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A common cis-acting sequence in the DiGeorge critical region regulates bi-directional transcription of *UFD1L* and *CDC45L*

Amit Kunte[1], Kathryn Ivey[1], Chihiro Yamagishi, Vidu Garg, Hiroyuki Yamagishi, Deepak Srivastava*

Departments of Pediatrics and Molecular Biology, University of Texas Southwestern Medical Center at Dallas, 6000 Harry Hines Boulevard, Rm. NA8.124, Dallas, TX 75390-9148, USA

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Abstract

Two to three megabase deletions on chromosome 22q11 are the cytogenetic findings most commonly associated with cardiac and craniofacial defects in humans. The constellation of clinical findings associated with these deletions is termed the 22q11 deletion syndrome. We had earlier described a patient with the 22q11 deletion phenotype who was hemizygous for an atypical 20 kb microdeletion in this region. The deletion included coding regions of two genes organized head-to-head, *UFD1L* and *CDC45L*, along with an 884 bp CpG-rich intervening region. Based on this genomic organization, we hypothesized that both genes may be co-expressed and co-regulated by sequences within this region. We demonstrate that expression of both genes is enhanced in a similar pattern in precursors of structures affected by the deletion. The intergenic region is sufficient to direct transcription most strongly in the developing pharyngeal arches and limb buds of transgenic mice and can also direct bi-directional transcriptional activation in a neural crest-derived cell line. Deletion analyses revealed that a 404 bp fragment closest to *UFD1L* is necessary and sufficient to direct this bi-directional transcriptional activity. These results reveal the presence of a conserved regulatory region in the 22q11 deletion locus that can direct simultaneous transcription of genes involved in ubiquitin mediated protein processing (*UFD1L*) and cell cycle control (*CDC45L*).

Keywords: DiGeorge; 22q11; Bi-directional promoters; *UFD1L*; *CDC45L*; Neural crest; CpG islands

1. Introduction

Deletions within the q11.2 region of chromosome 22 are associated with a variety of cardiac and craniofacial abnormalities that are characteristic of three clinical syndromes – DiGeorge syndrome (DGS), velocardiofacial syndrome (VCFS), and conotruncal anomaly face syndrome (CTAFS) (Emanuel et al., 1998). Based on their common phenotypic features and genetic etiology, these three clinical entities are referred to as the 22q11 deletion syndrome. Nearly 40 developmental defects are associated with this syndrome, the most common including malformations of the cardiac outflow tract and aortic arch, hypoplasia of the thymus and parathyroid glands, and the presence of a cleft palate. A common feature in the normal development of these structures is a contribution by cranial neural crest cells that migrate through the pharyngeal arches. Neural crest ablation in chick embryos causes cardiac defects similar to those seen in the 22q11 deletion syndrome (Kirby and Waldo, 1990). These observations suggest that development of neural crest-derived tissues is affected by deletion of segments of 22q11.2 and that one or more factors responsible for normal neural crest development are encoded in this region.

A variety of overlapping deletions within the commonly deleted DiGeorge critical region (DGCR), typically measuring 2–3 Mb, have been reported in patients with the 22q11 deletion syndrome. Interestingly, the phenotype does not correlate with the size or region of the deletion. In addition, a number of non-overlapping deletions have been reported in patients clinically diagnosed with the syndrome (Carlson et al., 1997; Novelli et al., 2000), some of which are outside the DGCR. Furthermore, no point mutations of any genes have been reported in patients who have the disease phenotype but do not have a 22q11 deletion.

Recent evidence in mice indicates that haploinsufficiency of *Tbx1*, a T-box family member that is expressed in the developing pharyngeal arches (Chapman et al., 1996) may be a major contributor to aortic arch defects in the 22q11 deletion phenotype. A subset of mice heterozygous for *Tbx1*

* Corresponding author. Tel.: +1-214-648-1246; fax: +1-214-648-1820.
E-mail address: dsriva@mednet.swmed.edu (D. Srivastava).
[1] These authors contributed equally to this work.

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display defects in aortic arch patterning that are reminiscent of defects seen in the 22q11 deletion syndrome (Jerome and Papaioannou, 2001; Merscher et al., 2001), although no other features of the syndrome are present. Furthermore, aortic arch defects seen in mouse chromosomal deletions syntenic to 22q11 can be rescued by a transgene carrying *Tbx1* (Lindsay et al., 2001; Merscher et al., 2001). The 22q11 deletion phenotype has, however, also been detected in patients with deletions that do not include *TBX1*. Additionally, as with other candidate genes, no patients have been identified with point mutations of *TBX1*.

These observations suggest a complex molecular mechanism underlying the pathogenesis of the 22q11 deletion syndrome. There are two popular explanations that may account for the observed discrepancies. The DGCR may contain common regulatory regions, such as locus control regions, that affect gene expression over a long range (Dallapiccola et al., 1996; Novelli et al., 2000). Such a region may affect expression of important distant genes even though they are not included in the deletion. Alternatively, the DGCR may contain multiple genes that function, either individually or in a combinatorial fashion, to regulate neural crest development (Lindsay and Baldini, 1998).

We had earlier described a patient, J.F., who demonstrated many of the clinical features commonly associated with the 22q11 deletion syndrome (Yamagishi et al., 1999). J.F. did not have the typical 22q11 deletion, but rather had only a 20 kb microdeletion that encompassed the first three exons of *UFD1L*, the first five exons of *CDC45L* and an 884 bp region between the two coding sequences. The Ufd1L protein has been implicated in post-ubiquitination processing of proteins (Hoppe et al., 2000; Johnson et al., 1995; Meyer et al., 2000) while Cdc45L orthologs are essential for the initiation of DNA replication (Loebel et al., 2000; Saha et al., 1998; Zou and Stillman, 1998). Support for a role of *Ufd1L* haploinsufficiency in the phenotype of J.F. came from the observation that *Ufd1L* gene expression is enhanced, during embryogenesis, in regions of the embryo derived from the cranial neural crest. Furthermore, expression of *Ufd1L* is downregulated in mice lacking dHAND, a basic helix–loop–helix transcription factor necessary for cranial neural crest development. Mice heterozygous for *Ufd1L* do not, however, have any detectable anomalies (Lindsay et al., 1999). This fact, in conjunction with the observation that both genes were disrupted in the patient J.F. led us to speculate that deletion of *UFD1L* alone is not likely to be responsible for the phenotype of J.F., and that combinatorial deletion of *UFD1L* and *CDC45L*, and/or the 884 bp intervening region may have contributed to the phenotype.

UFD1L and *CDC45L* are organized with their coding regions oriented in a head-to-head fashion and thus with their promoters presumably located in the 884 bp intervening sequence. This unusual genomic organization has been described previously for genes that are functionally related and co-regulated (Gaston and Fried, 1995; Guarguaglini et al., 1997; Schilling and Farnham, 1995). Here, we show that expression of *Ufd1l* and *Cdc45l* is enhanced in similar regions of the developing embryo. Furthermore, we demonstrate that the intergenic region contains the regulatory sequences necessary to direct strongest expression in the pharyngeal arches and limb buds of transgenic embryos, and that it directs bi-directional transcriptional activity in a variety of cell lines, including neural crest cells. The critical sequences necessary for transcription in both the *UFD1L* as well as the *CDC45L* directions localize to a 404 bp region upstream of the *UFD1L* coding sequence, suggesting that the two genes may be partly co-regulated in some cell types. We speculate that the two genes may be related functionally, either directly or indirectly, and therefore may play a combinatorial role during development.

2. Results

2.1. Coexpression of Ufd1l and Cdc45l

In order to compare the expression patterns of *Ufd1L* and *Cdc45L* in the precursors of tissues affected in the 22q11 deletion syndrome, we performed section in situ hybridization experiments for each mRNA on E9.5 mouse embryos. Hybridizations were performed on serial transverse sections at the level of the second pharyngeal arch and demonstrated a similar pattern of expression for the two genes (Fig. 1A–C,E–G). Both genes were expressed maximally in the neural crest-derived pharyngeal arch and aortic arch artery. Lower levels of expression were detected in other areas of the section, such as the heart mesenchyme and in the cleft between the second and third pharyngeal arches. Similar results were observed at E10.5 (data not shown). Whole mount in situ hybridization results were consistent with section results and showed that both *Ufd1l* and *Cdc45l* were expressed widely at low levels in the developing embryo, but there were specific regions where enhanced expression of both genes was detected, particularly the pharyngeal arches and limb buds (Fig. 1D,H).

2.2. The UFD1L–CDC45L intergenic region is a conserved CpG island

As *Ufd1l* and *Cdc45l* expression was enhanced in similar tissues during development, we analyzed the 884 bp intervening region separating the coding sequences for potential regulatory sequences. The sequence of the entire human *UFD1L–CDC45L* intergenic region (accession no. AC 000087; human cosmid 83c5) and the corresponding mouse sequence (accession no. NT 002584) were obtained from GenBank. Alignment of the human and mouse sequences revealed a number of stretches of conserved nucleotides throughout the region (Fig. 2A). The human sequence appeared to contain a region that fits the consensus for a TATA box in the direction of *UFD1L*. This sequence is not, however, conserved in the mouse intergenic region.

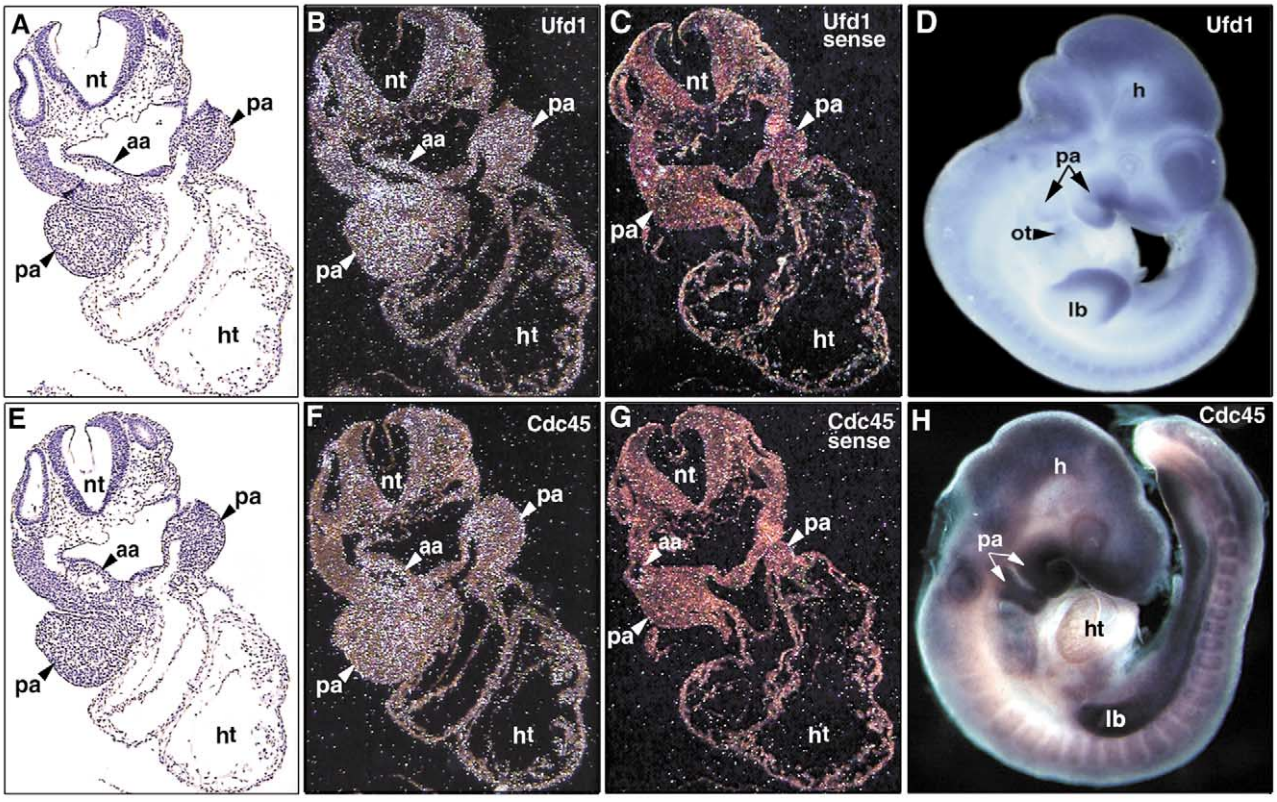

Fig. 1. Gene expression of *UFD1L* and *CDC45L*. Transverse sections of wild-type E9.5 mouse embryos, processed for in situ hybridization with antisense *Ufd1L* (B) and *Cdc45L* (F), display ubiquitous low level expression with the highest levels of each seen in the pharyngeal arches (pa). Expression in the aortic arch artery (aa) is also enhanced. Bright field views of each section are shown (A,E) along with sections hybridized with sense probes (C,G). Mouse whole mount in situ hybridization for *Ufd1L* (D) and *Cdc45L* (H) is also shown for comparison. nt, neural tube; ht, heart; h, head; lb, limb bud; ot, outflow tract.

TATA-less promoters have been frequently reported (Burbelo et al., 1988; Gaston and Fried, 1995; Gavalas et al., 1993; Ikeda et al., 2000; Zhang et al., 1998) and are usually driven by initiator elements (Smale, 1997). Transcription from such genes often initiates from multiple sites in either direction (Lavia et al., 1987). Conserved potential binding sites for a number of transcription factors such as AP-2, Sp1, Ets family proteins, CCAAT binding factors, p53, and interferon response factor-1 were also observed at various places in the intergenic region (Fig. 2A), as determined by the TESS algorithm (Schug and Overton, 1997).

In order to determine the transcription initiation sites for both *UFD1L* and *CDC45L*, we attempted to clone the $5'$ ends of both cDNAs by performing a $5'$ rapid amplification of cDNA ends (RACE) procedure on RNA harvested from HeLa cells (a human cervical carcinoma cell line). The products of the RACE reaction were analyzed by Southern blotting (Fig. 2B). A broad band was detected for the *UFD1L* RACE reaction, indicating heterogeneous products sized between 200 and 300 bp. This reflects putative start sites between positions 50 and 150 of the intervening region, with the *UFD1L* end designated as position zero. For the *CDC45L* RACE reaction, two bands were detected. The lower, broad major band reflected the presence of products sized between approximately 100 and 200 nucleotides, indicating putative start sites between positions 730 and 830. The upper, minor band was approximately 300 bp in length, indicating a putative start site around position 630.

The RACE products were cloned and sequenced in order to determine the exact positions of the putative start sites described above. As expected, a variety of products were obtained from both the *UFD1L* and *CDC45L* reactions, some of which are likely to be artifacts of premature termination of reverse transcription. For *UFD1L*, the longest sequences obtained were found to initiate at position 107 (A) or position 108 (C). We cannot differentiate between the two as the Moloney Murine Leukemia Virus (MMLV) reverse transcriptase used in the reaction adds a string of 3–5 dCs at the end of the template. The sequence surrounding this site fulfills the known minimal criteria for initiator sequences (Smale, 1997). RNAse protection analyses for the *UFD1L* gene demonstrated multiple protected bands (data not shown), further suggesting the possibility of multiple initiation sites. Sequencing of clones from the *CDC45L* RACE reaction yielded a large number of sequences initiating at distinct sites. Based on the length of the sequences and the GC content of the region, we estimated that a majority of these products most likely represent artifacts of premature termination during reverse transcription.

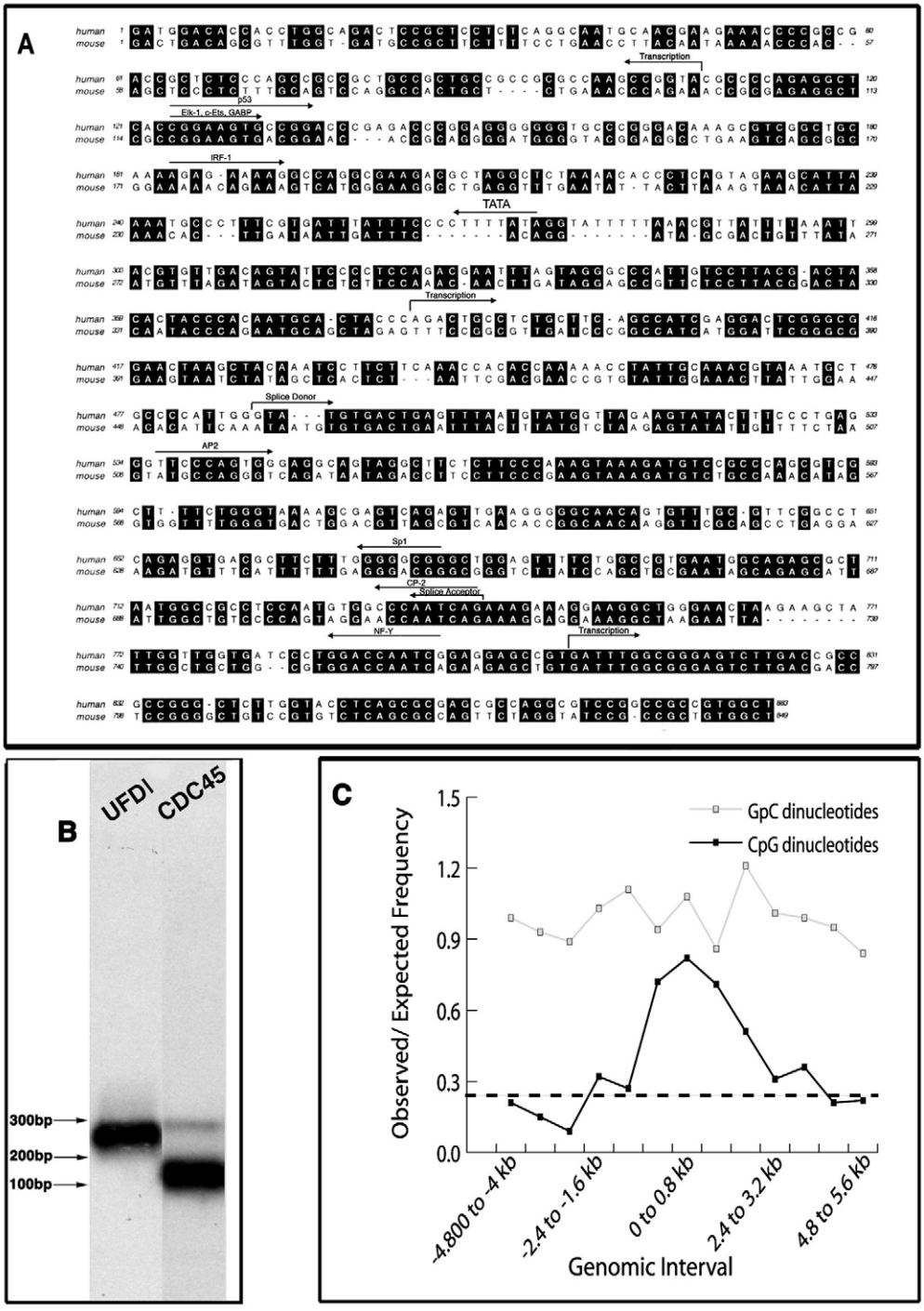

Fig. 2. Analysis of the *UFD1L–CDC45L* intergenic sequence. (A) Alignment of the human and mouse intergenic sequences demonstrates stretches of conserved residues, shaded in black. Position 1 denotes the *UFD1L* end. Transcription start sites, splice sites, and conserved transcription factor binding sites are indicated. (B) Southern analysis of the *UFD1L* and *CDC45L* 5′-RACE products was performed using sequences from the entire intervening region as probe. A broad band was detected for *UFD1L* corresponding to multiple start sites with the longest at position 108, as revealed by sequencing of the product shown. A major broad band was also detected for *CDC45L* with the longest fragment sequenced starting at position 808. The minor product indicated an additional transcriptional initiation site at position 381, by sequencing. (C) Ratio of observed frequency of CpG dinucleotides to the expected frequency of CpG dinucleotides, based on random nucleotide composition, was calculated for 884 bp segments of genomic DNA. This ratio was plotted as a function of genomic intervals including the intervening region and 884 bp intervals on either side of it. The 0–0.8 kb segment represents the intergenic region. Points on the *x*-axis indicate consecutive 884 bp segments on either side of the intergenic region. Negative numbers indicate distances from the *UFD1L* end and positive numbers indicate distances from the *CDC45L* end of the intergenic region. A similar analysis for the frequency of GpC dinucleotides serves as a negative control. The dotted line represents the frequency of CpG dinucleotides in bulk genomic DNA.

Among sequences corresponding to the lower band on the Southern blot, the largest sequence was found to initiate at position 808 (G). More than one sequence was also found to initiate at position 835 (G)/834 (C). RNAse protection assays yielded protected fragments compatible with initiation sites in the regions determined by sequencing (data not shown). Among sequences corresponding to the larger band, the most common start site was at position 381 (A), which matches the consensus sequence for an initiator site. All the transcripts initiating at position 381 lacked nucleotides 487–740, which are presumably removed by a splicing event. These results indicate that transcription of *CDC45L* initiates from multiple sites in the 884 bp intervening region. This observation is consistent with previous studies on transcriptional initiation from TATA-less promoters (Smale, 1997).

Analysis of the human sequence revealed a high G + C content. The G + C content of the entire 884 bp intervening sequence was 55.2%, while the first and last 200 bp within the region had a G + C content of 69.5 and 60.2%, respectively (Fig. 2C). The average G + C content of bulk genomic DNA is 40%. This suggested to us the possibility that this region might be a CpG island. We therefore calculated both the observed frequency of CpG dinucleotides as well as the expected frequency assuming random base composition, and used the ratio of the two values as an indicator of the relative CpG content of a given DNA segment (Fig. 2C). This was performed for the entire 884 bp intervening region and a number of contiguous 884 bp segments of genomic DNA on either side of the intergenic region. The intervening region had an observed/expected ratio of 0.82 while the ratio for bulk genomic DNA is 0.2–0.25 (Lavia et al., 1987). The ratio dropped steeply to a ratio resembling bulk genomic DNA on either side of the intergenic region, indicating that the region is indeed a CpG island. The frequency of GpC dinucleotides serves as a control and remained near an observed/expected ratio of 1 throughout the sequence analyzed.

2.3. The UFD1L–CDC45L intergenic region contains regulatory elements sufficient for bi-directional transcription

Based on the above-mentioned observations and previously available information about *UFD1L* and *CDC45L* mRNA sequence, we amplified the region from nucleotides 54–826 of the 884 bp intervening region between the coding sequences of the two genes and tested this sequence in a number of reporter gene assays. In order to test whether the *UFD1L–CDC45L* intervening region contained regulatory elements in addition to a core promoter, we generated transgenic mice carrying the *UFD1L–CDC45L* intergenic region upstream of a β-galactosidase reporter with a minimal promoter (*hsp68-lacZ*), the reporter being in the direction of *UFD1L* transcription. Whole mount X-gal staining of E10.5 F0 transgenic embryos revealed a pattern of expression similar to the expression of the *Ufd1L* and *Cdc45L* genes in 80% of embryos (Fig. 3A). Expression was detected maximally in the pharyngeal arches and palatal precursors, which are both structures containing neural crest-derived cells. Expression was also detected in the limb bud, the otocyst, and throughout the embryo at lower levels. In this assay, the presence of a heterologous promoter would

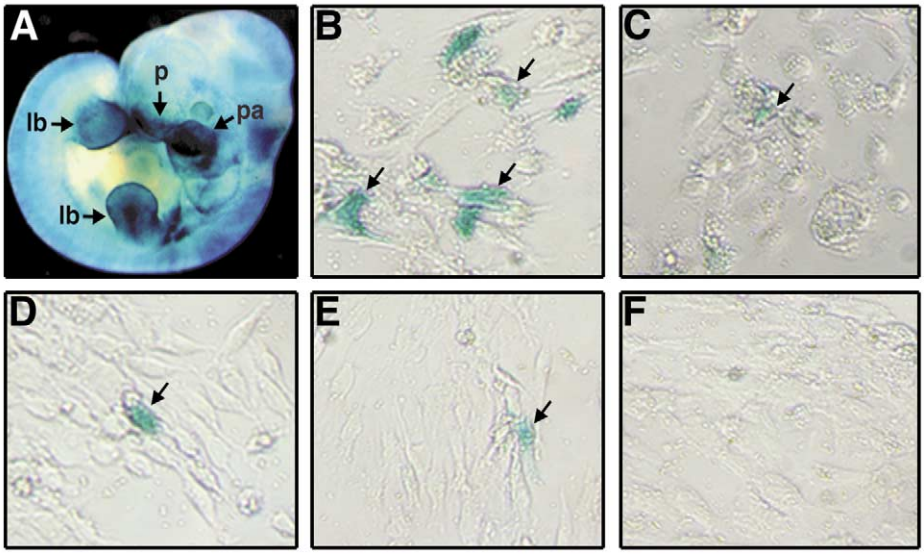

Fig. 3. Bi-directional transcriptional activity of the *UFD1L–CDC45L* intervening sequence in neural crest cells. (A) Transgenic E10.5 mouse embryo harboring a *lacZ* reporter downstream of residues 54–826 of the intervening region oriented in the direction of *UFD1L* expression. Note enhanced expression in the pharyngeal arches (pa), palatal precursors (p), and limb buds (lb). (B–F) Neural crest primary culture was transfected with hsp68lacZ downstream of residues 54–826 (B), or hsp68lacZ alone (C). A promoterless lacZ reporter driven by the intergenic region in the *UFD1L* orientation (D) or *CDC45L* orientation (E) revealed activity in both directions, when compared to a promoterless lacZ construct (F). Blue staining marks cells expressing lacZ. Arrows indicate lacZ-expressing cells.

result in a summation of enhancer activity in both directions.

As the *UFD1L–CDC45L* intervening region contained enhancer activity in mouse embryos but was less than 1 kb, further dissection and analysis of this region was performed in primary or immortalized cell cultures. Examination of the activity of this region in neural crest cell lineages was performed by transfecting chick primary neural crest cells with the same reporter construct. The total number of lacZ positive cells that migrated off the neural folds was counted in wells transfected with the above-mentioned construct and wells transfected with the empty *hsp68-lacZ* construct. An average of three independent experiments demonstrated a consistently higher proportion of positive cells in wells transfected with the construct carrying the intergenic region, the ratio being approximately 2.5:1 (Fig. 3B,C). Transfection of a promoterless *lacZ* gene did not produce any lacZ positive cells (Fig. 3F). In contrast, transfection of constructs carrying the intergenic region cloned in the 'forward' and 'reverse' orientations into a promoterless vector produced 15 and 12 positive cells per well, respectively (Fig. 3D,E). Finally, one well transfected with the intergenic region upstream of *hsp68-lacZ* was double stained for HNK, which is a marker of migrating neural crest cells. Out of 144 lacZ positive cells, 93 (64.5%) were also positive for HNK, indicating that the reporter is indeed active in migrating neural crest cells.

We next sought to determine whether the entire intervening region was sufficient to direct bi-directional transcriptional activation in a variety of cell lines. This was done by subcloning the fragment including residues 54–826 into a dual reporter vector (DRV) in the 'forward (F)' orientation (Fig. 4A inset; see Section 4, Experimental procedures). This vector contains a multiple cloning site flanked by a firefly luciferase reporter gene in one orientation and a renilla luciferase reporter gene in the other orientation. To identify cell lines that normally express *Ufd1l* and *Cdc45l* and would thus express the appropriate trans-acting factors necessary for the expression of these genes, Northern blot analysis was performed on RNA derived from multiple cell lines (Fig. 4B). *Ufd1L* and *Cdc45L* mRNA transcripts were

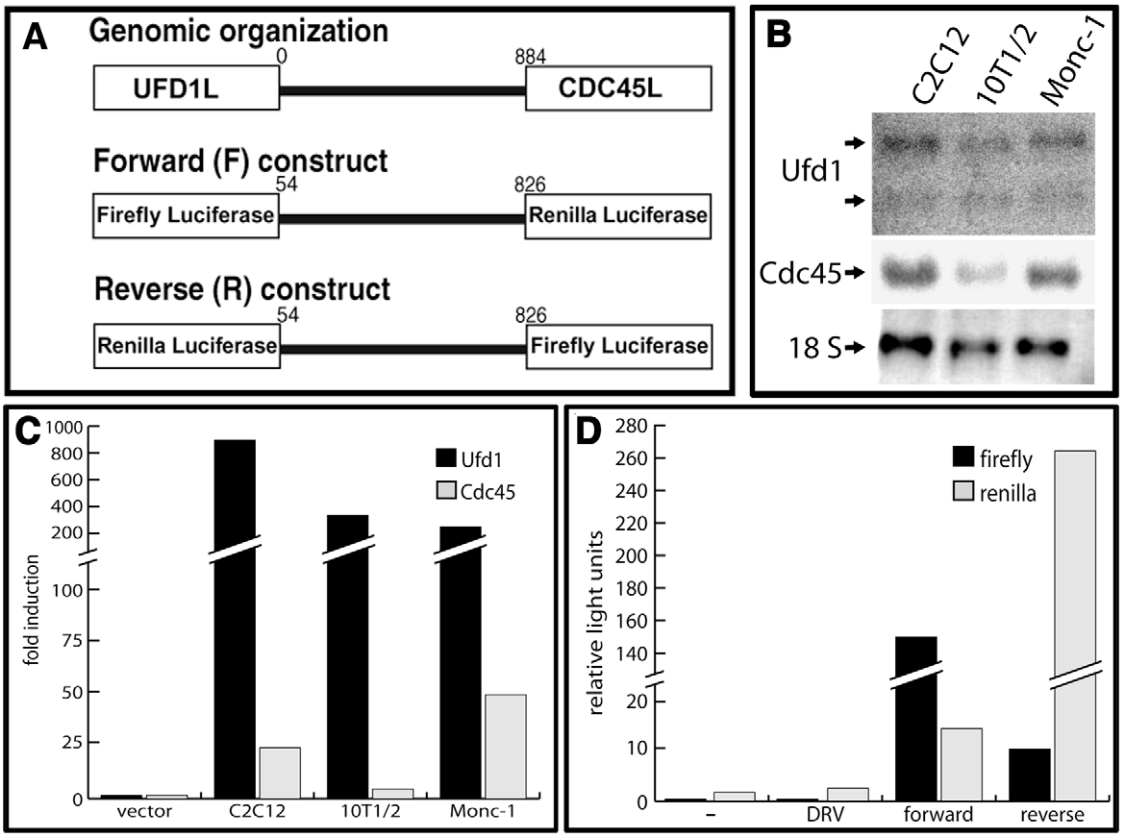

Fig. 4. Bi-directional transcriptional activity of the *UFD1L–CDC45L* intervening sequence in cell lines. (A) Schematic representation of the genomic organization of the *UFD1L* and *CDC45L* genes and the types of reporter constructs that were generated. (B) Northern blots showing endogenous expression of *UFD1L* and *CDC45* in three cell lines. 18S rRNA levels are shown as a loading control. (C) Dual luciferase assays in C2C12, 10T1/2, and Monc-1 cells transfected with DRV containing residues 54–826 of the intervening region driving expression in the direction of *UFD1L* or *CDC45L*. Luciferase activity is plotted as fold induction over cells transfected with empty vector. β-Galactosidase activity was used to normalize for transfection efficiency. (D) Dual luciferase assay in C2C12 cells, either untransfected (−), transfected with empty vector (DRV), or transfected with the DRV containing residues 54–826 of the intervening region in either the forward (*UFD1L* as firefly luciferase) or reverse (*CDC45L* as firefly luciferase) orientation. Activity is reported in relative light units and normalized to the activity of cotransfected β-galactosidase.

identified, at varying levels, in a mouse skeletal myoblast cell line (C2C12 cells; ATCC no.CRL-1772), a mouse fibroblast cell line (C3H/10T1/2 cells; ATCC no. CCL-226) and a mouse neural crest cell line (Monc-1 cells) (Rao and Anderson, 1997). The dual reporter constructs were tested in a transient transfection assay in each of the cell lines described above, enabling simultaneous detection of transcriptional activity in the *UFD1L* and/or the *CDC45L* direction. In all the three cell lines, transcriptional activity was detected in both directions. Consistently, the fold activation was higher in the *UFD1L* direction (~600-fold in C2C12, ~350-fold in 10T1/2, and ~270-fold in Monc-1 cells) compared to the *CDC45L* direction (~20-fold in C2C12, five-fold in 10T1/2, and 45-fold in Monc-1 cells) (Fig. 4C).

In order to be certain that the observed difference in transcriptional activity did not reflect a difference in the activity or stability of the two different luciferases, or a difference in transcription due to vector construction, we reversed the orientation of the fragment in the DRV in order to generate a 'reverse (R)' construct (see Fig. 4A and Section 4, Experimental procedures). We tested this construct in a similar transient transfection assay in C2C12 cells. The activity of the renilla luciferase (*UFD1L* direction) was now found to be much higher than that of the firefly luciferase (*CDC45L* direction), indicating directional specificity of the *UFD1L–CDC45L* regulatory regions (Fig. 4D). In summary, the entire *UFD1L–CDC45L* intervening region was sufficient to drive transcription of both genes and this activity was an order of magnitude higher in the direction of *UFD1L* transcription.

2.4. A 404 bp regulatory region is necessary and sufficient to direct bi-directional transcriptional activation of UFD1L and CDC45L

In order to further dissect the *UFD1L–CDC45L* bi-directional transcriptional activity, we generated progressive deletions of approximately 200 bp in the 826 bp fragment starting from either direction. Each of these shorter fragments was then subcloned into the DRV, in both orientations, and tested for transcriptional activity by transient transfection into C2C12 cells. The *UFD1L* end of the intervening region was designated as position zero and the *CDC45L* end as position 884. The two different orientations were designated as 'forward (F)' or 'reverse (R)' as depicted in Fig. 4A.

The activities of the various fragments were first compared by measuring, as activity of firefly luciferase, the fold induction of transcription over a promoterless fragment (Fig. 5A). In a series of deletions starting from the *CDC45L* end of the fragment, keeping the *UFD1L* end constant, we found that activity in the *UFD1L* direction was not affected more than two-fold by deletion of approximately 400 bp and was over 800-fold greater than control (Fig. 5A). Further deletion of 200 bp decreased the transcriptional activity by an order of magnitude. Some residual transcriptional activity was main-

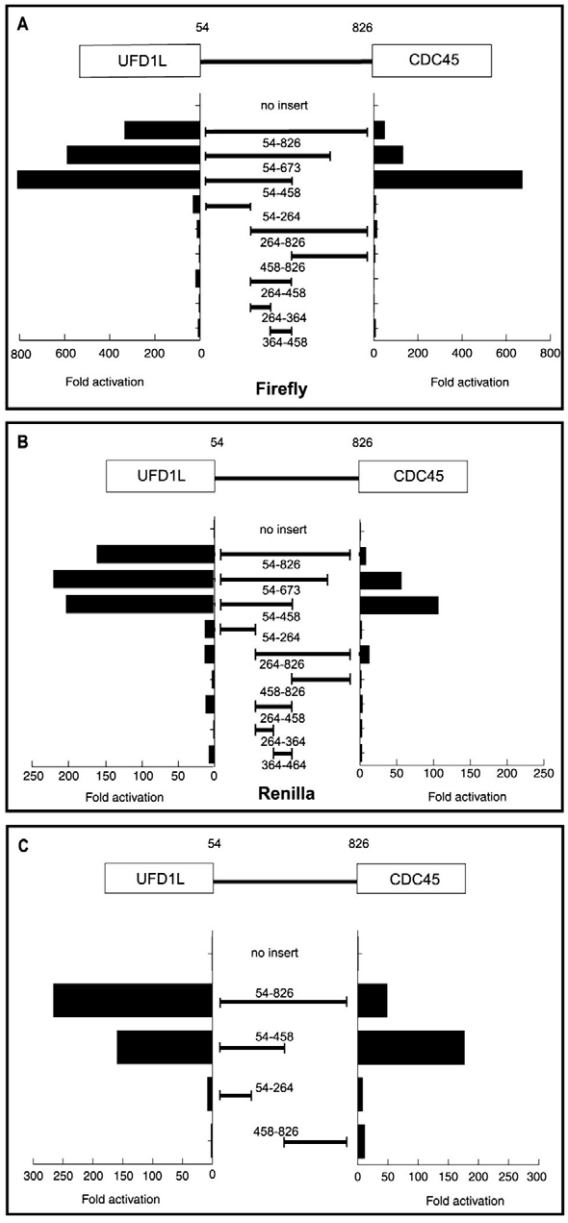

Fig. 5. Deletion analysis of the *UFD1L–CDC45L* intervening region. Fragments of the intervening region were cloned into the DRV in both orientations and tested for bi-directional activity in C2C12 cells. Transcriptional activity in both the *UFD1L* as well as the *CDC45L* directions is represented either by firefly luciferase activity (A) or renilla luciferase activity (B). Fold induction of transcription over basal activity of a promoterless construct is plotted on the value axis. β-Galactosidase activity was used to normalize for transfection efficiency. Data is an average of experiments done in duplicate. Black lines between the graphs indicate the particular nucleotides used in the construct. (C) Selected fragments of the intergenic region were cloned into the DRV in the 'reverse' orientation (Materials and methods). Transcriptional activity in the *UFD1L* direction is measured as renilla luciferase activity while activity in the *CDC45L* direction is measured as firefly luciferase activity. Fold induction of transcription over basal activity of a promoterless construct is plotted on the value axis. β-Galactosidase activity was used to normalize for transfection efficiency. Black lines between the graphs indicate the particular nucleotides used in the construct.

tained (30-fold over control). A fragment containing residues between 264 and 458 bp from the *UFD1L* end of the intergenic region similarly activated at only a 20-fold higher level compared to control.

Unexpectedly, deletion of 200 bp from the *CDC45L* end resulted in an increase in activation in the *CDC45L* direction from 50- to 130-fold. Deletion of 200 bp more resulted in a further increase in transcriptional activity up to 670-fold. The activity of this 404 bp fragment in the *CDC45L* direction was an order of magnitude higher than the activity of the full-length intervening region. Also, the fold increase of luciferase activity for the 54–458 bp fragment was approximately equivalent in either direction (~800 and 670-fold over control in the *UFD1L* and *CDC45L* directions, respectively). Deletion of the next 200 bp decreased activity to approximately ten-fold, which is below that seen with the full-length fragment. The 264–458 bp fragment did not, however, reproduce the increase seen with the 54–458 bp fragment, nor did fragments containing nucleotides 264–364 or 364–458.

In a complementary series of deletions from the *UFD1L* end of the intergenic region, activity in the *UFD1L* direction was diminished by an order of magnitude after deletion of the first 200 bp. Deletion of the next 200 bp did not cause a significant change. In the *CDC45L* direction, transcriptional activity after deletion of the first 200 bp was lower than that observed with the full-length fragment (~50- and 15-fold for the 826 and 673 bp constructs, respectively). The activity was only six-fold greater than control after deletion of a further 200 bp, which is a level of activity significantly below that seen with the full-length fragment. All the above findings regarding relative activities of all fragments were reproduced when the activity was measured as renilla luciferase (Fig. 5B). Thus, a 404 bp fragment of the intergenic region near the *UFD1L* end is capable of driving maximal transcription in both directions and further deletion of this fragment causes a loss of this ability.

Since a neural crest cell defect is thought to be the major contributor to the pathogenesis of the 22q11 deletion syndrome, we tested the activity of the deletion constructs indicated in Fig. 5C by transient transfection into Monc-1 cells. The results confirmed, in neural crest cells, the major conclusions drawn from the above experiments. Transcription in the *UFD1L* direction was unaffected by deletion of approximately 400 bp from the *CDC45L* end and diminished by further deletions. A deletion from the *UFD1L* end abolished transcriptional activity in the *UFD1L* direction. Transcriptional activity in the *CDC45L* direction increased after deletion of 400 bp from the *CDC45L* end but was abolished after deletion of a further 200 bp. The activity was greatly diminished, compared to the full-length fragment, after deletion of 400 bp from the *UFD1L* end. Thus, even in neural crest cells, nucleotides 54–458 are capable of driving maximal transcriptional activity in both directions and further deletion of this fragment disrupts the necessary regulatory regions.

3. Discussion

3.1. Bi-directional transcriptional activity of the UFD1L–CDC45L intervening region

We have described here an analysis of the intervening region between the *UFD1L* and *CDC45L* coding sequences, which is located on chromosome 22 in the DGCR. The entire region has the ability to direct transcription simultaneously in both directions. This ability was present in a variety of cell lines, including a neural crest cell line. Deletion analyses helped us narrow the region necessary for maximal bi-directional transcriptional activity to a 404 bp fragment closest to *UFD1L*. Further deletions of this fragment caused a loss of activity. This indicates the presence of critical regulatory elements for *UFD1L* transcription in the 264–458 bp fragment that are required in addition to the core promoter, which is present in the 54–264 bp fragment.

The 458–826 bp fragment has low transcriptional activity in the direction of the *CDC45L* gene, though it contains the observed initiation site at position 808. This indicates either the absence of a positive regulatory element or the presence of a repressive element in the 458–826 bp fragment. Though we cannot distinguish between these possibilities, it is possible to conclude that all the regulatory elements necessary for bi-directional transcriptional activation reside in the 54–458 bp fragment of the intervening region. It is also worth noting that the fold activation shown by the 54–458 bp fragment is similar in both directions. This is evident when activity in either direction is measured using either firefly luciferase (Fig. 5A), or renilla luciferase (Fig. 5B). We consider this finding to be indirect evidence that the two genes are co-regulated, possibly by the sharing of bi-directional regulatory elements. Such bi-directional elements responsive to factors such as Ets (Gaston and Fried, 1995; Ikeda et al., 2000), Sp1, and E2F (Guarguaglini et al., 1997) have been previously described in the context of similarly organized promoters.

3.2. The UFD1L–CDC45L intergenic region is a CpG island

CpG islands are commonly associated with the 5′ end of both housekeeping as well as some tissue-specific genes (Bird, 1986). It has been proposed that CpG islands frequently mark coding sequences that are organized head-to-head and contain bi-directional promoter activity (Lavia et al., 1987). This hypothesis has been borne out by a large number of studies. A number of genes that have been found to be organized in this manner encode proteins that are members of the same structural complex, including: (a) histone 2A and histone 2B (Osley et al., 1986), (b) α_1 and α_2 subunits of collagen 4 (Burbelo et al., 1988), and (c) α and β subunits of the human mitochondrial trifunctional protein involved in fatty acid metabolism (Orii et al., 1999). In other cases, genes encoding enzymes that

participate in sequential steps of an enzymatic pathway have been found to be organized in this fashion, such as genes in the purine biosynthetic pathway (Gavalas et al., 1993). Yet other genes have been found to respond to a common cellular need, such as growth induction (Guarguaglini et al., 1997; Schilling and Farnham, 1995) and are thus co-expressed. The characterization of the *UFD1L–CDC45L* intervening region as a CpG island is a further indication that the genes may be co-regulated. Such bi-directionally oriented genes that are co-regulated either share a common bi-directional start site and thus have a common promoter in the true sense or have closely spaced transcription initiation sites and share regulatory elements (Gaston and Fried, 1995; Ikeda et al., 2000; Orii et al., 1999). Our analysis supports the latter possibility in the case of *UFD1L–CDC45L*.

3.3. Relevance to the 22q11 deletion phenotype

Recent evidence indicates that *Tbx1*, a member of the T-box family of transcription factors (Chapman et al., 1996), may be a major contributor to aortic arch defects in mice that are similar to those seen in the 22q11 deletion syndrome (Jerome and Papaioannou, 2001; Lindsay et al., 2001; Merscher et al., 2001). Although *Tbx1* is not expressed in the neural crest-derived component of the pharyngeal arches, it is found in the pharyngeal endoderm and mesoderm, where it is regulated by a sonic hedgehog-dependent signaling pathway (Garg et al., 2001). However, the lack of *TBX1* point mutations in patients with the 22q11 deletion phenotype and observation of the disease phenotype in patients bearing deletions that exclude *TBX1* suggest a role for other genes in this syndrome.

The occurrence of a phenotype resembling that of the 22q11 deletion syndrome has been reported in mice homozygous for a null mutation in *Crkl*, a neural crest enriched, SH2 and SH3 domain-containing adapter protein that is located in the 22q11 deletion locus (Guris et al., 2001). This finding supports the hypothesis that other genes within the DGCR, that are involved in neural crest development, may also contribute to the 22q11 deletion phenotype. Our results regarding the presence of bi-directional transcriptional activity, a characteristic genomic organization and similar embryonic expression patterns suggest that *UFD1L* and *CDC45L* might be, at least in part, co-regulated. They may also thus be functionally related in neural crest-derived tissues, either directly in a molecular pathway or indirectly by responding to the same cellular needs. Clarification of these possibilities awaits elucidation of the precise cellular functions of the UFD1L and CDC45L proteins during development. Current studies implicate *UFD1L* in post-ubiquitination processing of proteins (Hoppe et al., 2000; Johnson et al., 1995; Meyer et al., 2000). *CDC45L* orthologs have been shown to be essential for the initiation of DNA replication in yeast and metazoans (Loebel et al., 2000; Saha et al., 1998; Zou and Stillman, 1998). Although these functions are seemingly unrelated, the fact that a number of cell cycle regulators are regulated by ubiquitin-dependent proteolysis may be a clue to a possible functional relationship between these proteins (Koepp et al., 1999; Tyers and Jorgensen, 2000). Elucidation of the biochemical pathways in which *UFD1L* and *CDC45L* function may thus help determine their contribution to neural crest development and to the 22q11 deletion phenotype.

4. Experimental procedures

4.1. Radioactive section in situ hybridization

A 415 bp *Cdc45L* mouse cDNA fragment was amplified by reverse-transcriptase-polymerase chain reaction (RT-PCR) using whole mouse embryo RNA (upper, 5′-ACC-ACTTCATCCAGGCTCTC-3′; lower, 5′-GGTGCTTTC-TGCTGCCTTCT-3′) under the following conditions: 94°C for 5 min; 94°C for 30 s, 60°C for 30 s, 72°C for 30 s (35 cycles); 72°C for 7 min. A plasmid containing the 700 bp open reading frame of mouse *Ufd1L* cDNA was utilized. ^{35}S-labeled antisense riboprobes for *Cdc45L* and *Ufd1L* were synthesized with T7 or SP6 RNA polymerase, respectively (MAXIScript; Ambion Inc., Austin, TX). Radioactive section in situ hybridization was performed on paraffin-embedded serial sections of E9.5 and E10.5 mouse embryos.

4.2. Cloning of the intervening human genomic DNA between UFD1L and CDC45L

To obtain human genomic DNA containing *UFD1L* and *CDC45L*, a 388 bp fragment was amplified from the 3′ flanking sequence of *UFD1L* by PCR (primers: 5′-GGT-GGGTAGACAGCCTTCAT-3′; 5′-GGAATGACACTGG-GACAGAC-3′) and used to screen a human P1 artifical chromosome (PAC) genomic library (kindly provided by R. Schultz). A genomic DNA fragment of 884 bp containing the intergenic region between *UFD1L* and *CDC45L* was amplified by PCR from the PAC clone (primers: 5′-AAAAAGCTTAAACCCCGCCGACC-GCTCTC-3′; 5′-ACTAAGCTTTCAAGACTCCCGCCA-AATCA-3′).

4.3. Generation of transgenic mice

Residues 54–826 of the human *UFD1L–CDC45L* intergenic region were cloned upstream of the *hsp68-lacZ* reporter gene (Kothary et al., 1989). DNA for pronuclear injection was linearized, gel-purified and eluted using a QIAEXII kit (Qiagen, Valencia, CA). Fertilized eggs from B6C3F1 female mice were collected for pronuclear injection. Injected eggs were implanted into ICR female mice and foster mothers were sacrificed to collect F0 embryos at embryonic day (E) 10.5. DNA from yolk sacs of embryos was used for genotyping by PCR-amplification of the *lacZ* gene. Primer sequences are available upon request.

4.4. β-Galactosidase staining

Methods for analysis of transgenic mice were described previously (Yamagishi et al., 2000). Briefly, embryos were dissected in 4°C phosphate buffered saline (PBS) and fixed in 2% paraformaldehyde/PBS with phenol red for 30 min on ice. After rinsing with PBS, embryos were incubated overnight at room temperature in 0.1% X-gal, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 1 mM magnesium chloride, 0.002% NP-40, 0.1% sodium deoxycholate, PBS, pH 7.0. After staining, the embryos were rinsed in PBS and post-fixed at 4°C overnight in 4% paraformaldehyde, 0.1% glutaraldehyde, PBS.

4.5. Neural crest cell culture and transfection

The region of the cranial neural folds from the otic vessicle to somite 3 of stages 9–11 chick embryos was dissected and treated with 0.5 mg/ml of collagenase type II in PBS at 37°C for 2–3 min to remove the mesenchyme surrounding the neural tube. Isolated neural tube was washed in Dulbecco's modified Eagle's medium (DMEM) (GibcoBRL, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS), then placed on gelatin coated culture plates. The dissected neural tubes were cultured at 37°C for 2 h in DMEM with 10% FBS, to allow neural crest cells to begin migrating from the neural tube onto the culture plates. Plasmids containing empty vector or residues 54–826 of the *UFD1L–CDC45L* intervening region upstream of the hsp68-lacZ or AUG-βGal reporter were transfected into the migrating neural crest cells using FuGENE6 (Roche, Indianapolis, IN) according to the manufacturer's instructions. After culture at 37°C for 24 h, the neural crest cells were fixed and stained for β-galactosidase activity as described above.

4.6. RNA isolation and Northern blot analysis

Total RNA was extracted with TRIzol reagent (Life Technologies, Carlsbad, CA). Two micrograms of total RNA were size-fractionated on a 1% agarose/formaldehyde gel and transferred to a nylon membrane (Amersham Pharmacia Biotech, Piscataway, NJ). Ethidium bromide staining of the 18s rRNA band was used to ensure integrity and equal loading of the RNA samples. Blots were hybridized to ^{32}P-radiolabeled mouse *Cdc45L* (400 bp 3′ UTR) and *Ufd1L* (700 bp open reading frame (ORF)) DNA probes in Rapid-hyb (Amersham Pharmacia Biotech) at 62°C for 16 h. The blots were washed twice at 62°C with 2× SSC, 0.1% sodium dodecyl sulphate (SDS) and once at 62°C with 0.1× SSC, 0.1% SDS preheated to 62°C.

4.7. Determination of transcriptional start sites for UFD1L and CDC45L

5′-RACE was performed using SMART RACE cDNA Amplification Kit (Clontech, Palo Alto, CA) and total RNA extracted from HeLa cells (human cervical cancer cell line) with TRIzol reagent (Life Technologies). The *UFD1L* gene-specific primer was 5′-CTTCCCTCCTTTCTCCACATCT-GACCTGTC-3′ and the *CDC45L* gene-specific primer was 5′-TGGCTCTGGACCACCTCGTAGAACTCTTTG-3′. 5′-RACE products were size-fractionated on a 1.2% agarose gel and transferred to a nylon membrane (Amersham Pharmacia Biotech). Blots were hybridized to a ^{32}P-radiolabeled 772 bp intergenic region DNA probe in Rapid-hyb (Amersham Pharmacia Biotech) at 62°C for 16 h. The blots were washed twice at 62°C with 2× SSC, 0.1% SDS and once at 62°C with 0.1× SSC, 0.1% SDS preheated to 62°C. Products of both 5′-RACE reactions were also TA cloned (Invitrogen, Carlsbad, CA) and sequenced to determine the transcriptional start sites.

RNAse protection analyses were performed using the RPAIII kit (Ambion) according to guidelines provided by the manufacturer. 5, 10, 15, or 50 μg of HeLa cell RNA were used as input. Probes spanning 0–458 and 458–884 bp of the intergenic region were synthesized by in vitro transcription using the MaxiScript SP6/T7 kit (Ambion). The probes were synthesized antisense to both *UFD1L* as well as *CDC45L* and used in RNAse protection assays.

4.8. Construction of UFD1L/CDC45L fragments

Fragments of the *UFD1L*/*CDC45L* intervening region were amplified by PCR using the original 884 bp genomic DNA as template with appropriate pairs of the following primers. The 54 bp 5′ and 826 bp 3′ primers are located in known 5′ untranslated regions of *UFD1L* and *CDC45L* as reported in the GenBank database.

- 54: bp5′: 5′-AAAAAGCTTAACCCCGCCGACCGCT-CT-3′;
- 264 bp5′: 5′-ATGCCCTTTCGTGATTTATTTCC-3′;
- 364 bp5′: 5′-CGACTACACTACCCACAATGCACT-3′;
- 458 bp5′: 5′-TCAAACCACACCAAAAACCT-3′;
- 264 bp3′: 5′-GGAAATAAATCACGAAAGGGCAT-3′;
- 364 bp3′: 5′-AGTGCATTGTGGGTAGTGTAGTCG-3′;
- 458 bp3′: 5′-AGGTTTTTGGTGTGGTTTGA-3′;
- 673 bp3′: 5′-CCCCAAAGAAGCGTCACCTC-3′;
- 826 bp3′: 5′-TCAAGACTCCCGCCAAATCAAAAAA-GCTT-3′.

Individual PCR products were cloned into a DRV (kindly provided by A. Strauss, Vanderbilt University) by blunt-end ligation into a SmaI site. This vector was constructed by ligating bases 77-1498 of pRL Null Runilla (Promega, Madison, WI) into the KpnI site of pGL3 Basic (Promega) such that the two luciferase orthologs (runilla and firefly) were transcribed in opposite directions with a polylinker separating their 5′ ends. Clones containing each insert in either direction were then used for transfection into multiple cell lines. Constructs containing firefly luciferase in the

direction of *UFD1L* were designated 'forward' while constructs containing renilla luciferase in the *UFD1L* direction were designated 'reverse' (Fig. 4A inset).

4.9. Cell culture and transfections

The mouse fibroblast cell line, C3H/10T1/2, (ATCC no. CCL-226) or the mouse skeletal myoblast cell line, C2C12, (ATCC no. CRL-1772) was cultured in DMEM (GibcoBRL) and supplemented with 10% FBS (GibcoBRL) and penicillin–streptomycin (GibcoBRL). The cells were refed and subcultured according to ATCC guidelines. Monc-1 cells were kindly provided by D. Anderson (California Institute of Technology, CA) and cultured on fibronectin-coated plates as described previously (Rao and Anderson, 1997). C2C12 and C3H/10T1/2 cells were transfected in 100 or 60 mm plates by the calcium phosphate method as described by Sambrook et al. (1989). Five micrograms (100 mm plate) or 3 μg (60 mm plate) of experimental or control plasmid was cotransfected with equal amounts of hsp68-lacZ plasmid. β-galactosidase activity was used to normalize for transfection efficiency. Monc-1 cells were transfected by electroporation using 10 μg of each plasmid. The cells were trypsinized and resuspended in chilled PBS at a density of 10^7 cells/ml. Ten micrograms of reporter and hsp68-lacZ plasmid was electroporated into 10^7 cells (300 V at a capacitance setting of 975 μF). Cells were kept on ice for 10 min before re-plating and then grown for 36–48 h.

4.10. Luciferase assays

Cells were harvested in passive lysis buffer (Promega) according to the manufacturer's instructions. Assays were performed using the Dual Luciferase Assay System (Promega) on a luminometer (Rosys Anthos Lucy 2) using a 10 s integration time for each reading. Assays for β-galactosidase activity were performed using an ONPG (*o*-nitrophenyl-galactopyranoside; Sigma, St. Louis, MO) assay protocol as described by Sambrook et al. (1989).

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