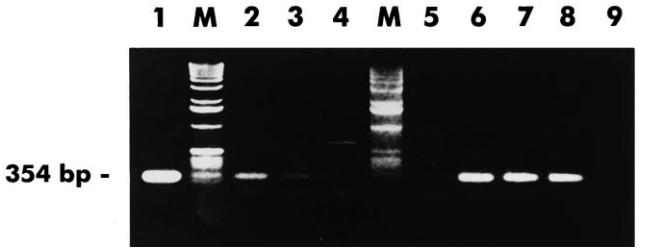

Fig. 4. PCR amplification products obtained using primers from the MYCL3 sequence. PCR product from the cDNA31 (lane 1, positive control), ×3000.11 (lane 2), total human (lane 3), CHO (lane 4), yeast (lane 5, negative control) DNA. Lanes 6–8: yWXD340, 3191 and 1674 YAC DNA. Lane 9: PCR control containing no DNA. M: marker DNA.

gene where no *Eco*RI sites are detected) from total human DNA (lanes 2 and 5) and DNA from X only (lane 3)and the 5×cell line (lane 6). In addition, a band of approximately 10.5 kb, attributable to the MYCL1 gene, was detected on total human DNA (lanes 2 and 5).

Conversely, when we hybridised the cDNA31 probe to the same membrane blot, one band of approximately 4 kb was detected on ×3000.11 DNA (Fig. 3, B, lane 4) on total human DNA (lanes 2 and 5), 5× (lane 6, CTR) and X only DNA (lane 3). In addition to the 6.5 kb MYCL2 fragment, a band of 5.5 kb was found in all the DNA analysed with the exception of the ×3000 DNA (lane 4). Considering that the cDNA31 probe, used in this hybridisation, encompasses the last portion of the published MYCL2 sequence, we assume this band due to its hybridisation to the next *Eco*RI fragment. In both cases, under this stringency, *Eco*RI digested hamster DNA (lane 1) did not show any hybridisation signal.

Taking all the results together, we can conclude that the processed MYCL2 maps to the long arm of the X chromosome at the band q22-23 and it is therefore distinct from the sequence found on Xq27. During our last search in databases before the submission of the paper, we found that a PAC clone (dj320j15) from the Xq22-23 region shows sequence homology with the MYCL2 gene, confirming that our sequence, MYCL3, is distinct from both MYCL1 and MYCL2 genes.

3.3. Genomic sequencing and sequence analysis

In order to determine the degree of homology between the selected cDNA31 and the corresponding genomic sequence, we sequenced its genomic counterpart. By using primers 31F1 and 31R1 (reported in Table 1) designed at the 3′ and 5′ ends of the cDNA sequence, we obtained a unique PCR fragment of the expected size using ×3000.11 or yWXD340 DNA (Fig. 4).

From the sequence analysis of the PCR product generated from the YAC340 with these primers, we found that a high degree of homology (93.9%) is maintained between the X DNA and exon 3 of the MYCL1 gene. The additional primer np5834 designed from the MYCL1 sequence at the 5′ of the primer 31FI, when used in combination with primer 31RI, produced a PCR fragment of the correct size that, by sequencing, proved to maintain the MYCL1 homology.

From this region, homologous to the MYCL1 gene, we extended the sequence by cloning fragments via inverse PCR at the 3′ and the 5′ ends using the YAC yWXD340 as a template.

At the 3′ end, we cloned and sequenced 483 nt, the proximal 446 of which are 91.5% homologous to MYCL1. The remaining 37 nt were without homology with any sequence in the databases. At the 5′ end, we extended the sequence of 2114 nt, the first 458 nt with 90.4% sequence homology of with MYCL1. Then the homology with the MYCL1 gene is interrupted. Computer analysis of the remaining 1656 nt showed that the region between nt 1227 and 1492 bears significant homology to several ESTs (62.2–91.2% homology in 17 ESTs) which is not related to known repetitive sequences. However no ORFs with similarity to any protein in any database were detected through computer analysis. In addition, attempts to amplify RT-PCR products, using primers designed in this region of the EST homology combined with others in the MYCL3 sequence, were always negative. Northern blots containing mRNAs from different foetal and adult human tissues were also hybridised with our sequence, but the bands obtained were compatible with those pertaining to the MYC family members already described. The sequence we produced (2824 nt) is available in the database (an Y16968). The region between nt 1657 and 2787 is homologous to the MYCL1 gene (nt 5681 and 6814) corresponding to the 3′ UTR of exon 3. A comparative analysis of the MYC-related sequence found in Xq27.2 revealed that it is characterised by AU-rich regions that are considered to confer selective mRNA instability [23]. A consensus sequence (AUUUA), typical of a number of short-lived mRNAs [24], has been recognised at position 2674 and 2710. The presence of a 14 nt direct repeat (AAGAGTCTTGTGTG), occurring at nt 1642 and 2806 on the Xq27.2 MYC-related sequence and flanking the region of homology to the MYCL1 gene, has also been identified. This motifs may be related to the mechanism responsible for sequence reintegration in the genome [25].

4. Discussion

After having completed the Xq27 physical map, we focused on specific regions of the Xq27 band to start identifying genes. We chose YACs carrying human DXS probes conserved in mouse DNA and we focused on subregions of the q27 band having a high GC content [26] in order to maximise the possibility of finding genes.

We present here the finding of a sequence related to the MYCL1 proto-oncogene, selected from cDNAs obtained from different tissues, using the cDNA direct selection approach [15]. We excluded the possibility that the MYCL3 sequence was the MYC homologous L2 previously mapped to the long arm of the X chromosome in q22-qter by Morton et al. [22], as we located it in Xq22-23 by using a panel containing a combination of DNAs from hybrids carrying different portions of the human X chromosome. Other sequences have been identified with comparable homology to some portions of C-MYC and they represent pseudogenes [25] or truncated genes as B-MYC [27] or S-MYC [28], whose functions remain unknown. The existence of a region homologous to several ESTs just upstream of the MYCL3 sequence raises the possibility that this sequence has been transposed at the 3′ end of an active gene, to which it could confer new properties as has been described for the urokinase gene 3′ UTR region, containing sequences that can exert both positive and negative influences on the expression of chimeric reporter genes [29]. However, we were unable to find any evidence of MYCL3

transcription, neither by RT-PCR nor by Northern analysis. Taking these results together, it is very likely that the MYCL3 sequence detected in Xq27.2 represents another member of the MYC-related pseudogene family.

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