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Connexin 31 (*GJB3*) is expressed in the peripheral and auditory nerves and causes neuropathy and hearing impairment

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Mutations in the connexin 31 (*GJB3*) gene have been found in subjects with dominant and recessive deafness and in patients with erythrokeratodermia variabilis. We report here a dominant mutation in the *GJB3* gene (D66del) in a family affected with peripheral neuropathy and sensorineural hearing impairment. A wide range of disease severity for peripheral neuropathy, from asymptomatic cases to subjects with chronic skin ulcers in their feet and osteomyelitis leading to amputations, was detected in D66del patients. Mild, often asymmetrical, hearing impairment was found in all but one patient with mutation D66del of this family and the same mutation was present in an independent family ascertained because of hearing impairment. We have found mouse connexin 31 (*Gjb3*) gene expression in the cochlea and in the auditory and sciatic nerves, showing a pattern similar to that of *Gjb1* (connexin 32), of which the human ortholog (*GJB1*) is involved in X-linked peripheral neuropathy. This expression pattern, together with auditory-evoked brainstem anomalous response in D66del patients, indicates that hearing impairment due to *GJB3* mutations involves alterations in both the cochlea and the auditory nerve. Peripheral neuropathy is the third phenotypic alteration linked to *GJB3* mutations, which enlarges the list of genes that cause this group of heterogeneous disorders.

INTRODUCTION

Gap junction channels permit the rapid exchange of ions, secondary messengers and small metabolites between neighboring cells. They are formed by connexin protein subunits, which constitute a highly conserved multigenic family. Each connexin has four transmembrane domains linked by one cytoplasmic and two extracellular loops, with cytoplasmic C- and N-terminal ends. Six connexin molecules assemble to form a half-channel or connexon, which docks with its counterpart in an adjacent cell to make a complete intercellular channel (1).

Mutations in connexin genes have been found in several disorders. *GJB1* mutations are associated with X-linked Charcot–Marie–Tooth peripheral neuropathy (2) and three other connexin genes have been identified as deafness-causing genes (*GJB2*, *GJB3* and *GJB6*) (www.iro.es/cx26deaf.html) (3). Mutations in *GJB3* cause dominant (4) and recessive (5) non-syndromic deafness and erythrokeratodermia variabilis (6,7). Association between deafness and peripheral neuropathy has been reported only for patients with mutations in *GJB1* (8). Furthermore, the pathological basis of hearing impairment due to mutations in the *GJB3* gene is unknown. We describe here a dominant mutation in the *GJB3* gene (D66del) in a family affected with peripheral neuropathy and sensorineural hearing impairment. We have detected mouse connexin 31 (Cx31, *Gjb3*) gene expression in the cochlea and in the auditory and sciatic nerves in a pattern similar to *Gjb1* [connexin 32 (Cx32)]. Peripheral neuropathy is the third phenotypic alteration linked to *GJB3* mutations.

RESULTS

D66del mutation in *GJB3*, peripheral neuropathy and hearing impairment

To evaluate a putative involvement of Cx31 in peripheral neuropathy, we have performed single strand conformation polymorphism (SSCP) analysis of *GJB3* in 103 unrelated patients affected with peripheral neuropathy. These patients

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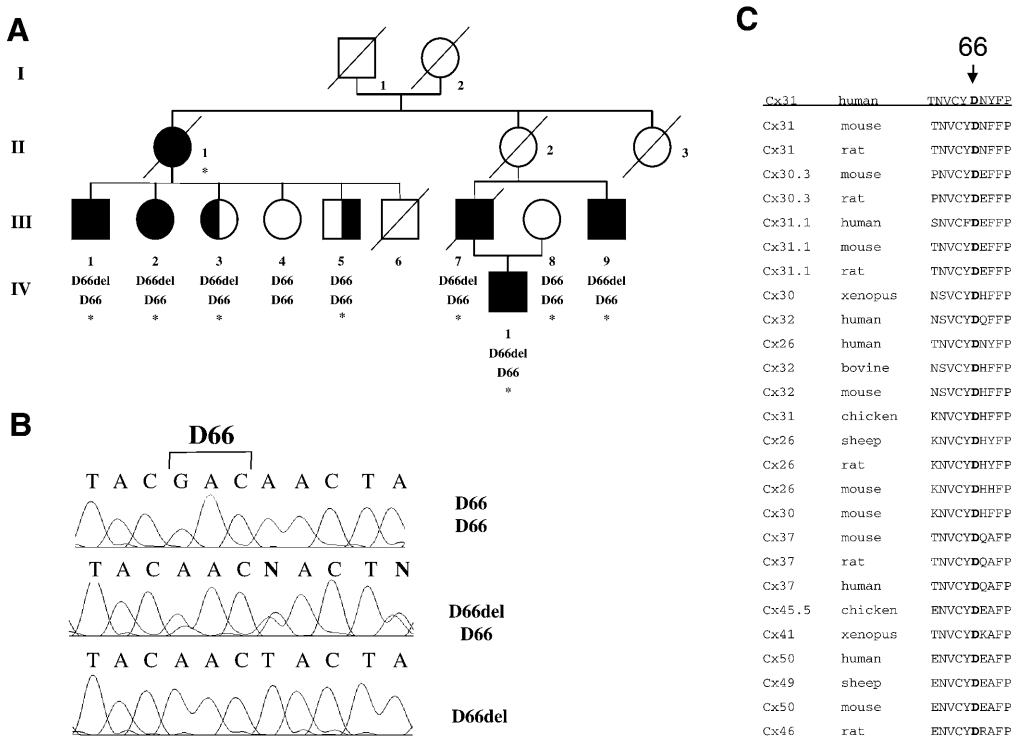

Figure 1. *GJB3* mutation in a family affected with peripheral neuropathy and hearing impairment. (**A**) Squares indicate males and circles females; members of the family affected by hearing impairment are shown in black at right and those affected by peripheral neuropathy in black at left. The presence of the D66del mutation is indicated. Family members with diabetes are marked with an asterisk. (**B**) Sequence of a control subject at the site of the mutation (genotype D66/D66); sequence of an affected individual showing heterozygosity for the mutation (D66del/D66); and sequence obtained by subcloning the mutant *GJB3* allele (D66del) from an affected individual. (**C**) Multiple alignment of Cx31 with other gap junction proteins at the site of the mutation. Amino acid 66 is shown in bold.

were previously found to be negative for mutations in *GJB1* (Cx32) and for the 1.5 Mb duplication which causes *CMT1A* (9). We detected several abnormal SSCP band patterns, seven of which turned out to be polymorphisms (10). Sequencing of an abnormal fragment from one patient (III-7; Fig. 1A) revealed a 3 bp deletion (GAC) beginning at nucleotide 196 on one allele, leading to the loss of aspartic acid at position 66 (Fig. 1B), a residue that is conserved across all species and all different connexins (Fig. 1C). Patient III-7 had been diagnosed with severe symmetrical, motor and sensory demyelinating polyneuropathy (Fig. 2A; Tables 1 and 2) and suffered from distal chronic trophic ulcerations and osteomyelitic changes requiring bilateral foot amputation.

We have studied eight relatives of this patient and have found the D66del mutation in five family members (III-1, III-2, III-3, III-9 and IV-1). Patient IV-1 never complained of neurologic symptoms, but clinical (pes cavus and hammertoes) and electrophysiological examination was consistent with a symmetric motor and sensory polyneuropathy. Patients III-1, III-3 and III-9 denied having symptoms of neuropathy and patient III-2 referred numbness and paresthesia in the four limbs. Electrophysiologic studies demonstrated minor abnormalities (in either the amplitude, latency or conduction velocity), indicating that the four patients have minor degrees of peripheral nerve involvement. In addition, the aunt of the proband (patient II-1) suffered from motor and sensory axonal polyneuropathy; this patient required the amputation of one foot. Although DNA from patient II-1 was not available for analysis, from the results of her family members it can be extracted that she had the *GJB3* D66del mutation.

Since mutations in the *GJB3* gene have been reported in patients suffering from deafness (4) and despite the fact that none of the family members available for study complained of hearing difficulties (except subject III-5, who has diabetes mellitus type II with neuropathic complications and does not carry mutation D66del), audiometric studies were performed in the members of this family (Fig. 2B). The audiogram of patient III-1 showed a unilateral severe sensorineural hearing loss at all frequencies, mainly affecting high frequencies (there was no response at 8000 Hz). The audiogram of patient III-9, who had suffered from acoustic trauma, showed a hearing threshold increase in all frequencies at the right ear, with a scotoma at 4000 Hz in both ears. Patient III-2 showed normal threshold below 2000 Hz, impaired threshold above 2000 Hz in the right ear and impaired threshold above 4000 Hz in the left ear. Patient III-3 presented a low pathological response at 1000 Hz with threshold at 30 dB in the right ear. The audiogram of III-4 was normal. Patient IV-1, who had suffered from repetitive middle-ear infection during infancy, presented a mixed hearing loss (conductive and sensorineural). Patients II-1 and III-7 had hearing problems, but audiograms were not available. Auditory-evoked brainstem response studies showed,

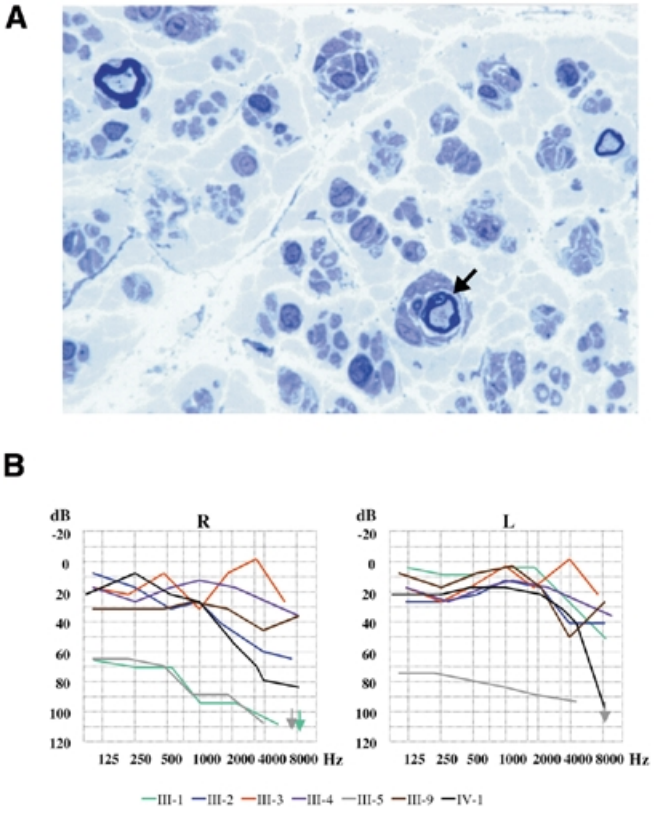

Figure 2. Phenotypic characteristics of patients with mutation D66del in the *GJB3* gene. (**A**) Sural nerve biopsy of patient III-7 showing severe demyelination, with remyelination, occasional onion bulb formation (arrow) and scattered axonal sprouting. (**B**) Audiograms of family members III-1, III-2, III-3, III-4, III-9 and IV-1. Normal auditory thresholds should be upper 25 dB at all frequencies (Hz) in both ears. Low levels of hearing were detected for middle-high frequencies. L, left ear; R, right ear; dB, decibels; Hz, Hertz.

in patient III-1, a bilateral lengthening of intervals I-III and I-V; in patient III-2, a bilateral lengthening of intervals I-III and unilateral of interval I-V; and in patient IV-1, a lengthening of intervals I-III and I-V at the left ear and a delay in wave I at the right ear (data not shown). These results are indicative of bilateral mixed cochlear and auditory nerve alteration.

Although mutations in *GJB3* have also been described in patients with erythrokeratodermia variabilis (6,11), none of the patients in the family described here have skin abnormalities and there was no family history of them. Mutation D66del was not found in 200 Spanish and Italian control subjects. The analysis of 260 unrelated deaf subjects with different patterns of inheritance of hearing impairment lead to the identification of a small nuclear family with mutation D66del. The two patients (mother and daughter) with mutation D66del had (unilateral or bilateral) high frequency hearing impairment (not shown). Neither of them complained of neurological symptoms and clinical examination was normal (Table 1). Motor and sensory sural nerve (Table 2) and median nerve electrophysiological parameters (sensory conduction velocity 62.1 m/s, distal latency 1.4 ms, amplitude 65.1 mV; and motor conduction velocity 54.1 m/s, distal latency 3.5 ms and amplitude 8.0 mV) were normal in patient AGV.

Cx31 gene is expressed in mouse peripheral nerves and cochlea

Since the reported family has both peripheral neuropathy and hearing impairment, we investigated the expression pattern of *Gjb3* in the mouse sciatic and auditory nerves and in the cochlea. We generated specific RNA probes for the mouse *Gjb3* gene and performed whole mount mRNA *in situ* hybridization. We found expression of *Gjb3* in the cochlea, the auditory nerve and the sciatic nerve (Fig. 3). *Gjb3* is expressed in the spiral limbus and spiral ligament as described recently by Xia *et al.* (12) (Fig. 3A). The section of the adult sciatic nerve after mRNA *in situ* hybridization demonstrated *Gjb3* expression in Schwann cells in a pattern similar to *Gjb1* (13) (Fig. 3G).

DISCUSSION

We have identified a deletion of three nucleotides in the *GJB3* gene, which should lead to the deletion of aspartic acid at position 66 of Cx31 in patients with different levels of severity of peripheral neuropathy and/or hearing impairment. The D66del mutation affects a residue conserved across all species and across all different connexins (Fig. 1C). Interestingly, deletion of aspartic acid at position 66 has also been found in the *GJB1* (Cx32) gene in a family with X-linked Charcot–Marie–Tooth neuropathy (9). Also, the same residue in connexin 26 (*GJB2*) has been reported mutated (D66H) in three families with Vohwinkel's syndrome (mutilating keratoderma and deafness) (14) and in a family with palmoplantar keratoderma and deafness (15). The strong conservation of this residue in all connexins and the identification of disease-causing mutations affecting residue 66 in three connexin genes (*GJB2*, *GJB3* and *GJB1*) indicate that this residue should be important for the function of connexins. Aspartic acid 66 lies in the first extracellular domain, which plays a role in multimer assembly and docking with other connexons (16). Mutation D66del could selectively impair the ability of Cx31 to form heteromeric as well as homomeric connexons, or could change the conformation of Cx31, altering the gating properties of the connexon, as has been demonstrated for some mutations in Cx32 (17).

A wide range of clinical severity (for both neuropathy and hearing impairment) seems to be associated with mutation D66del. From the data presented here it is clear that patients III-7 and IV-I have a severe sensitive and motor neuropathy, and nerve biopsy clearly showed the demyelinating characteristics of the neuropathy of patient III-7. The other patients with mutation D66del of this family have only electrophysiological minor degrees of neuropathy. A young patient from another D66del family ascertained because of hearing impairment did not present neuropathy alterations.

Despite the fact that *GJB3* has been related to hearing impairment, only one of the families with mutation D66del described was originally referred due to hearing problems. The detailed audiological examination of members of the family with peripheral neuropathy revealed that members with mutation D66del have a mixed (cochlear and auditory nerve) form of hearing impairment. Interestingly, four members of the family studied here have diabetes mellitus, which does not

Table 1. Summary of clinical data of subjects of families with mutation D66del in the *GJB3* gene

Subject	D66del	Age (yr)	Arreflexia/ hyporreflexia	Sensory symptoms	Chronic skin ulcers	Hearing impairment
III-1	+	54	–	–	–	+
III-2	+	51	+	+	–	+
III-3	+	49	–	–	–	–
III-4	–	48	–	–	–	–
III-5	–	48	+	–	+	+
III-7	+	50	+	+	+	+
III-9	+	48	–	–	–	+
IV-1	+	24	+	–	–	+
AGV	+	42	–	–	–	+

+, present; –, absent.

Table 2. Summary of electrophysiological findings in subjects of families with mutation D66del in the *GJB3* gene

Subjects	Mixed nerve conduction (median nerve)			Sensory nerve conduction (sural nerve)			Motor nerve conduction (peroneal nerve)		
	A (μV)	DL (ms)	CV (m/s)	A (μV)	DL (ms)	CV (m/s)	A (mV)	DL (ms)	CV (m/s)
III-1	6.6	4.0	55.0	4.3	3.3	42.4	4.1	5.3	39.3
III-2	9.2	3.5	57.1	7.1	3.0	46.6	2.4	3.7	47.4
III-3	8.0	3.8	45.4	ND	ND	ND	2.9	3.7	49.0
III-4	18.0	3.0	61.6	12.1	2.8	50.0	6.2	3.3	54.2
III-7	NR	NR	NR	NR	NR	NR	0.9	7.3	29.7
III-9	ND	ND	ND	10.5	4.1	43.1	7.6	4.1	42.4
IV-1	NR	NR	NR	5.9	3.4	41.1	1.2	7.7	31.4
AGV	ND	ND	ND	20.9	2.6	53.8	ND	ND	ND
Control (mean values in age-matched controls)	26.0 ± 9.7		70.0 ± 3	24.7 ±7 (20–40 years) 18.9 ± 5.2 (40–60 years)		51.8 ± 4.5 (20–40 years) 49.0 ± 4.1 (40–60 years)	6.0 ± 2.0	3.5 ± 0.6	47.6 ± 2.5

A, amplitude; DL, distal latency; CV, conduction velocity; NR, no response; ND, not done.

segregate with the mutation reported here. Patient III-5, who does not have the *GJB3* D66del mutation, suffered from symmetric sensorineural deafness, secondary to type II diabetes mellitus, which evolved over a long time, associated with a severe peripheral neuropathy. It is well known that the prevalence of hearing impairment in the diabetic population is 3 to 4-fold that of the general population and the severity of the hearing loss is related to the length of evolution of the disease and the presence of a neuropathy complication (18). The hearing loss related with diabetes is typically bilateral and symmetric, as shown by the audiogram of patient III-5 (Fig. 2B) (19).

Mutation D66del shows variable penetrance, as occurs for other missense/amino acid deletion mutations in connexin genes (3). It is possible that genetic modifiers or environmental factors, such as alcohol, diabetes and others, could contribute to the serious form of the neuropathy presented in the two patients who underwent amputation of their feet. A thiamine deficiency polyneuropathy was excluded in patient III-7, but alcohol by itself or other factors could contribute to the severe phenotype of this patient.

In summary, we have identified a new *GJB3* mutation associated with peripheral neuropathy and hearing impairment and have demonstrated that *GJB3* is not only expressed in the cochlea, but also in the auditory and peripheral nerves. This expression pattern, together with auditory-evoked brainstem anomalous response, indicates that hearing impairment due to *GJB3* mutations involves alterations in both the cochlea and the auditory nerve. It is interesting to note that the pattern of expression of *Gjb3* is comparable to that found for *Gjb1* and the neuropathological changes of patient III-7 are similar to those detected in patients with *GJB1* mutations (2). *GJB3* maps close to the chromosome 1p region, which contains the *CMT2A* locus (20,21), but it is unlikely that it is the *CMT2A* gene, as has been shown by negative mutation analysis in affected subjects from *CMT2A* families (10). This report adds *GJB3* to the list of genes already known to cause peripheral neuropathy, an extremely heterogeneous and variable disorder (22).

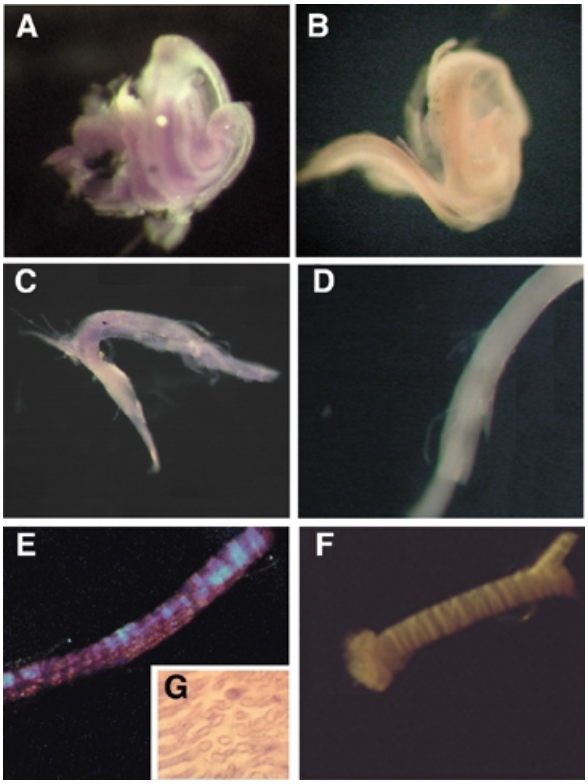

Figure 3. Mouse *Gjb3* is expressed in the peripheral nerve and the cochlea. The *Gjb3* mRNA was detected by whole-mount *in situ* hybridization of DIG-labeled probes to (**A**) cochleas (**C**) auditory nerve of P1 mice and (**E**) sciatic nerve of adult mice. Negative control for non-specific hybridization in (**B**) cochlea, (**D**) auditory nerve and (**F**) sciatic nerve. (**G**) Micrography of a sciatic nerve section showing *Gjb3* expression in Schwann cells.

MATERIALS AND METHODS

Patients and family

The pedigree of the family with peripheral neuropathy and the D66del mutation is shown in Figure 1. The proband (III-7) was a 50-year-old, non-diabetic male, with a medical history of obesity, chronic alcohol consumption and sleep apnea syndrome. He was referred to the Princeps d'Espanya Hospital when he was 47 years old with a 5-year history of numbness and paresthesia in the four limbs. On neurological examination deep tendon reflexes were absent, he had bilateral pes cavus, and muscle strength was normal in the four extremities except for the inability to walk on his heels. There was severe hypoesthesia to pin and touch in a sock and glove distribution and distal vibratory sense loss in the four limbs (Table 1). Blood biochemical and hematological parameters and cerebrospinal fluid analysis were normal. Thiamine levels in serum were normal. One year later the patient suffered from distal chronic trophic ulcerations on both feet with superimposed osteomyelitic changes, requiring bilateral foot amputation. His 24-year-old son (IV-1; Fig. 1) had never complained of neurologic symptoms and was not an alcohol drinker. However, the physical examination showed mild bilateral pes cavus and hammertoes; deep tendon reflexes were preserved in the upper limbs and knees, but were weak at the ankles, and sensation was intact. The aunt of the proband (patient II-1) suffered from insulin-dependent diabetes mellitus since the age of 39 and at the age of 51 was diagnosed with motor and sensory axonal polyneuropathy. She required the amputation of one foot. Patients III-1, III-3 and III-9 denied having symptoms of neuropathy and patient III-2 referred numbness and paresthesia in the four limbs. Electrophysiologic studies were performed in patients III-1, III-2, III-3, III-4, III-7, III-9, IV-1 and AGV.

Otoscopy was normal in all patients. Audiograms were performed in patients III-1, III-2, III-3, III-4, III-5, III-9 and IV-1. Auditory-evoked brainstem responses were performed in available patients (III-1, III-2 and IV-1) with mutation D66del.

Mutation analysis

The single coding region of *GJB3* was PCR-amplified using the two sets of the following pairs of primers: 5′-acctattcattcatacgatgg-3′ and 5′-gagtgtgcagcaggtagagg-3′; and 5′-ctacctgttcagcctcatctt-3′ and 5′-cctgcatttcccattggcag-3′. PCR was performed in a 25 µl total volume containing 100 ng of genomic DNA, 7.5 pmols of each primer, 250 µM of each dNTP, 1.5 mM $MgCl_2$ and 1 U of *Taq* DNA polymerase. The conditions for the reactions were 94°C for 5 min, 35 cycles of 94°C for 30 s, 58°C for 30 s and 72°C for 40 s, and a final extension of 72°C for 7 min. Variation from wild-type sequence was detected by SSCP/heteroduplex analysis as described by Sala and Espinosa-Parrilla (23). Direct automatic sequencing of variant fragments was performed with the same primers on an automatic genetic analyzer.

Whole mount *in situ* hybridization

Cochlea and auditory nerve from C57BL/6 post-natal day 1 (P1) mice and sciatic nerve from adult mice were dissected. The cochlea duct was exposed. Cochlea and nerves were fixed in 4% paraformaldehyde in 1× phosphate-buffered saline. Dorsal and lateral walls of the cochlea duct were removed to ensure penetration of the RNA probe in the sensory epithelium. To generate the RNA probe, a 511 bp fragment of the mouse *Gjb3* gene (positions 1021–1532 of GenBank accession no. X63099) was cloned into the pGEM-T Easy Vector (Promega). Sense and antisense probes were made by *in vitro* transcription and labeling with digoxigenin (Roche Molecular Biochemicals). *In situ* hybridization was performed as described by Nieto *et al.* (24) using 50 ng/µl of the probe in each hybridization experiment.

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