Chromosomal mapping and expression of the human *cyr61* gene in tumour cells from the nervous system

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Abstract

Aims—To characterise the human *cyr61* gene (*cyr61H*) and determine its chromosomal locality. To compare expression of *cyr61H* in human tumour cell lines with that of two other structurally related genes, *novH* (nephroblastoma overexpressed gene) and CTGF (connective tissue growth factor), that are likely to play a role in the control of cell proliferation and differentiation.

Methods—To isolate the human *cyr61* gene, placental genomic and HeLa cDNA libraries were screened with murine *cyr61* cDNA. The nucleotide sequence of the complete *cyr61H* cDNA was established. Both Southern blotting of a panel of somatic cell hybrids and in situ hybridisation on chromosomes were performed to map the *cyr61H* gene. Expression of *cyr61H*, *novH*, CTGF, and *novH* was analysed by northern blotting in both human neuroblastomas and glioblastoma cell lines.

Results—Genomic and cDNA clones encompassing the *cyr61H* gene were isolated and characterised. Comparison of mouse and human *cyr61* sequences indicated that their genomic organisation is highly conserved. Alignment of coding sequences highlighted the conservation of *cyr61* regions that might be critical for its biological function. The data showed that the *cyr61H* gene is assigned to chromosome 1p22.3 and that different levels of *cyr61H*, CTGF, and *novH* mRNA have been detected in several human tumour cell lines derived from the nervous system.

Conclusions—The human *cyr61* gene belongs to an emerging family of genes including CTGF/*fisp12* and *nov*. The murine *cyr61* encodes an extracellular cysteine rich protein that exhibits chemotactic activity, promotes attachment and spreading of cells, and potentiates the mitogenic effect of growth factors. Assignment of the *cyr61H* gene to chromosome 1p22.3 will allow studies to determine whether human pathologies derived from the nervous system or from other tissues are associated with chromosomal abnormalities involving this region. Although the coding regions of *cyr61H*, CTGF, and *novH* are highly homologous, a growing body of evidence suggests that expression

of these genes is regulated differentially, and that a balance between expression of these genes might represent a key element in determining the stage of differentiation and/or the malignant potential of tumour cells.

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Keywords: human *cyr61*; CTGF; *nov*; chromosome mapping; neuroblastomas; glioblastomas; nervous system tumours

The murine *cyr61* gene was characterised as a growth factor inducible, immediate early gene in mouse fibroblasts.¹ The CYR61 protein is a secreted, cysteine rich, heparin binding protein that associates with the cell surface and the extracellular membrane.² The chicken homologue of *cyr61* (CEF10) had been identified as an immediate early gene whose expression is induced by production of active v-src in ts NY72-4 Rous sarcoma virus infected chicken embryo fibroblasts (CEF). Enhanced CEF10 RNA levels were also detected in serum treated normal fibroblasts.³

The CEF10/*cyr61* genes share extended nucleotide sequence similarities with two groups of genes (*fisp12*/CTGF (connective tissue growth factor) and *nov* (nephroblastoma overexpressed gene)),⁴⁻⁹ encoding proteins likely to play a role in cell growth and/or differentiation.⁴⁻¹⁰ These genes also share more distant sequence similarities with two drosophila genes, twisted gastrulation and short gastrulation, which interact with decapentaplegic to regulate dorsal-ventral patterning.¹¹⁻¹² A comparative analysis of the primary structures of CTGF/*fisp12*, *cyr61*/CEF10, and NOV established that these proteins contain 38 conserved cysteine residues and the following four structural motifs: (1) an insulin growth factor binding protein (IGFBP) module, highly homologous to the core N-terminal IGF binding domain of previously described IGF binding proteins; (2) a Von Willebrand factor type C repeat (VWC) module likely to be involved in oligomerisation and represented in Von Willebrand factor; (3) a thrombospondin type I repeat (TSPI) module, represented in thrombospondin, and thought to be involved in the interaction with extracellular matrix molecules; and (4) a carboxy-proximal motif (CT) proposed to represent a dimerisation domain.¹³ Although the functionality of these domains remains to be established, it is tempting to propose that their conservation is related to the biological function(s) of these proteins.

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Figure 1 (A) Organisation of the human *cyr61* gene. Partial restriction map of λ Hu11 and of the plasmid subclones pBB6.5 and pEE6.2. Probes: pBB6.5 (6.5 kb BamHI fragment), pEE6.2 (6.2 kb EcoRI fragment). The position of exons in λ Hu11 and the pBB6.5 and pEE6.2 subclones was mapped following Southern blotting with exon specific oligonucleotides as probes. The black boxes represent human exonic regions. Exons were numbered according to the nucleotide and amino acid sequences of the murine *cyr61* gene. B, BamHI; E, EcoRI; H, HindIII; Xa, XbaI; Xo, XhoI. (B) Comparison of the murine, chick, and human *cyr61* open reading frames (ORFs). Amino acid sequences from the human and murine *cyr61* and chicken CEF10 proteins have been aligned to give maximal homology using the Clustal method.⁴⁰ Conservative substitutions are indicated by a single line. The * and ° symbols represent predicted phosphorylation sites for protein kinase C and casein kinase II, respectively.

In spite of their highly conserved organisation, the immediate-early CEF10/*cyr61* and *fisp12*/CTGF genes encode positive regulators of growth with distinct biological activities and are subject to different regulatory signals.

Both CTGF and CYR61 proteins exhibit chemotactic activities.^{4,14} CYR61 has been shown to promote cell adhesion and potentiate the mitogenic effects of growth factors such as β fibroblast growth factor (β FGF) and platelet derived growth factor B (PDGF B).¹⁴ CTGF,

Figure 2 Predicted secondary structure according to Garnier *et al*²⁰ of (top) murine CYR61 (*cyr6*-mouse), (middle) chicken CEF10 (*p19336*), and (bottom) human CYR61 (*cyrhu*).

Figure 3 Detection of *cyr61H* specific sequences in human-rodent hybrid cell line DNA. Samples (10 μ g) were digested with *Eco*R1 restriction endonuclease, electrophoresed, transferred on to nylon membranes and hybridised with the ³²P labelled *pcyr61* cDNA probe (2.0 kb *Eco*R1 fragment). Lane 1, human-mouse hybrids positive for human sequences; lane 2, human-mouse hybrids negative for human sequences; lane 3, mouse DNA; lane 4, human DNA; lanes 5 and 6, human-hamster hybrids positive for human sequences; lane 7, human-hamster hybrids negative for human sequences; lane 8, hamster DNA.

which was reported to act as a growth factor,⁴ is a downstream target of transforming growth factor β (TGF β).^{15 16} We have shown previously^{10 17} that, in contrast to the other members of this gene family, *nov* is not an immediate-early gene. Its expression is associated with cell quiescence and is downregulated on induction of cell proliferation following treatment with serum and oncogenic transformation.

Because altered expression of *nov* has been shown to be associated with avian and human neuroblastoma^{6 7} (also Chevalier *et al*, unpublished), it is conceivable that an unbalanced expression of this gene's family may constitute a key point in the proliferation of tumour cells.

The observation that *cyr61* expression correlates with chondrogenesis during embryonic development¹⁸ and that *nov* expression is detected during embryogenesis of muscle, nervous tissue, and cartilage¹⁹ (also Chevalier *et al* and Kocalkovski *et al*, unpublished) suggested that alterations of the expression pattern of these genes could be involved in several pathologies.

nov and CTGF genes have been assigned to chromosomes 8q24.1 and 6q23.1, respectively.²⁰ In this report we show that the human *cyr61* gene (*cyr61H*) is conserved, maps to chromosome 1p22.3, and is expressed differentially in tumours and tumour cell lines derived from the nervous system.

Methods

CELL CULTURE AND TISSUES

The neuroblastomas analysed were isolated and characterised in the IGR, Villejuif (Bénard *et al*, unpublished data). Pathological diagnosis determined one undifferentiated neuroblastoma (stage IV), two ganglioneuroblastomas (stage III), and three ganglioneuromas (local).

Glioma cell lines were established from fresh tumour specimens.²¹ The tumours were obtained from patients between 4 and 71 years of age. Classification of the tumours was performed according to WHO guidelines. The tumour cell lines analysed in this study are derived from tumours of WHO grade 3 or 4 and are referred to as high grade hereafter.²² The cell lines were maintained at 37°C in Earle's modified minimal essential medium (MEM) containing 10% fetal calf serum in an 8% CO₂ humidified atmosphere.

NUCLEOTIDE SEQUENCING

The 2.0 kb *Eco*RI insert derived from 16λH recombinant phage was further subcloned in Bluescript pBS/KS plasmid (Stratagene Cloning Systems, La Jolla, California, USA) to generate pCYR61H and sequenced by the dideoxy chain termination method²³ in the presence of (α-³⁵S) dATP and T7 polymerase (Pharmacia, Orsay, France). Sequence data treatments were performed using the computer facilities at Infobiogen (Villejuif, France).

DNA AND RNA PURIFICATION, SOUTHERN AND NORTHERN BLOTTING

Procedures for DNA and RNA purification from tissues and cell cultures and for Southern and northern blotting are described elsewhere.²⁴ The Southern blots were hybridised to the 2.0 kb *Eco*RI fragment derived from the pcyr61H clone. The northern blots were hybridised either to the 1.0 kb *Nco*I-*Hind*III fragment derived from the *cyr61*H clone, to the 3.5 kb *Bgl*II-*Bam*HI fragment

from the pBH7 novH clone (probe pBH7/BB),²⁵ or to the 700 bp *Pst*I fragment derived from the pS6 CTGF clone pS6 (probe pS6/PSP07).²⁵

IN SITU HYBRIDISATION

The details of chromosome preparation and banding, probe labelling, and hybridisation have been described previously.^{26, 27} Probes labelled with 11-UTP (Bio-Rad Laboratories, Ivry sur Seine, France) were detected by indirect immunofluorescence using a fluorescein conjugated antibody. Probes were annealed with total DNA in order to avoid hybridisation of repeated sequences contained in the genomic pBB6.5 and pEE6.2 probes. The final concentration of total DNA in the hybridisation mixture was 20 µg/ml, slides were annealed at 37°C for 10 minutes.

Results

Screening of a normal human genomic placental library (Clontech, Montigny le Bretonneux, France) and of a HeLa cell cDNA library (Clontech), using murine pcyr61 cDNA¹ as a probe, allowed us to isolate several λ recombinant clones whose representatives are λHu11 genomic DNA and λl61H cDNA (fig 1). As reported previously for the murine *cyr61* gene,²⁸ the entire *cyr61*H coding region spans about 3 kb of DNA (fig 1).

Comparison of the pcyr61H cDNA nucleotide sequence with the murine pcyr61 and the chicken CEF10 cDNA sequences³ revealed an overall identity of 82% and 77%, respectively. The consensus sequences TTATAAA, which

Table 1 Analysis of human markers: chromosomes, enzyme PGM1, and CYR61H *Eco*RI sequences (6.2 kb) in 25 independent human-rodent hybrids

Chromosomes																										<i>EcoRI</i> 6.2 kb	<i>PGM1</i>
Hybrids	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	X	Y			
<i>Man-mouse</i>																											
L.53K	/	+	/	+	+	-	+	+	-	-	+	-	-	-	-	-	+	-	-	/	-	-	/	*	+	+	
L.53N	-	-	+	/	-	+	-	+	-	-	-	+	/	-	-	-	+	-	+	+	+	-	-	*	-	-	
LA.56E	-	+	+	/	+	+	+	-	-	-	+	-	-	-	/	/	+	-	+	+	+	/	+	*	-	-	
LA.56G	+	+	+	-	+	+	+	+	-	+	/	+	+	-	-	+	+	+	+	+	+	+	+	*	+	+	
LA.56I	-	+	+	/	+	-	+	+	-	-	-	+	+	/	-	+	+	+	+	-	+	+	+	*	-	-	
LA.56S	-	+	+	-	-	+	+	-	-	-	+	+	-	-	-	-	+	-	-	-	/	-	+	*	-	-	
LA.56U	+	+	-	+	+	-	+	+	-	/	+	+	-	-	+	+	+	+	+	+	+	+	/	*	+	+	
<i>Man-hamster</i>																											
V.106	-	-	-	-	/	-	-	-	-	+	-	-	-	-	+	-	-	-	/	+	+	/	+	+	-	-	
CH.106IV	-	-	-	-	-	+	-	+	-	-	+	+	-	-	-	-	-	-	-	+	/	+	+	*	-	-	
CH.BLD	-	-	-	+	+	+	-	+	-	+	+	-	+	+	-	-	-	-	+	-	-	/	*	-	-	-	
CH.BLE	-	-	+	/	-	+	-	+	/	-	-	-	/	+	+	+	-	/	+	-	-	-	-	*	-	-	
CH.BLH	+	-	+	+	-	+	-	/	+	/	+	+	+	+	+	+	-	+	+	+	+	+	+	*	+	+	
CH.BLI	+	-	-	+	/	-	-	+	-	-	+	+	-	-	-	-	-	+	+	-	-	-	+	*	+	+	
CH.BLJ	-	-	-	+	+	+	+	/	-	-	+	+	+	+	+	+	-	+	-	+	/	+	/	*	+	+	
CH.BLK	-	-	+	-	+	+	-	-	-	/	+	-	+	-	+	-	-	/	-	/	+	+	+	*	+	+	
CH.BLN	+	-	-	+	+	+	+	-	+	-	+	-	-	-	-	+	-	/	+	+	+	+	+	*	+	+	
CH.56F	-	-	-	-	-	-	-	+	-	-	-	-	-	+	+	+	-	-	+	-	+	+	+	*	-	-	
CH.56X	+	+	+	/	+	+	-	+	+	-	-	+	-	-	-	-	-	+	+	-	-	+	*	+	+		
CH.34E	-	-	+	/	+	-	-	+	+	-	+	+	-	+	-	+	/	-	+	+	+	-	*	-	-	-	
CH.34G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	/	-	-	-	-	+	*	-	-		
CH.34S	-	-	-	-	-	+	-	/	-	+	+	+	-	+	-	/	-	-	-	+	+	+	+	*	-	-	
CH.34V	+	-	+	+	+	-	-	+	+	-	+	+	+	+	/	/	-	+	+	+	-	-	*	-	+	+	
CH.34X	+	-	/	-	-	+	-	+	+	/	+	+	+	-	+	+	-	+	-	-	+	-	*	-	+	+	
CH.34FU	-	-	+	+	-	-	-	+	-	-	-	-	-	+	-	/	-	-	/	+	-	-	*	+	-	-	
CH.34GT	-	-	-	+	-	+	-	+	/	+	-	+	+	+	-	-	-	+	-	/	+	-	*	-	-	-	
<i>Discordant</i>																											
CYR61H(%)	8	40	48	32	26	48	36	41	32	43	29	52	35	63	35	38	52	14	39	50	59	26	43	44		0	

Hybrid cell lines have been described by Nguyen *et al.*³¹ Karyotype analyses of each cell line and reference enzyme marker studies were done at the time of DNA preparation. In some cases, hybrid content differed from the initial description by Nguyen *et al.* Analyses for phosphoglucosyltransferase 1 (PGM1) a chromosome 1 reference marker was performed according to Van Someren *et al.*⁴¹ Chromosomes: +, chromosome detected at least in 30% of cells; -, chromosome not detected; /, chromosome detected in fewer than 30% of cells, not scored for mapping; *, chromosome absent in the human parental strains. Enzyme PGM1 and CYR61H *Eco*RI sequence: +, the human marker is present in hybrid cells; -, the human marker is absent in hybrid cells.

Figure 4 In situ hybridisation of biotinylated *cyr61H* specific probes on human metaphase chromosomes. Chromosomes were counterstained with propidium iodide. (A) Hybridisation with the pBB6.5 probe. (B) Hybridisation with the pEE6.2 probe. Arrows indicate fluorescent spots in 1p22.3. (C) Schematic representation of the relative position of the *cyr61* gene and a longitudinal map (mapped in relation to chromosome banding) of chromosome 1.

confer instability to transcripts,²⁹ are represented in the human *cyr61* gene at positions 1896–1903 and 1990–1997, and only one of the 49 bp repeats present in the murine *cyr61* 3' untranslated region²⁸ is partially conserved in the human gene (39/49 bp). The predicted amino acid sequence of the longest open reading frame of p*cyr61H* revealed that the 381 amino acid CYR61H protein was 91% and 81% identical to murine CYR61 and chicken CEF10. Therefore, p*cyr61H* is likely to represent the human homologue of the murine *cyr61* gene and the chicken CEF10 gene. Prediction of post-translational modifications revealed that CYR61/CEF10 proteins were likely to undergo several specific phosphorylations in addition to the common ones (fig 1). It is worth noting that, in exon 4, the stretch of amino acids from positions 166 to 227, which lies between the VWC and TSP1 modules,¹³ is the least conserved between the CYR61H, CYR61, and CEF10 proteins. As shown in fig 2 the predicted secondary structure and hydrophobicity profile³⁰ of these three proteins revealed significant topological differences.

Therefore, these observed differences might confer particular biological properties to each of the three CYR61H, CYR61, and CEF10 proteins.

Comparison of the CYR61H protein sequence with that of other related proteins of the same family also revealed a high degree of conservation of the four putative IGFBP, VWC, TSP1, and CT domains.

As a first step in our search for rearrangement or alterations of the *cyr61H* gene in human pathologies, we have performed a physical mapping of the *cyr61H* gene.

Southern blot hybridisations with the p*cyr61H* probe were performed on a series of 25 rodent-human hybrid DNA samples³¹ digested with EcoRI. The human *cyr61* specific sequences were detected in a 6.2 kb fragment (fig 3). The same probe also revealed two *cyr61* specific fragments of 7.6 kb and 2.5 kb in murine and hamster DNA, respectively. In human-rodent hybrids a positive correlation was observed between *cyr61H* sequences and the phosphoglucomutase 1 gene (PGM1), a well known chromosome 1 reference marker.³² Among 25 independent hybrids analysed, 11 were positive and 14 were negative for these two markers. No discordant result was observed between *cyr61H* specific sequences and PGM1 (table 1). The percentage of discordant results were 8% for chromosome 1 and between 8% and 63% for the other chromosomes (table 1). According to the exclusion criterion (marker absent/chromosome present), all chromosomes other than chromosome 1 could be excluded for the presence of *cyr61* specific sequences. In hybrids negative for *cyr61H* sequences, only chromosome 1 was absent. Taken together, these data indicate that the *cyr61H* gene is localised on chromosome 1.

Non-radioactive in situ hybridisations were performed using both pBB6.5 and pEE6.2 as probes on metaphase chromosomes obtained from human lymphocyte cultures of normal donors (fig 4). The frequency of metaphases with chromosomes showing one fluorescent spot on one chromatid was about 60%. These fluorescent spots were observed systematically in the distal part of band p22.3 of chromosome 1, therefore assigning the *cyr61H* gene to this band.

Alterations and deletions of the distal part of chromosome 1p have been associated with several human tumours of neuroectodermal origin, including neuroblastoma.³³ Concentrations of *cyr61H* mRNA varied from one type of neuroblastoma to another (fig 5). While *cyr61H* expression could be detected easily in ganglioneuromas (322, 321, and 320) and in ganglioneuroblastomas (345 and 175), a much lower concentration of *cyr61H* RNA was detected in neuroblastoma 104. Variations of CTGF and novH expression were observed also in these tumours. Except in neuroblastoma 104, in which *cyr61H*, CTGF, and novH were expressed at very low concentrations, no obvious correlation could be drawn with respect to the relative expression of these three genes.

Figure 5 Expression of *cyr61H*, *CTGF*, and *novH* in human neuroblastoma and glioma cell lines. Total RNA samples (20 µg) were electrophoresed in 1% agarose gels, transferred to Nytran⁺ membranes (Schleicher and Schuell, Strasbourg, France) and hybridised with ³²P labelled *cyr61H*, *CTGF*, or *novH* specific probes. The amount of RNA transferred in each lane was normalised following hybridisation with a human GAPDH probe (Clontech). (A) Neuroblastomas; (B) glioma cell lines.

Different concentrations of *cyr61H* mRNA species were detected also in human glioblastoma derived cell lines (fig 5). The expression of *cyr61H* was detectable in all glioblastoma cell lines studied but one (167). It is worth noting that neither *CTGF* nor *novH* could be detected in this glioma cell line.

Two different *cyr61H* mRNA species were detected in the different neuroblastomas and glioblastomas. In addition to the major 2.5 kb mRNA species, a less abundant 3.5 kb mRNA species was detected in a subset of these cell lines. As already reported²⁵ for the 3.5 kb and 7.0 kb *CTGF* mRNA species which are expressed in these cell lines, no obvious correlation was observed between the concentrations of the 2.5 kb and 3.5 kb *cyr61H* mRNA species expressed. Because the complete *cyr61H* cDNA probe detected only a 6.2 kb DNA fragment and only one chromosomal localisation was revealed by in situ hybridisation, it was concluded that the 3.5 kb mRNA species is most likely the result of an alternative splicing event. The observation that the 3.5 kb mRNA species of both *cyr61H* and *CTGF* are

expressed in the same glioblastomas might be of biological relevance.

Discussion

Although alterations of chromosome 1p have been associated with several human tumours of neuroectodermal origin including neuroblastoma,³³ melanoma,³⁴ breast cancer,³⁵ and small lung carcinoma³⁶ only a few human tumour pathologies have been correlated thus far with abnormalities of the 1p22 region. A (1;10)(p22;q12) constitutional translocation was reported in a patient with stage 4S neuroblastoma.³⁷

A larger survey of neuroblastoma samples would be required to establish a correlation between *cyr61H* expression and the stage of neuroblastoma malignancy. However, it is worth noting that *cyr61H* expression was greatest in ganglioneuromas, which represent a benign stage of neuroblastoma and are composed of fully mature ganglion cells embedded in nerve fibres.³⁸ Therefore, considering that the *CYR61* protein plays a role in cell proliferation and adhesion,¹⁴ it is possible that genetic alterations of 1p22 that affect *cyr61H* expression might participate in the development of these tumours.

Downregulation of *cyr61H* expression was also reported in rhabdomyosarcomas.³⁹ Analysis of subtractive hybridisations performed between human primary myoblasts and an embryonal rhabdomyosarcoma cell line has led to the isolation, from myoblasts, of a fragment of cDNA (A33210)³⁹ whose sequence is 100% identical to positions 544–847 of the *cyr61H* gene. Thus, it appears that inhibition of *cyr61H* expression might also be of importance in the maintenance or progression of these tumours.

In human glioblastoma cell lines different levels of *cyr61H*, *CTGF*, and *novH* expression have been observed. Because *CTGF*, *cyr61*, and *nov* might have an antagonistic effect on cell growth,¹⁰ it is possible that a balance between expression of these three genes is required to modulate the proliferation and/or differentiation state of the cells.

Because *cyr61* expression has been shown to correlate with chondrogenesis during mouse development,¹⁸ it would be worth examining whether *cyr61H*, *nov*, and *CTGF* are altered in human pathologies derived from cartilage or bone, and whether abnormalities of the chromosomal regions 1p22.3, 8q24.1, and 6q23.1 are observed in such pathologies.

Addendum

Sequence data from this article has been deposited with the EMBL/GenBank Data Libraries under Accession number Y11307. While this manuscript was being submitted, Jay *et al*⁴² reported an approximate localisation of the human *cyr61* to chromosome 1p22–p31.

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