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Down Syndrome Critical Region Gene 2: Expression during Mouse Development and in Human Cell Lines Indicates a Function Related to Cell Proliferation

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The isolation of the genes located in chromosome 21 and the characterisation of their function are essential steps towards the understanding of the physiopathological mechanisms involved in Down syndrome. We have used two complementary approaches to characterise the function of the novel gene DSCR2 (Down Syndrome Critical Region gene 2): the isolation and characterisation of the mouse gene homologue to the human DSCR2 gene, and the analysis of the expression of the gene in different human cell lines. We have isolated and characterised a 1012 bp of a mouse cDNA having a high homology to the human DSCR2 gene. The predicted mouse dscr2 protein has an identity of 85.4% as compared to the human protein, indicating that the DSCR2 protein has been conserved during the evolution. However, the amino acid sequence is not homologous to other known proteins, or to known protein domains. The dscr2 gene is expressed throughout all the stages of the mouse embryo development. In the adult mouse the gene is expressed in testis, kidney, liver, brain, heart, skeletal muscle, and pancreas. The expression analysis of the DSCR2 gene in different human tumour derived cell lines indicates that the gene is expressed in all proliferating cell lines tested. The levels of the DSCR2 mRNA correlate with cellular growth of T98G and Jurkat cells in response to different treatments. The expression pattern throughout the foetal development together with the correlation observed with the cell cycle indicates a possible function for the DSCR2 gene related to cell proliferation.

Down syndrome is one of the most common birth defects usually caused by the trisomy of chromosome 21 (1). Down syndrome is characterised by a specific phenotype featured by mental retardation and other major congenital malformations such as those of the heart and of the gastrointestinal tract (2). It has also been described an increased incidence of leukaemia in Down syndrome patients (3, 4). The region of the chromosome 21 located between the markers D21S55 and MX1, also known as the Down syndrome critical region-2 (DCR-2) has been associated with different features of Down syndrome through genotype-phenotype correlation's of partial trisomy 21 patients (6–10). It has been postulated as the minimal region for 6 facial and dermatoglyphic features (9) and, in some patients, the region is apparently determinant for the pathogenesis of mental retardation, the congenital heart disease and the duodenal stenosis (8). From this DCR-2 region of chromosome 21 we had previously isolated a novel human gene called DSCR2 (Down Syndrome Critical Region gene-2), official name proposed and approved by the Human Gene Nomenclature Committee for the alias c21LRP. The novel human DSCR2 gene is located between STS markers D21S343 and D21S268 and has no apparent strong homology to other known genes (5).

As a result of the Human Genome Project a rapidly increasing number of transcribed sequences from human and other model species are becoming available (11). Most of the sequences correspond to new genes whose function is not known. These uncharacterised new genes are currently annotated using different software such as GeneQuiz (12), Genotator (13) or Rummage-DP (14), using homology to proteins of known function and signatures that identify protein families. However these approaches frequently fail to assign the function to an important percentage of novel genes, generating a large quantity of unannotated

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genes concerning to their functions (12). In these cases, experimental approaches have to be designed in order to search for the possible function of these unannotated genes.

No function was so far known for the DSCR2 (c21LRP) gene. Therefore, in the present work we have used two different complementary approaches to investigate the possible function of the DSCR2 gene: the isolation and characterisation of the mouse gene homologous to the human DSCR2 gene, and the analysis of the expression of the gene in 7 different human tumour derived cell lines. This work is part of a project to characterise the function of the genes present between markers ETS2 and SH3BGR, located in the Down syndrome chromosomal region-2 from the human chromosome 21, and potentially responsible for some of the DS features (5, 15–17).

MATERIALS AND METHODS

Clones, cell lines and reagents. cDNA clones were obtained through the UK-HGMP-Resource Centre from the IMAGE Consortium (18). The following human cell lines were purchased from the ATCC: RAJI (Burkitt lymphoma), HL60 (promyelocytic leukemia), HeLa (cervix epitheloid carcinoma), HepG2 (hepatocellular carcinoma), HT29 (colon adenocarcinoma), MCF-7 (breast adenocarcinoma) and T98G (glioblastoma). Jurkat E6.1 (acute T leukemia) was purchased from ECACC. RPMI 1640 and the DMEM media were obtained from Biological Industries and the fetal calf serum (FCS) was obtained from GIBCO-BRL. 12-O-tetradecanoylphorbol 13-acetate (TPA) and propidium iodide were from Sigma Chemical (St. Louis, MO).

Cell cultures. The cell lines Jurkat, Raji and HL60 were grown in complete RPMI 1640 medium, containing 10% heat-inactivated foetal calf serum (FCS), 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin, at 37°C in a humidified atmosphere at 5% carbon dioxide. The cell lines HeLa, HepG2, HT29, MCF-7, and T98G were grown in complete DMEM medium, containing 10% FCS, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin, at 37°C in plates in a humidified atmosphere at 10% carbon dioxide. T98G cells were synchronised in a quiescent state by serum starvation for 72 h (19). Jurkat cells were treated with 100 nM TPA for 24 h to inhibit cell growth (20). Cell cycle was analysed by cytofluorimetric assay of the DNA content using a FACScalibur Cytometer (Benton Dickinson) after staining with propidium iodide (21). Data analysis was performed using the ModFitLT v2.0 software (Verity Software House) to obtain the percentage of cells in the G0/G1, S, and G2/M cell cycle phases.

In silico screening, sequencing and analysis of the cDNAs sequences. The human DSCR2 sequence has been used to detect mouse EST sequence homologues in the mouse EST GenBank database (NCBI) using the BLAST 2.0 software (22). IMAGE cDNAs clones (602333 and 0775603) were requested and subsequently sequenced in a Licor sequencer using the Thermosequenase cycle sequencing kit (Amersham) with the primers T3 and T7, according to the manufacturer's protocol. Sequence assembling was done using SeqMan software from DNASTAR package (Lasergene). The ORF obtained from the newly determined sequence (AJ238270) was predicted using EditSet (DNASTAR) and analysed with the ATGpr software (http://www.hri.co.jp/atgpr) to detect the ATG of translation start (23). Comparisons of the nucleotide and amino acid sequences to public databases have been performed using BLAST algorithms (24) and other prediction applications were carry out as described in Vidal-Taboada *et al.* (1998).

Expression analysis by Northern blots. Total RNA from different adult mouse tissues (brain, heart, liver, skeletal muscle, pancreas, lung, kidney, testis and uterus) and from complete mouse embryos at different development stages (11.5, 12.5, 14.5, 15.5, 16.5, 18.5 dpc) were isolated using the Tripure reagent (Boehringer Mannheim). RNA from human cell lines was also obtained using the Tripure reagent. 10 μg of RNA from each sample were electrophoresed and transferred to a nylon membrane (Schleicher & Schuell) as described (25). The IMAGE 602333 cDNA clone insert, or the human DSCR2 gene, was radioactively labelled by random priming using the RediPrime II kit (Amersham) and the probe was purified using Sephadex G-50 columns (Pharmacia). Northern blots were prehybridised in hybridisation solution (50% formamide, 5× SSC, 5× Denhard's solution, 1% SDS) for 2 h. The hybridisation was performed at 42°C overnight using a probe concentration of 10^6 cpm/ml. Washes were done using 0.1× SSC 0.1% SDS as follows: 2× 5 min at room temperature, 2× 15 min at 37°C and finally at 55°C for 15 min. The hybridised filter was exposed to an imaging plate BAS-MP (Fujifilm) for 2 days, and the image captured in a Molecular Imager FX (Bio-Rad). Image data were processed using the Quantify One 4.0 software (Bio-Rad).

In silico gene expression analysis. The possible repetitive sequences present in the mouse DSCR2 gene sequence was masked using the Repeat Masker software (http://ftp.genome.washington.edu/cgi-bin/RepeatMasker). The resulting masked sequence was used to search the mouse EST sequences division of the GenBank database using the BLASTN algorithm (22). 58 mouse and rat EST sequences (E value $>$ e -50) were detected in the BLAST search and were subsequently classified by their tissue origin and E value. The frequency of the expression in the different cDNA libraries was estimated as the number of EST homologous to the DSCR2 gene related to the total number of EST sequenced of each cDNA library and was expressed as EST homologous per million of EST sequenced.

RESULTS

Identification and Sequencing of the Mouse DSCR2 Gene

With the goal to contribute to identify the possible function of the DSCR2 gene (Down syndrome Critical Region gene 2), also known as the alias C21-LRP (Chromosome 21-Leucine Rich Protein) (5), we initially decided to characterise the mouse homologue of the human DSCR2 gene. We expected that the subsequent comparison of the degree of conservation between the human and mouse sequences would allow the detection of the presence of conserved protein domains that could indicate a potential function for this gene. Thus, the nucleotide sequence from the human DSCR2 cDNA (AJ006291) was used to search the public ESTs databases using the BLASTN software. Several ESTs sequences from mouse were detected with a high homology to the human DSCR2 cDNA sequence. The IMAGE EST 602333 (AA145057) from a mouse testis library was selected as a possible mouse homologue of the human DSCR2 gene based in its high nucleotide homology and its pattern of expression, since testis is the human tissue where the expression of the DSCR2 mRNA is higher (5). This EST was purchased and the cDNA insert was sequenced to completion. The analysis of the sequence obtained revealed that the cDNA lacked the 5′ end of the mRNA and part of the cod-

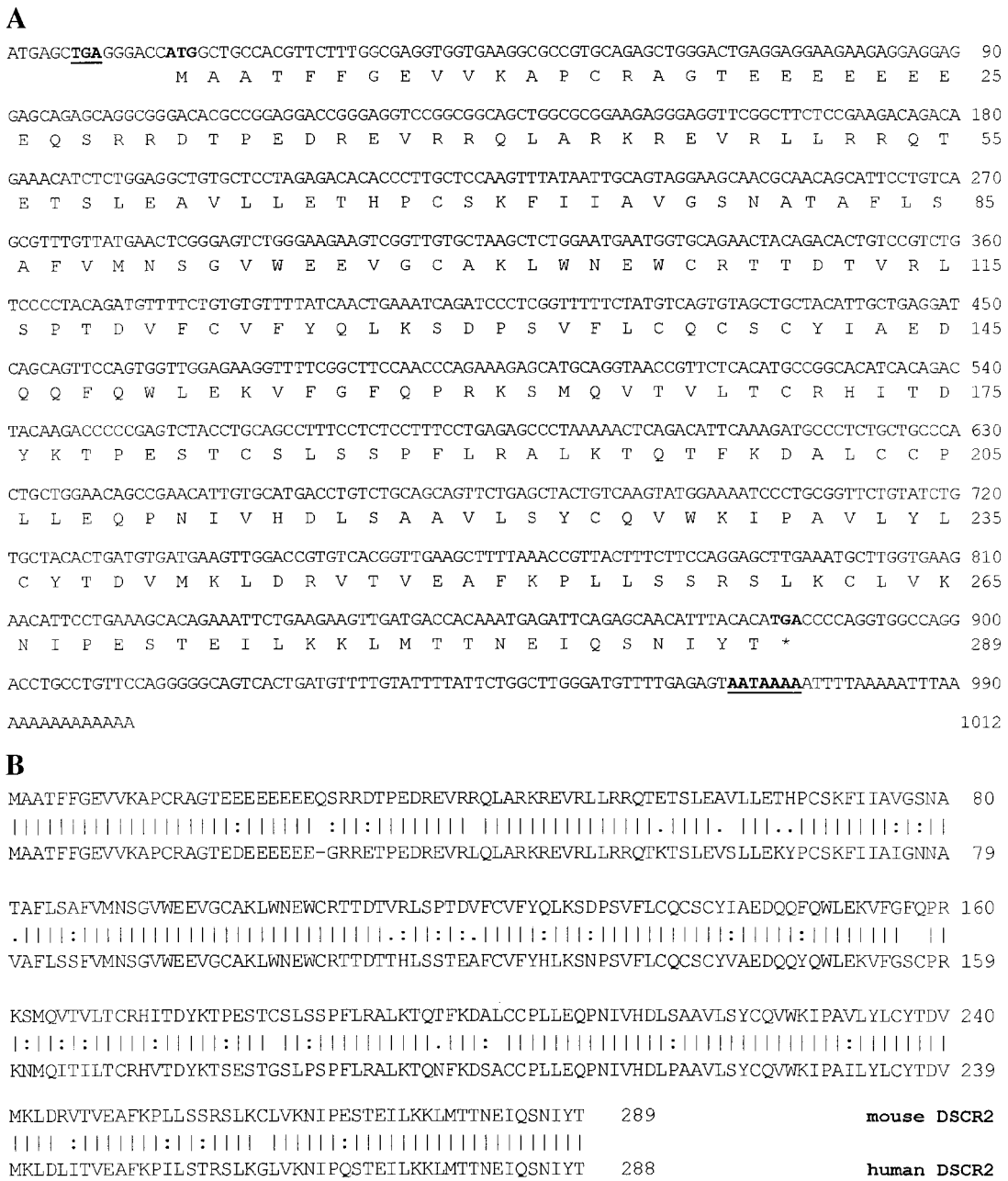

FIG. 1. Characterisation of the mouse dscr2 cDNA and the conservation of the predicted mouse protein to the human DSCR2 protein. (A) Nucleotide sequence corresponding to the mouse dscr2 cDNA (AJ238270) and the amino acid sequence of the protein encoded. The stop codon that is in frame with the ORF just 5′ the predicted translation start and the poly(A) signal are underlined. (B) Alignment of the DSCR2 amino acids sequences from mouse (top) and human (bottom). The two regions predicted as potential transmembrane domains are shown underlined.

ing region. Subsequently, daily searches against the mouse ESTs database were performed using the newly obtained sequence. In one of this searches we detected the mouse IMAGE EST 0775603 (AA275781) that expanded the 5′end of the mouse sequence previously obtained. This EST was purchased and sequenced to completion.

The newly obtained complete sequence of the cDNA 0775603 (AJ238270) has 1012 bp in length, contains a poly(A) consensus signal (699–975) and has a coding region of 870 bp (16–885) (Fig. 1). The analysis of the region around the translation start at position 16 agrees with the Kozak consensus (26) and gives the best score using the ATG prediction software ATGpr.

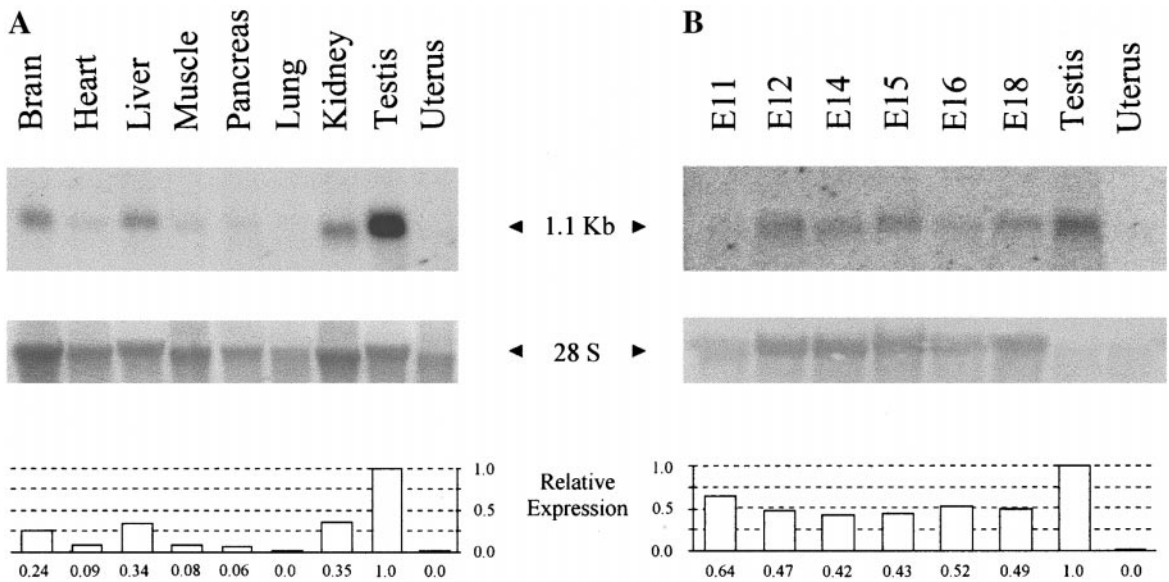

FIG. 2. Northern analysis of the mouse DSCR2 gene in different mouse tissues: (A) RNA from 9 different adult mouse tissues as indicated. (B) RNA from 6 different mouse embryo developmental stages (E11, E12, E14, E15, E16, E18) and RNA from adult testis and uterus as high and low DSCR2 expression controls. The normalised results of both Northern analyses, taking as a reference the 28S RNA, are shown at the bottom.

There is also a stop codon before this start codon (ATG) that is in the same reading frame of the predicted protein (Fig. 1A). All these results indicate that the ATG at position 16 is the more consistent start codon for the mouse dscr2 gene. The mouse dscr2 cDNA sequence has a homology of 81.1% with the human DSCR2 cDNA in the coding region. The 3′ end of the cDNA is less conserved due to the presence of insertions or deletions in this region resulting in an overall homology of 77.4% between the mouse and human DSCR2 cDNAs. The mouse gene encodes for a potential protein of 289 amino acids of which 35 are strongly basic, 37 are strongly acidic, 101 are hydrophobic, and 89 are polar amino acids. The mouse dscr2 product has a calculated molecular weight of 33 kDa, a predicted isoelectric point of 6.2. The major difference with the human DSCR2 protein is the insertion of one amino acid (Q) at position 27. The identity between the mouse and the human DSCR2 proteins is 85.4% and the similarity is 96.9% (Fig. 1B). The high identity observed indicates that the DSCR2 protein has been highly conserved during the evolution of human and mouse.

Features of the Mouse dscr2 Gene Predicted from the Analysis of the Amino Acid Sequence

The protein sequence predicted was subsequently used to search the public protein databases Swissprot and trEMBL in order to find protein homologies. No significant matches were detected. Searches for protein motifs against Blocks, Emotifs, pFam, Prints, Prodoms and Prosite database did not detect known protein motifs or protein domains with statistical significance. Homology searches based in the amino acids properties, using the PropSearch software, detected homology with proteins that have been implicated in signal transduction or in the control of the cell cycle at the G1/SCC (start) transition, such as the TGF-beta receptor type II (tgr2_pig), the mouse G1/S-specific cyclin D1 (cgd1_mouse), the ADP-Ribosyl Cyclase 1 (cd38_human), and the G1/specific cyclin D2 (VIN-1 Proto-oncogene, cgd2_rat).

Prediction of the subcellular location, based in the presence of signal peptides in the protein sequence, using the PSORT II software failed to detect signal peptides in the sequence of the DSCR2 protein. Due to this fact, the PSORT program predicts a potential location in the cytoplasm (69.6%) or in the mitochondria (17.4%). Prediction of the secondary structure using different transmembrane (TM) helix prediction software such as DAS, HMMTop, TopPredict2, and TM-Pred indicates the possible existence of one or two possible TM region that are conserved in the human and mouse DSCR2 proteins (Fig. 2). The majority of the algorithms used detect a potential TM helix core between amino acids 72 and 89 in the mouse and human protein sequences and another possible TM helix between amino acids 218 and 237. There are two possible hypothetical models based in these data. In the first model, the protein would have a unique TM domain (72–92) with the N-terminus in the intracellular compartment and with a long C-terminus located extracellularly. In the second model, the protein would

TABLE 1

In Silico Gene Expression Analysis Obtained Using the Mouse DSCR2 Compared to the Public ESTs Databases

Organism	Tissue	ESTs (GenBank Accession No.)	ESTLib ID (total number of ESTs)	Frequency (per million)
Mouse (embryo)	ES Cells	AV100803, AV099655, AV095900, AV098429, AV095619	1882 (9632)	519
	Fertilised Egg	C85757	1119 (3314)	301
	2 cells	AA474421	862 (14813)	68
	8 cells	AU020345, AU019991	1381 (3443)	581
	16 cells	AU044003	1532 (3195)	313
	3.5 dpc blastocyst	C77690	1021 (5692)	176
	10 dpc	AV115021	1884 (13955)	72
	10.5 dpc	AV147859, AV148434, AV144033, AV135863, AV141525	1891 (16293)	307
	12 dpc	AV156335, AV165590, AV160113	1906 (5441)	551
	12.5 dpc	AA239967	593 (11458)*	87
	13 dpc	AV166575, AV170190	1904 (9064)	221
	13.5–14.5 dpc	W53931, W71157	437 (41150)*	49
	19.5 dpc	AA060121, W41440	431 (26102)*	77
Mouse (adult)	Brain (hypocampus)	AV152406	1896 (6359)	157
	Heart	AA434747, AA415958	855 (8393)*	238
	Kidney	AA275781, AA140502	539 (7942)	252
	Liver	AA237211	598 (9801)*	102
	Mammary gland	AA718870, AI503574, AA544355, AA794703, AI180585	868 (39637)*	76
	Pancreas	AV055240, AV054717	1870 (13589)	147
	Stomach	AV081765, AV073653, AV077183	1878 (10023)	299
	Testis	AA145057, AV047894	483 (6056) 1784 (9208)	137
Rat (adult)	Brain	AI227730, AA943423	1273 (6469)	155
	Eye	AI715199, AI764007	1387 (8150)**	123
	Heart	AA799850, AI410657, AI009339, AA926093, AA925642, AA956406	1086 (4144)** 1186 (3547)** 1284 (4024)**	512
	Lung	AI170064	1085 (4023)	249
	Placenta	AI170064	1091 (5054)	198
	Skeletal muscle	AA849137	1089 (4183)	239

Note. *Normalised cDNA libraries. **Subtracted cDNA libraries.

have two TM domain positions (72–92 and 218–237) with the N-terminus and C-terminus located intracellularly and with the domain present between the two TM domains located extracellularly.

Expression of the Mouse dscr2 Gene in Embryo and Adult Tissues by Northern Analysis

In order to determine the expression pattern of the mouse dscr2 gene a Northern blot of 9 adult tissues and 6 embryo stages was performed using as a probe the insert of the EST 0775603 cDNA. The results of the Northern analysis in adult tissues are shown in Fig. 2A. The mouse dscr2 mRNA has an estimated length of 1.13 kb and is highly expressed in testis in comparison with brain, liver and kidney. It is also expressed at lower levels in heart, skeletal muscle and pancreas (Fig. 2A).

The Northern analysis of complete mouse embryo indicates that the gene is expressed from E11 to E18 (Fig. 2B). The quantitative results, after normalisation of the density of the hybridised bands with the corresponding 28S RNA band, are shown in the lower panel of Fig. 2B. The results indicate that there are no drastic changes in the levels of expression of the dscr2 gene during the development of the complete mouse embryo.

In Silico Gene Expression Analysis of the Mouse dscr2 Gene

The results of an *in silico* gene expression analysis using BLASTN searches of the mouse EST databases provides qualitative expression data (Table 1). The *in silico* gene expression analysis indicates that the dscr2 gene is expressed during all the stages of the mouse embryo development, from the stem cells and the fertilised egg to the E19.5 development stage. It also indicates that the dscr2 gene is expressed in other tissues such as the mammary gland and the stomach (Table 1). Therefore the *in silico* expression data is consistent qualitatively with the expression pattern of the dscr2 gene determined experimentally.

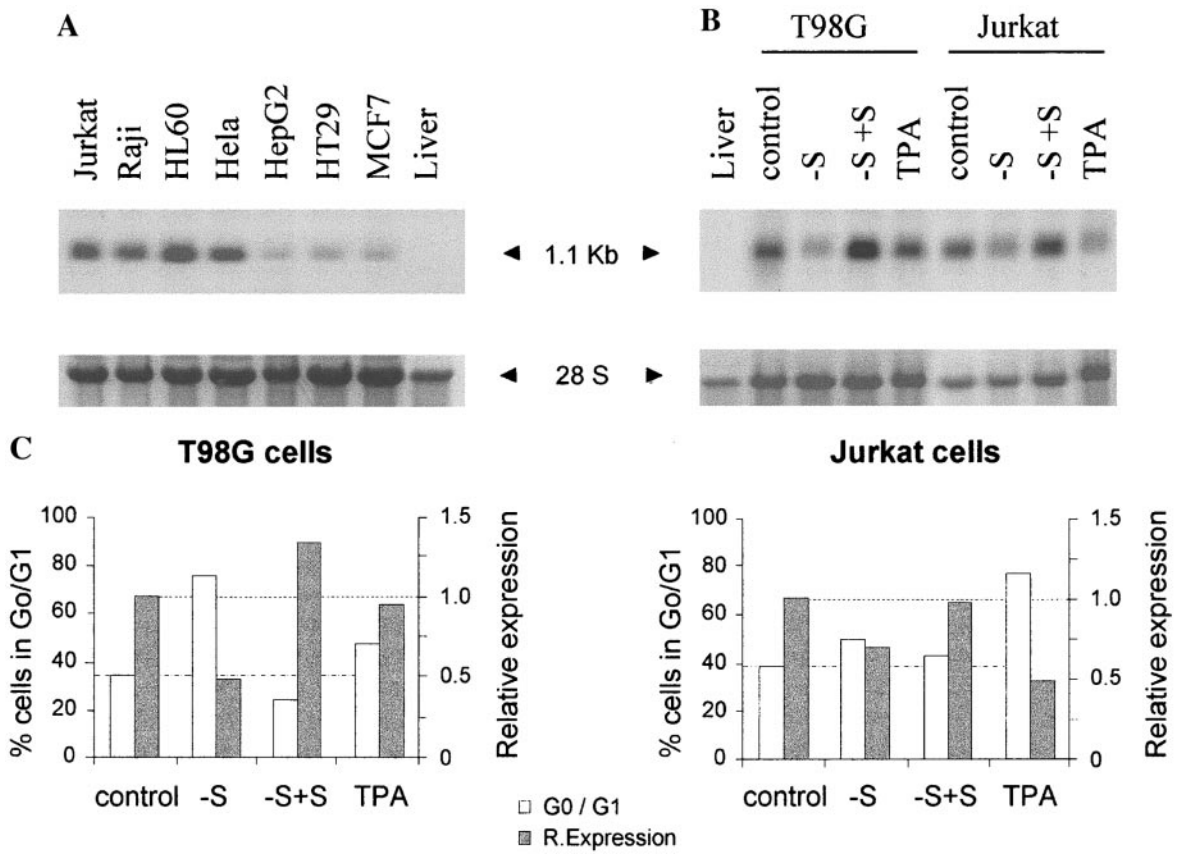

FIG. 3. Northern analysis of the human DSCR2 gene in different cancer cell lines under different culture conditions: (A) RNA from 7 different human cancer cells lines as indicated. (B) RNA from T98G and Jurkat cell cultures at different conditions as follows: In complete medium for 72 h (control), in serum-free medium for 48 h (−S), in complete medium for 24 h after 72 h of culturing in serum-free medium (−S + S), and in complete medium with 100 nM TPA for 24 h (TPA). RNA from human liver has been used as a control of tissue cells in quiescence. (C) Percentage of cells in G0/G1 stage in the cultures of T98G and Jurkat cells at the conditions indicated above (open bars), and the normalised results of the Northern blot shown in the part B of this figure (solid bars), using the 28S RNA as internal control of normalisation. The results obtained are relative to the expression in T98G or Jurkat controls.

Expression of the DSCR2 Gene in Different Human Cell Lines

A parallel approach to contribute to search for the function of the DSCR2 gene was started studying the expression of the gene in different human cell lines. In order to study the expression of the DSCR2 gene in proliferating cells of different origin, a Northern analysis was performed using RNA from 7 different human cell lines (Fig. 3A). RNA from human liver was used in the Northern analysis as a control of the expression in a tissue that has a high proportion of non-proliferating cells (Fig. 3). The normalised results indicate that the DSCR2 mRNA is expressed in all of the growing cell lines tested. The expression of the DSCR2 mRNA is higher in HL60, HeLa, Jurkat, and Raji, as compared to the HepG2, HT29, and MCF-7 cell lines. The DSCR2 mRNA is expressed in the hepatocellular carcinoma cell line HepG2 but no expression is detected by Northern analysis in non-proliferating human liver as previously described (5).

To further investigate if the expression of the gene could be linked to cell proliferation we have compared the mRNA levels between proliferating and quiescent cells. Therefore, cultures of T98G and Jurkat cells were submitted to different proliferation conditions and the RNA was isolated and analysed through Northern analysis (Figs. 3B and 3C). T98G cells were grown in the absence of FCS in the culture medium during 72 h to synchronise the cells at G0/G1. Subsequently, proliferation was induced by addition of FCS and the RNA was obtained after 24 h of incubation. Treatment of the cultured cells with TPA during 24 h was used to induce quiescence of Jurkat cells. Cell cycle analysis was performed in all these treatments to confirm the stage of cell cycle of the cells. In the case of the T98G cells, the depletion of FCS in the culture medium induces a decrease in the expression of the DSCR2 mRNA of 51% in comparison with the control. Subsequent addition of FCS to the culture results in an increase of the expression of 173% relative to the quiescent cells. In the case

of Jurkat cells, the depletion of FCS in the culture medium also reduces the levels of expression of the DSCR2 mRNA, and the posterior addition of FCS restores the expression to the control levels. The results of the treatment of Jurkat cells with TPA indicate that the expression of the DSCR2 mRNA decreases to the same levels of cultured T98G cells in absence of FCS. However, TPA treatment of T98G cells does not have any effect in the expression levels of the mRNA, indicating that TPA probably does not directly regulate the expression of the DSCR2 gene. The results of the cytofluorometric analysis of cell cycle of both cell lines and the results of the Northern analyses at different conditions (Fig. 3C) show that the levels of DSCR2 mRNA correlate with cellular growth in T98G and Jurkat cells.

DISCUSSION

In this work, we report the identification and the characterisation of the mouse homologue of the human DSCR2 gene, also known as c21LRP, and the expression analysis in different human cell lines demonstrating that the expression correlates with the proliferation status. The mouse cDNA identified and characterised has a high homology with the human DSCR2 cDNA. The mouse dscr2 encodes for a protein sequence that has a high identity with the human DSCR2 protein sequence indicating that the DSCR2 gene has been conserved in these two species during the evolution (Fig. 1B). It also indicates a need in the conservation of the structure of the DSCR2 protein suggesting a potentially important conserved function. The secondary structure predictions of the mouse protein sequence using different algorithms detects the same two transmembrane domains that have been predicted in the human protein sequence (5), suggesting a possible location of the protein associated to the membrane. It is possible to predict two potential alternative topological models, either with a single or with two TM domains. The structure of the model based in one TM domain is quite common in different families of transmembrane proteins, whereas the model based in two TM domains is less frequent and only has been described in several membrane channels (27). Despite the identification of the two potential TM motifs, the classical approaches to determine the possible function of the gene based in the amino acids sequence homology to known proteins have failed (5).

Therefore, we have initiated approaches based on the study of the expression of the DSCR2 gene as an alternative source of information contributing to the characterisation of the possible function of this gene. The results obtained indicate that the expression pattern of the dscr2 gene is similar in the different tissues of adult mouse as compared to adult human tissues. This gene is expressed at higher levels in the testis and at lower levels in brain and in liver in both species (Fig. 2A (5)). The dscr2 mRNA is also expressed at lower levels in kidney, heart, muscle and pancreas as derived from the Northern analysis using RNA from mouse tissues. The expression results obtained in the mouse embryo through Northern analysis indicate that the dscr2 mRNA is expressed during the stages from E11 to E18 of the development (Fig. 2B) and agree with the expression of the gene described in the human embryo tissues (5). The results obtained in an *in silico* analysis of the expression also indicates that the gene is expressed during all stages of the mouse embryo development starting from the one cell embryo stage (Table 1). However, these *in silico* results are only qualitative in the case of the ESTs obtained from normalised or subtracted cDNA libraries.

In a parallel approach based in the study of the expression pattern of the gene in human cell lines, we report that the gene is expressed in all of the proliferating human cell lines that we have tested (Fig. 3A). The DSCR2 gene also seems to be regulated through changes in the cell cycle as it can be deduced from the Northern analysis of cells cultured in conditions that induce quiescence or proliferation of the cells (Fig. 3B). Thus, the DSCR2 gene seems to be expressed in the cells and tissues that have a high rate of proliferation such as the fetal tissues, the adult testis or the cancer cell lines. The changes observed in the levels of expression with the induction of quiescence or proliferation in the cells agree with the idea that the expression of the DSCR2 gene changes during the cell cycle. In addition, the amino acid sequence has a potential homology to proteins involved in cell cycle. In conclusion, all these results indicate that the function of the DSCR2 gene product could be related to cell proliferation. This possible role in cell proliferation would also agree with the relative high levels of expression of the DSCR2 gene in testis, a tissue that has a high level of cell proliferation (28).

In summary, the location of the DSCR2 gene in the Down syndrome critical region-2, together with the detected expression through embryonic development, and the correlation with cell proliferation place this gene as a potentially interesting candidate to explain some of the features present in Down syndrome patients. The opportunity is now open to demonstrate through additional experiments, such as transfection of cells and focus assay the specific mechanism through which the DSCR2 gene is related to cell proliferation. The results obtained so far also open up the possibility to develop new oriented strategies, such as knock-out and transgenic mouse models, in order to determine the function of the DSCR2 gene and the potential mechanism through which this gene may be involved in Down Syndrome.

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