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Expression and regulation of the *Msx1* natural antisense transcript during development

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ABSTRACT

Bidirectional transcription, leading to the expression of an antisense (AS) RNA partially complementary to the protein coding sense (S) RNA, is an emerging subject in mammals and has been associated with various processes such as RNA interference, imprinting and transcription inhibition. Homeobox genes do not escape this bidirectional transcription, raising the possibility that such AS transcription occurs during embryonic development and may be involved in the complexity of regulation of homeobox gene expression. According to the importance of the *Msx1* homeobox gene function in craniofacial development, especially in tooth development, the expression and regulation of its recently identified AS transcripts were investigated *in vivo* in mouse from E9.5 embryo to newborn, and compared with the S transcript and the encoded protein expression pattern and regulation. The spatial and temporal expression patterns of S, AS transcripts and protein are consistent with a role of AS RNA in the regulation of Msx1 expression in timely controlled developmental sites. Epithelial–mesenchymal interactions were shown to control the spatial organization of S and also AS RNA expression during early patterning of incisors and molars in the odontogenic mesenchyme. To conclude, this study clearly identifies the *Msx1* AS RNA involvement during tooth development and evidences a new degree of complexity in craniofacial developmental biology: the implication of endogenous AS RNAs.

INTRODUCTION

Until very recently, the significance of natural antisense (AS) transcripts was underestimated. AS transcripts in prokaryotes have been known for at least two decades (1,2) while a limited number of cases have been reported in eukaryotes. Recent data have established their importance in eukaryotes (3–5). Indeed, these transcripts are involved in gene expression regulation (5) as exemplified by RNA interference, presently applied in numerous knockdown strategies (6). Natural AS transcripts are grouped into two classes: *cis* AS transcripts which are transcribed with the sense (S) transcripts from a unique gene locus, and *trans* AS transcripts which are transcribed from a different locus. Rare data are available on the physiological impact of AS RNAs during development. For example, in *Caenorhabditis elegans let4* controls the timing of post-embryonic cell division and fate [for review see (7)]. In mouse, miR196a negatively regulates *Hoxb8* and restricts *Hox* homeogene expression pattern (8). Interestingly, these two AS RNAs belong to the specific subclass of small RNAs also named microRNA (21–23 nt).

Recent studies have highlighted another subclass of AS RNAs: the long *cis* AS (containing >100 bp). *In silico* studies based on expressed sequence tag database have predicted that they may constitute ∼15% of the mouse genome (9) and >20% of the human genome (4). Long *cis* AS transcripts have been shown to exert regulatory functions on protein expression at various levels such as epigenetic imprinting, RNA maturation, edition and translation inhibition (5,10). However, the functional data on AS transcripts have been essentially generated *in vitro*. Very few long AS transcripts have been analyzed in the specific context of mammalian development. Some expression patterns have been reported: for instance, *Hoxa11* AS and

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S transcripts show complementary expression territories in developing limbs (11).

The mouse *Msx1* locus has been shown previously to undergo a bidirectional convergent and overlapping transcription (12). *Msx1* AS transcript belongs to the long *cis*-AS subclass (2184 nt, in mouse), and was proposed to control protein expression. This assertion was based on (i) *in vitro* evidence of protein down-regulation by the AS transcript, and (ii) *in vivo* expression patterns in newborn mouse osteoblasts (12). Indeed, in the mandibular bone *Msx1* AS transcript evidenced a complementary expression pattern to the *Msx1* S transcript. More specifically, the *Msx1* AS transcript is expressed in the latest stage of osteoblast maturation (osteocytes) whereas *Msx1* S transcript is expressed in preosteoblasts and osteoblasts (12). According to these observations, it was hypothesized that *Msx1* AS transcript expression is instrumental in the regulation of osteoblastic cell differentiation. It would control the progressive reduction of Msx1 homeoprotein expression and therefore induce the interdependent cell cycle exit and differentiation (12). This assertion is supported by several *in vitro* and *in vivo* data on Msx1 homeoprotein. *In vitro*, Msx1 overexpression blocks myoblasts differentiation (13), induces myotubes dedifferentiation into myoblasts and promotes transdifferentiation into osteoblasts (14). Consistently, *in vivo*, Msx1 expression is reversely correlated with cell differentiation progression (15,16).

During embryonic development, Msx1 is instrumental in various systems, notably in the craniofacial complex where it acts as a transcriptional repressor (17–21). *Msx1* expression is observed early in development in neural crest cells and their derivatives, including first branchial arch ectomesenchymal cells devoted to tooth formation (17,22,23). In mice, the first evidence of tooth morphogenesis occurs at embryonic day 11.5 (E11.5) as a thickening of the dental epithelium, called the dental lamina (24). However, before this morphological event and as early as E10.5, the respective incisor and molar fields are already determined. Oral epithelium induces a site-specific combination of homeobox genes expressions in the subjacent dental mesenchyme, and these combinations are organized as an 'odontogenic homeobox code' (25–27). *Msx1* has been shown to be a key element within this code, specifying the incisor presumptive region at E10.5 (25,28). After E11.5, tooth morphogenesis is already initiated and the dental lamina epithelium progressively invades the subjacent mesenchyme and forms the dental bud (E12.5–E13.5). Thereafter, dental development progresses through the cap (E14.5) and bell stages (E16.5), characterized by tooth-specific morphogenesis and cell differentiation. Tooth morphogenesis is orchestrated by successive organization centers, called primary and secondary enamel knots (29). This overall process of tooth morphogenesis is driven by sequential and reciprocal interactions between dental epithelium and mesenchyme [for review see (30,31)]. Msx1 plays a central role in these epithelial–mesenchymal interactions as evidenced by molar tooth development arrest at the bud stage in mice lacking Msx1 (23,32,33).

Finally, during tooth development, Msx1 is important for two major processes, firstly, the dental field determination and secondly, the tooth morphogenesis. Msx1 is, therefore, an exemplary model to analyze cell–cell communication leading early determination and subsequent morphogenesis in mammals. Based on this conclusion and on the recent discovery of the endogenous AS transcript of *Msx1* (12), the present study aimed to delineate the timing and impact of this AS transcript in these established developmental cascades; firstly, to determine whether *Msx1* AS transcript is expressed prior to the late terminal differentiation stages (12), and secondly, whether *Msx1* AS transcript is instrumental in epithelial–mesenchymal interactions leading to the two distinct steps of odontogenesis, namely, tooth field determination (E10.5) and tooth morphogenesis (E11.5–E16.5). Using different approaches we have proposed a novel role for *Msx1* AS in tooth development.

MATERIALS AND METHODS

Embryo collection

Timed matings were set up such that noon of the day on which vaginal plugs were detected was considered as embryonic day 0.5 (E0.5). Wild-type mouse embryos used for *in situ* hybridization and explants cultures were collected from matings of CD-1 mice (Elevage Dépré, Saint Doulchard). *Msx1/nLacZ* heterozygous embryos and *Dlx2/LacZ* transgenic embryos were collected from CD-1 females crossed with *Msx1/nLacZ* heterozygous males (23) and *Dlx2/LacZ* transgenic males (34), respectively. Pregnant mice were sacrificed by cervical dislocation and the embryos collected from E9.5 to E18.5. Newborns, 1- and 2-day-old mice were sacrificed by head cutting.

Msx1/nLacZ and Dlx2/LacZ embryos whole mount β-galactosidase staining

β-galactosidase activity was evidenced by standard histochemical methods as described previously (35). Briefly, the embryos were harvested, fixed for 30 min in a 1× phosphate-buffered saline (PBS) containing 4% paraformaldehyde (PFA), washed in 1× PBS and finally stained overnight at 32°C in a PBS 1× solution containing 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 2 mM magnesium chloride, 0.1% Nonidet P-40 and 400 μg/ml of X-Gal substrate (all products from Sigma, Saint Quentin Fallavier). The embryos were then fixed overnight in the same fixative solution and rinsed in PBS 1× before being either photographed, wax embedded and sectioned, or used for whole mount *in situ* hybridization.

In situ hybridization experiments

In situ hybridization experiments were performed for both *Msx1* transcripts and for *Dlx2* and *Barx1* S transcripts. Four probes were designed to detect *Msx1* transcripts as presented in Figure 2. Two probes, E2A and E2B, are localized in *Msx1* exon 2 and are complementary for the AS and S transcripts, respectively (Figure 2). These probes were generated as described previously (12) from a SphI/SphI *Msx1* exon 2 960 bp DNA fragment cloned in a bluescript plasmid (18). The E1 probe, complementary to the S transcript, was obtained by EcoRI digestion and T7 transcription of a bluescript plasmid containing the XbaI/EcoRI 368 bp fragment of *Msx1* exon 1 (Figure 2). This fragment was obtained by PCR amplification. The I probe, complementary to the

AS transcript, was obtained by EcoRI digestion and T7 transcription of a bluescript plasmid containing the SmaI/SmaI 935 bp fragment of *Msx1* intron (Figure 2). The *Dlx2* probe was generated as described previously from a bluescript derivative (named E61) (35). The *Barx1* probe was generated, using EcoRI digestion and T7 transcription, from a bluescript vector containing a 951 bp *Barx1* DNA fragment (kindly gifted by J. -F. Brunet).

Whole mount digoxigenin-labeled hybridizations were carried out as described previously (35). Briefly, after proteinase K and triethanolamine/acetic anhydride pretreatments, samples were fixed in 4% PFA before overnight hybridization at 65°C with probe at 1 μg/ml. After post-hybridization washes, digoxigenin immunodetection was realized with an antidigoxygenin antibody coupled to alkaline phosphatase (AP) (Roche Diagnostics, Meylan) diluted at 1/2000. The AP enzymatic activity was finally revealed using NBT/BCIP solution (Roche) according to the manufacturer's instructions.

Radioactive (^{35}S-UTP) *in situ* hybridizations were performed on 7 μm paraffin frontal sections as described previously (36). Sample sections floated onto polylysine (Sigma) coated slides were pretreated with proteinase K (Sigma) and 0.25% (vol/vol) acetic anhydride (Sigma) to reduce the background. Hybridization was carried out overnight in a humidified chamber at 60°C. The slides were then washed twice at high stringency (20 min at 60°C in 2× SSC, 50% formamide and 10 mM DTT), and treated with 40 mg/ml RNAse A (Sigma) for 15 min at 37°C to remove any non-specifically bound probe. The high stringency washes were repeated, followed by a further wash at 60°C in 0.1× SSC and 10 mM DTT. Sections were then washed in 0.1× SSC at room temperature and dehydrated in 300 mM ammonium acetate (Sigma) in 70%, 95% and absolute ethanol. The slides were air-dried and dipped in hypercoat LM-1 emulsion (Amersham-Biosciences, Orsay) and stored at 4°C in a light-tight box for 6–8 weeks. They were finally developed using Kodak D19 and fixed in Kodak UNIFIX (Kodak).

Digoxigenin-labeled *in situ* hybridizations were performed on 10 μm cryosections. Sections were hybridized overnight at 65°C in a humidified chamber with 1 mg/ml of probe in hybridization buffer. The slides were washed twice in 5× SSC, 50% formamide at 65°C, then in maleic acid buffer (MAB) at room temperature (all products were obtained from Sigma). They were incubated in MAB with 2% blocking reagent (Roche), 2% lamb serum (Gibco, Cergy-Pontoise) and a 1/500 dilution of antidigoxigenin antibody coupled with AP (Roche) at room temperature. The digoxigenin-labeled probes were detected as described previously.

Explants cultures

Mandible explants were dissected out from embryos under a stereomicroscope and, if necessary, treated with 2 U/ml Dispase (GibcoBRL) in order to separate the epithelium from mesenchyme. Intact mandibles or isolated mesenchyme were cultured as described previously (37). Briefly, all explants were cultured at 37°C, 5% CO_2 and 40% O_2 on 0.1 μm Nuclepore filters (Millipore) in a Trowell type organ culture dish containing DMEM (GibcoBRL) supplemented with 10% fetal calf serum (FCS; Gibco) and 1% penicillin–streptomycin (Gibco). After 12 h of *in vitro* culture, tissues were treated for 2 min with 100% ice cold methanol, fixed overnight in 4% PFA at 4°C, and processed for whole mount *in situ* hybridization analysis.

Recombinant protein bead implantation assays were done as described previously (38). Heparin acrylic beads (Sigma) were incubated in Fibroblast Growth Factor 8 (FGF8) protein (1 mg/μl; R&D). Affi-gel blue beads (BioRad) were incubated in Bone Morphogenetic Protein 4 (BMP4) protein (100 ng/μl; R&D). About 100 beads were washed with 1× PBS and soaked with 10 μl of growth factor solution for 1 h at 37°C for BMP4 and overnight at 4°C for FGF8. The beads were placed on top of the whole mandible or on top of isolated mesenchyme using fine forceps. Explants with beads were cultured for 12 h as described previously.

Cell culture and transfection assays

MD10H1 cell line was obtained by large T antigen immortalization of E18 mouse embryo first molar mesenchyme (39). These cells were used for transfection assays in triplicate using Exgen transfection system according to the manufacturer's protocol (Euromedex, Mundolsheim). Briefly, MD10H1 cells were plated out in α-MEM supplemented with 10% of FCS (GibcoBRL) at 5×10^4 cells/cm^2. After 24 h, cells were transfected with 0.5 μg of either *Dlx2* or *Barx1* expression vectors (provided by S. Harris and P. T. Sharpe, respectively) or with 0.5 μg of *Msx1* AS expression vectors. The latter one was generated by PCR amplification from a DNA template with the following primers (5′-TTA CAT CCT GGT GGT CTG AG-3′ and 5′-CCA GCA TGC ACC CTA CGC AA-3′). The amplified fragment corresponds to the entire AS transcript. Control transfection experiments were performed using empty expression plasmids. After 24 h, the medium was removed, the cells rinsed with 1× PBS and used for RNA analysis.

RNA analysis

RNA from the transfected cells, from the heads dissected from E9.5 to E18.5 embryos and from newborn to 2-day-old mice were extracted using TriReagent (Euromedex) as described previously (40) and used for RT–PCR. RT was carried out on 1 μg of total RNA with Superscript II using oligo(dT) primer according to the manufacturer's protocol (Invitrogen). The PCR was performed in 20 μl with 2 μl of the RT reaction and 10 pmol of the following primers for 30 cycles: *Msx1* S (forward primer: 5′-TCC TCA AGC TGC CAG AAG AT-3′; reverse primer: 5′-TCA GGT GGT ACA TGC TGT AG-3′), *Msx1* AS (forward primer: 5′-TTA TGT CCA CCT GCC CTT TC-3′; reverse primer: 5′-GGG CCC AAA GGA TTA TTG TT-3′), *Dlx2* (forward primer: 5′-TCC TAC CAG TAC CAA GCC A-3′; reverse primer: 5′-AAG CAC AAG GTG GAG AAG C-3′), *Barx1* (forward primer: 5′-CCA TGC CCG GCC CCG CAG GCG CAT C-3′; reverse primer: 5′-GAA TTC AGT CCT CGC AAT TTC GG-3′) and *GAPDH* (forward primer: 5′-TTC CAG TAT GAT TCC ACT CA-3′; reverse primer: 5′-CTG TAG CCA TAT TCA TTG TG-3′). In all analysis, RNA without RT served as control.

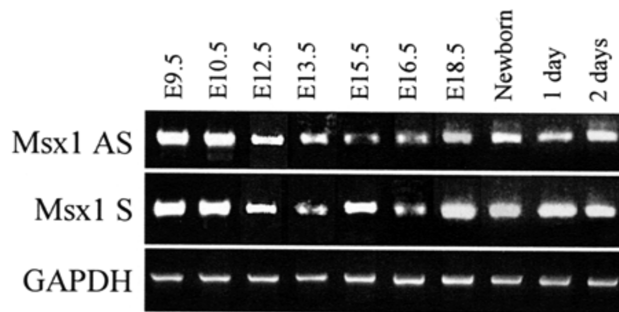

Figure 1. *Msx1* S and AS transcripts detection from E9.5 to 2 days post natal. The two *Msx1* transcripts were detected by semi-quantitative RT–PCR during this entire period.

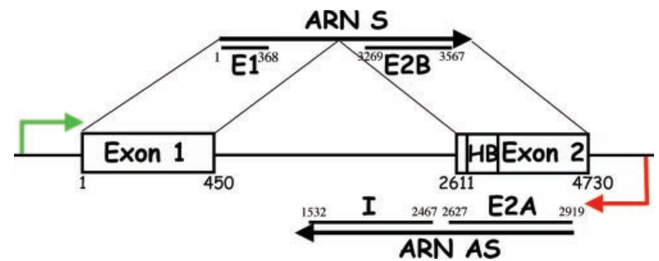

Figure 2. Schematic representation of the two *Msx1* transcripts and position of the four probes used *in situ* for hybridizations. *Msx1* S and AS transcripts organization in regard of the *Msx1* gene structure (accession number S73812) is schematically presented. The *Msx1* S (top) and AS (bottom) transcripts are shown. The S transcript correspond to exons 1 and 2 sequences. The AS transcript sequence is complementary to the exon2 and a major part of the intron. Positions of probes used for hybridization are given. Green and red arrows, respectively, represent S and AS promoter.

RESULTS

First evidence of *Msx1* AS transcript expression during development

The first evidence for endogenous *Msx1* AS transcripts was shown previously in postnatal mice (12). In order to determine if *Msx1* AS was expressed during embryonic development and in early postnatal life, semi-quantitative RT–PCR was performed on RNA from E9.5 to E18.5 embryos and from day 0 to day 2 newborn mice. These experiments showed that both *Msx1* transcripts were detected as early as E9.5 and throughout development until postnatal day 2, suggesting a potential role in embryogenesis (Figure 1).

Msx1 S and AS transcripts and protein expression from E9.5 to E11.5

Expression of both S and AS *Msx1* transcripts was analyzed during early embryonic development by whole mount *in situ* hybridization. The protein expression was analyzed at these same stages using *Msx1*/*LacZ* heterozygous embryos, in which the *LacZ* knock in enabled protein expression followed indirectly (23).

The *Msx1* AS transcript was described previously as a 2.1 kb long transcript which contains sequence overlapping the entire exon 2 and 1012 bp of the intron of Msx1 gene (Figure 2). Both the S and AS *Msx1* transcripts are complementary in exon 2 sequence. A set of four probes (E1, I, E2A and E2B) was used for *in situ* hybridization experiments. The S transcript probe (E1) was nested in the *Msx1* exon 1 since this sequence is specific to the S transcript (Figure 2). The AS transcript probe (I) was nested in *Msx1* intron since this sequence is specific to the AS transcript (Figure 2). With the original *Msx1* vector (18) containing part of exon 2 sequence, two probes were generated. E2A probe was complementary for the AS transcript whereas E2B probe was complementary for the S transcript (Figure 2). For all experiments the four probes were used. In all experiments, with the exception of E13.5–E16.5, detected expression using both S probes produced the same pattern of expression, as also seen with both the AS probes.

At E9.5, the *Msx1* AS transcript was not detected in mandibulary and maxillary regions (Figure 3B and E) but appeared to be expressed in brain. In contrast, the S transcript (Figure 3A and D) and protein (Figure 3C and F) were detected in the

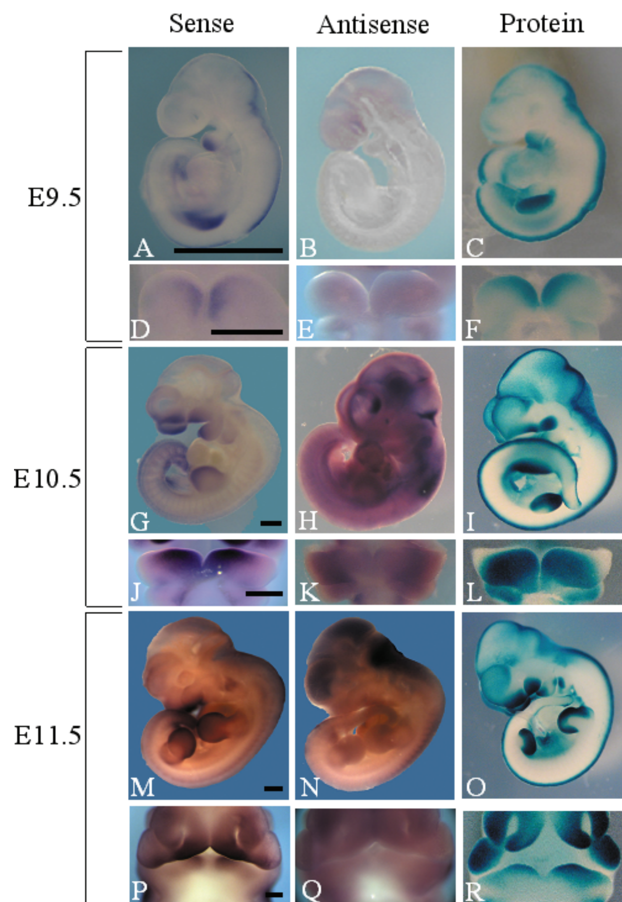

Figure 3. *Msx1* S and AS transcripts and Msx1 protein expression from E9.5 to E11.5 in mouse embryos. Both *Msx1* transcripts are detected by whole mount *in situ* hybridization on wild-type mice embryos. Msx1 protein expression is revealed by the β-galactosidase activity using *Msx1-nLacZ* heterozygous mouse embryos. (**A–F**) At E9.5, the *Msx1* AS transcript is not detected in the first branchial arch in contrast to the S transcript and the protein. (**G–L**) At E10.5, both S and AS *Msx1* transcripts are detected in complementary regions in the mandible. The *Msx1* S transcript and the protein are detected in the distal part of the mandible and the *Msx1* AS transcript in the proximal parts. (**M–R**) At E11.5, both *Msx1* transcripts are detected in progressively overlapping areas in mandible outlining two reverse gradients, proximal-distal for the *Msx1* AS transcript and distal-proximal for the *Msx1* S transcript and the protein. The bar scale represent 800 μm for whole embryos, and 500 μm for the mandible.

distal parts of the mandible primordium. In the facial region, *Msx1* AS transcript expression was first detected at E10.5 (Figure 3H and K). At this stage, both *Msx1* transcripts exhibited an interesting opposite pattern in the mandibular arch. *Msx1* AS transcript was detected in the proximal part of mandible (Figure 3H and K) whereas *Msx1* S transcript (Figure 3G and J) and protein (Figure 3I and L) were still detected in the distal part of mandible. *Msx1* AS transcript was detected in other anatomical sites such as the future brain and limb buds (Figure 3H). At E11.5, in the facial region, the territories of both *Msx1* transcripts and protein appeared to be enlarged and overlapping, corresponding to almost all the oral part of mandible (Figure 3M–R). Two opposite gradients of *Msx1* S and AS transcript expression patterns were observed. *Msx1* S transcript expression level appeared higher in the distal region (Figure 3P) and reversely the *Msx1* AS transcript was expressed more significantly in the proximal regions (Figure 3Q). Protein expression also appeared to be expressed at a higher level in the distal region (Figure 3R). *Msx1* AS transcript was still detected in the forming brain and in the limb buds at this stage, (Figure 3N).

Comparative expression of *Dlx2, Barx1, Msx1* S and AS transcripts at E10.5 in presumptive molar and incisor fields

The expression of *Dlx2*, *Barx1* and both *Msx1* transcripts were comparatively analyzed in the presumptive incisor and molar fields of E10.5 embryo mandible by whole mount *in situ* hybridization. The *Msx1* AS transcript was detected in the proximal regions of the E10.5 mandibular arch (Figure 4A) whereas the *Msx1* S transcript was detected in the distal part of the mandible (Figure 4C) adjacent to the *Dlx2* epithelial expression (blue in Figure 4C and D). The mesenchymal expression of *Dlx2* (Figure 4B) and *Barx1* (Figure 4D) was evidenced in the proximal area, as was the *Msx1* AS expression. However, the *Barx1* mesenchymal expression pattern appeared more restricted than that of the *Dlx2* and *Msx1* AS. In the epithelium, the *Msx1* AS transcript was not detected in the distal region of *Dlx2* epithelial expression (Figure 4B and blue LacZ staining in Figure 4C and D).

FGF8 regulates *Msx1* AS transcript expression in mandible explant culture

In order to determine whether *Msx1* AS transcript expression would be integrated in the epithelial signaling pathways that establish the morphogenetic fields in E10.5 mandible, epithelial induction of *Msx1* AS transcript mesenchymal expression was evaluated in mandibular explants cultures. Interestingly, 12 h after the removal of mandibular arch, a loss of *Msx1* AS transcript expression was observed in the mandibular explant (Figure 4E). To test the potential induction of *Msx1* AS transcript expression by epithelium signals, BMP4 and FGF8 soaked beads were applied to the mandibular mesenchyme explants. After 12 h of culture, *Msx1* AS transcript expression was detected around the FGF8 beads (Figure 4F), as was *Msx1* S transcript (Figure 4G), whereas BMP4 or BSA beads had no effect (Figure 4H and I). In contrast to the AS transcript, the *Msx1* S transcript expression was increased by BMP4 soaked beads (Figure 4J). To explore a potential inhibitory effect of

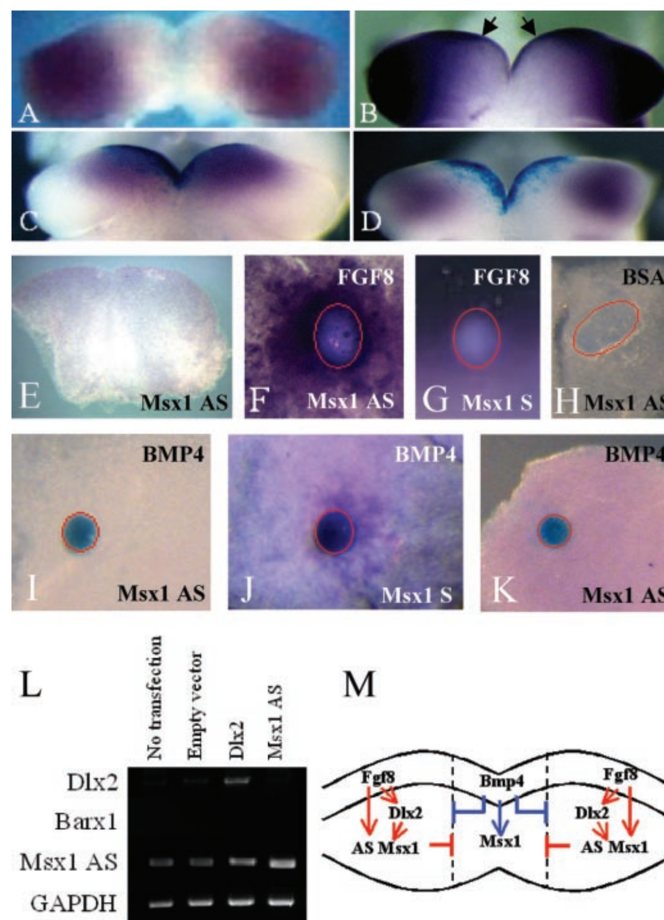

Figure 4. Analysis at E10.5 of *Msx1* AS transcript potential integration in mandibular dental field determination. (**A–D**) Comparison of the *Msx1* AS transcript expression pattern with *Dlx2* and *Barx1* transcripts in E10.5 mandible. The *Msx1* AS transcript (A), the *Dlx2* transcript (B) and the *Barx1* transcript (D) were detected in the same proximal regions of the mesenchyme by whole mount *in situ* hybridization whereas the *Msx1* S transcript (C) was detected in the distal part of mandible. In contrast, in the epithelium where *Dlx2* expression is seen distally there is no associated expression of either *Msx1* AS or *Barx1* transcripts expression [LacZ staining in (C) and (D) and arrows in (B)]. (**E–K**) The *Msx1* AS transcript mesenchymal expression pattern in E10.5 mandible is controlled by epithelial signals. (E) The *Msx1* AS transcript expression was absent when the mandible explant is cultured without oral epithelium. (F) The *Msx1* AS transcript mesenchymal expression was restored around an FGF8 soaked bead as was the *Msx1* S transcript expression (G). *Msx1* AS transcript expression was not restored around BMP4 or BSA soaked beads (H and I). In contrast to the AS transcript, the *Msx1* S transcript expression increased around the BMP4 soaked beads (J). No inhibitory effect on *Msx1* AS transcript expression is observed when BMP4 soaked beads are put in presence of epithelium (K). (**L**) The *Msx1* AS transcription activation by Dlx2. MD10H1 cells were transfected with either an empty vector, a Dlx2 expression vector, or an *Msx1* AS transcript expression vector. RNA extracted from these cells was reverse-transcribed and cDNA analyzed by PCR. Dlx2 overexpression appears to be able to induce a moderate increase of the *Msx1* AS transcript synthesis whereas *Msx1* AS transcript overexpression had no effect on either *Dlx2* or *Barx1* expressions. (**M**) Schematic representation of the regulation of both *Msx1* transcripts by epithelial BMP4 and FGF8 signaling. In proximal regions of the E10.5 mandible, FGF8 activate *Msx1* AS transcript expression directly or/and potentially through Dlx2 activation. The AS transcript is suspected to inhibit Msx1 homeoprotein expression in these proximal regions though a mechanism that remains to be elucidated. In the distal region, BMP4 activates *Msx1* S transcript and protein expression and inhibits Dlx2 expression. No direct effect of BMP4 on *Msx1* AS transcript expression was evidenced.

BMP4 on the *Msx1* AS transcript expression, mandibular explants were cultured for 12 h with BMP4 soaked beads in the presence of epithelium (Figure 4K). No significant change was observed in the *Msx1* AS transcript expression (Figure 4K).

Impact of Dlx2 and Barx1 overexpression on *Msx1* AS transcript expression in dental cells

As *Dlx2* and *Barx1* are coexpressed with *Msx1* AS transcript in the proximal parts of E10.5 mouse embryo mandible, a potential regulation between these genes was analyzed *in vitro* in undifferentiated mesenchymal MD10-H1 cells. In order to examine whether the *Msx1* AS transcript could be under the control of *Dlx2* and *Barx1* in the mesenchyme, the expression level of this transcript was analyzed after *Dlx2* or *Barx1* overexpression. The semi-quantitative RT–PCR results showed a small but reproducible increase in *Msx1* AS transcript steady state levels which was observed 24 h after the transfection with a *Dlx2* vector (Figure 4L). Quantitative analyses of *Msx1* AS transcript expression increase after Dlx2 overexpression were realized using two techniques: real-time PCR (Roche) and classical PCR fragments quantification on BET gel using Optimate 5.21 (Visiomic). Both techniques showed a moderate stimulation to 1.2 times that of *Msx1* AS transcript steady state expression. The impact of Dlx2 on *Msx1* AS transcript expression in these experiments is probably underestimated according to the low transfection efficiency obtained for MD10-H1 cells (only 35%). *Barx1* transcript expression level was not affected by Dlx2 overexpression (Figure 4L), nor *Dlx2* expression affected by Barx1 overexpression (data not shown). The controlled overexpression of *Msx1* AS transcript showed no effect on either *Dlx2* or *Barx1* expression levels (Figure 4L).

Msx1 S, AS transcripts and protein expression during tooth morphogenesis

All *in situ* hybridization experiments were carried out with the panel of four different probes (Figure 2). From E9.5 to E12.5, similar results were obtained with the two probes for *Msx1* AS transcript. The two probes for *Msx1* S transcript also gave identical results. At E11.5, both *Msx1* transcripts and the protein were detected in similar regions of the dental mesenchyme surrounding the dental lamina (Figure 5A–C). At E12.5, both *Msx1* transcripts and the protein were still detected in the same anatomical sites, more precisely in the mesenchyme surrounding the growing tooth buds (Figure 5D–F). At E13.5, the probes revealed different expression patterns. The *Msx1* S transcript was detected in the dental mesenchyme (Figure 5G and H) with both probes (E1 and E2B) and unexpectedly in the dental epithelium with the E1 probe (Figure 5G). The *Msx1* AS transcript was detected, with both AS probes, in the dental epithelium (Figure 5I and J), and also, at a low level, in the dental mesenchyme. At this stage, the protein was expressed only in the dental mesenchyme (Figure 5K). At E16.5, the *Msx1* S transcript was detected by the two probes in the dental mesenchyme (Figure 5L and M). At this stage, as observed previously at E13.5, an epithelial expression of *Msx1* S transcript was revealed only by the E1 probe (Figure 5L). The *Msx1* AS transcript was detected at E16.5 only in the dental epithelium with whatever probes used (Figure 5N and O). No protein expression was detectable at this stage (Figure 5P). In newborns, before the first sign of odontoblast differentiation, the S and AS transcripts were detected with E1 (Figure 5Q) and I (Figure 5T) probes, respectively, whereas the exon two probes (Figure 5R and S) gave no signal.

Msx1 S, AS transcripts and protein expression during early limb bud development

Msx1 transcripts and protein expression were comparatively analyzed during anterior limb bud development, from E9.5 to E14.5, by whole mount *in situ* hybridization and β-galactosidase staining (Figure 6). At E9.5, *Msx1* S transcript and protein were detected in the entire limb bud whereas *Msx1* AS transcript was absent (Figure 6A–C). At E10.5, similarly to the observed pattern in the mandible, both *Msx1* transcripts were detected with complementary expression patterns. *Msx1* S transcript and protein were detected in the area bordering the apical ectodermal ridge whereas the *Msx1* AS transcript was detected in a more proximal region, but immediately adjacent to the *Msx1* S transcript positive region (Figure 3G–I and Figure 6D–F). At E11.5, the *Msx1* AS transcript was still located in an area adjacent to the *Msx1* S transcript region (Figure 3M and N and Figure 6G and H). At E14.5, the *Msx1* AS transcript was detected in the proximal part of the interdigital regions (Figure 6J), whereas the protein was expressed with a proximal-distal gradient in the same interdigital areas and also in tip of digits (Figure 6K).

DISCUSSION

The *Msh*-like homeobox genes, orthologous to the Muscle Segment Homeobox gene of *Drosophila melanogaster*, constitute an ancient family described in numerous species ranging from coelenterates to mammals (20). Inside this family, a variable number of members have been reported for different species, corresponding to evolutionary duplications (20). These genes exhibit a simple genomic structure with two exons separated by a single intron (41). The *Msx1* gene appears to be the most conserved gene within the *Msx* family of mammals and avians (20). Interestingly, a bidirectional overlapping and convergent transcription was identified for this *Msx1* gene in mouse and human and might also be conserved in at least three other species: rat, bovine and chicken. Such a possibility was suggested by the existence of a conserved identical 60 bp sequence containing a TATA box and corresponding to the AS promoter region (12). According to the importance of Msx1 homeoprotein during development and the preliminary *in vitro* observation that the *Msx1* AS transcript has the ability to interfere with Msx1 homeoprotein expression (12), the investigation of the physiological significance of the bidirectional transcription of the *Msx1* locus during development appeared to be important. The experimental strategy was to investigate the presence of the *Msx1* AS transcript during mouse embryogenesis by RT–PCR, to analyze its expression pattern in comparison with the *Msx1* S transcript and the protein in tooth and other significant developing systems where *Msx1* signaling pathways are instrumental (42–44), and finally to analyze its potential regulation by epithelial signals.

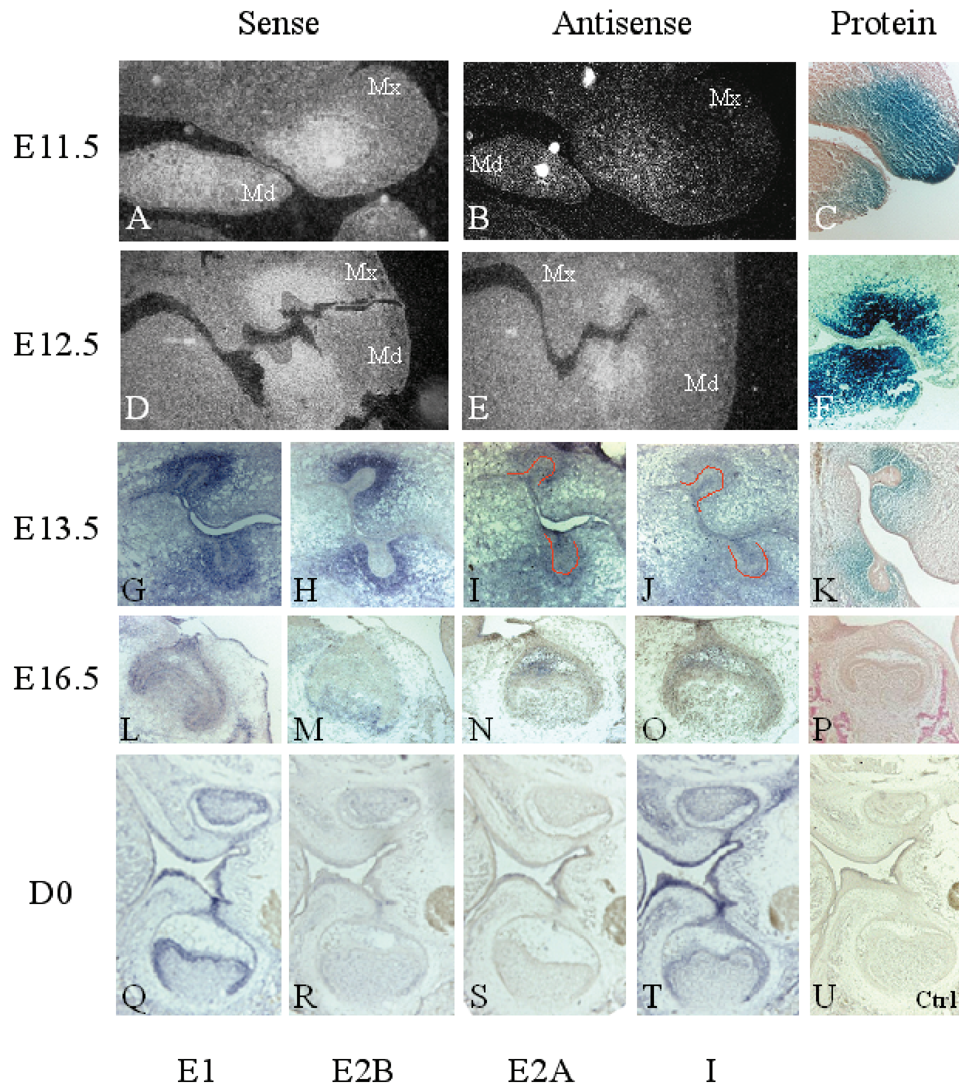

Figure 5. *Msx1* S and AS transcript detection and Msx1 protein expression during mouse tooth development from E11.5 to birth. Both transcripts of *Msx1* were detected by *in situ* hybridization in wild-type mouse embryonic serial sections in comparison with LacZ expression in *Msx1*/*LacZ* hereterozygotes. (**A–C**) At E11.5, both *Msx1* transcripts were detected whatever the probes used in the dental mesenchyme as was *LacZ*. (**D–F**) At E12.5, both *Msx1* transcripts were detected whatever the probes used along with LacZ in the dental mesenchyme but in a more restricted area than at E11.5, surrounding the tooth buds. (**G–K**) At E13.5, the expression patterns of the both *Msx1* transcripts were more complex. Using E1 probe, the *Msx1* S transcript was detected in both dental mesenchyme and epithelium (G). Using E2B probe, the *Msx1* S transcript is only detected in dental mesenchyme surrounding the tooth bud (H). Using E2A and I probes, the *Msx1* AS transcript is detected in both dental epithelium and mesenchyme (I and J). LacZ expression was only detected in the dental mesenchyme surrounding the tooth bud and the epithelium was clearly devoid (K). (**L–P**) At E16.5, the *Msx1* S transcript was detected in dental epithelium and mesenchyme with the E1 probe whereas it was only detected in mesenchyme with E2B probe. *Msx1* AS transcript detection was restricted to the dental epithelium independently of the probe used. No Msx1 fusion protein expression was detected at this stage. (**Q–T**) At birth, the *Msx1* S transcript was detected in dental epithelium and mesenchyme only with the E1 probe. The *Msx1* AS transcript was detected in both dental tissues only with the I probes. (**U**) Experimental control (Ctrl) corresponding to the use of unlabeled probe. (Md: mandible; Mx: maxilla).

Evidence for *Msx1* AS transcript expression during embryonic development

The *Msx1* AS transcript was originally identified in newborn and young adult mice (12), in association with skeletal growth. *Msx1* AS transcript was proposed to be instrumental postnatally in terminal differentiation, by inhibiting Msx1 protein expression (12,45). Indeed, Msx1 homeoprotein expressing cells were considered as undifferentiated cells, based on *in vitro* observations that Msx1 (i) inhibits master gene expression such as MyoD (46) and Runx2 (12), (ii) modulates the expression of cell cycle determinants such as cyclin D1 (47) and p19(INK4d) (48), (iii) induces dedifferentiation of myotubes (14), and (iv) is specifically present in progenitor cell niches in adults (49). Interestingly, the initial analysis of *Msx1* AS transcript expression during antenatal mouse development (Figure 1) revealed that, similar to the S transcript, the *Msx1* AS transcript was already expressed at E9.5 before the initial steps of skeleton morphogenesis and cell differentiation, and continuously up to birth. These data suggest that the *Msx1* bidirectional convergent and overlapping transcription may not only be linked to the control of cell terminal differentiation but also implicated in more complex processes during development, such as dental and skeletal morphogenesis.

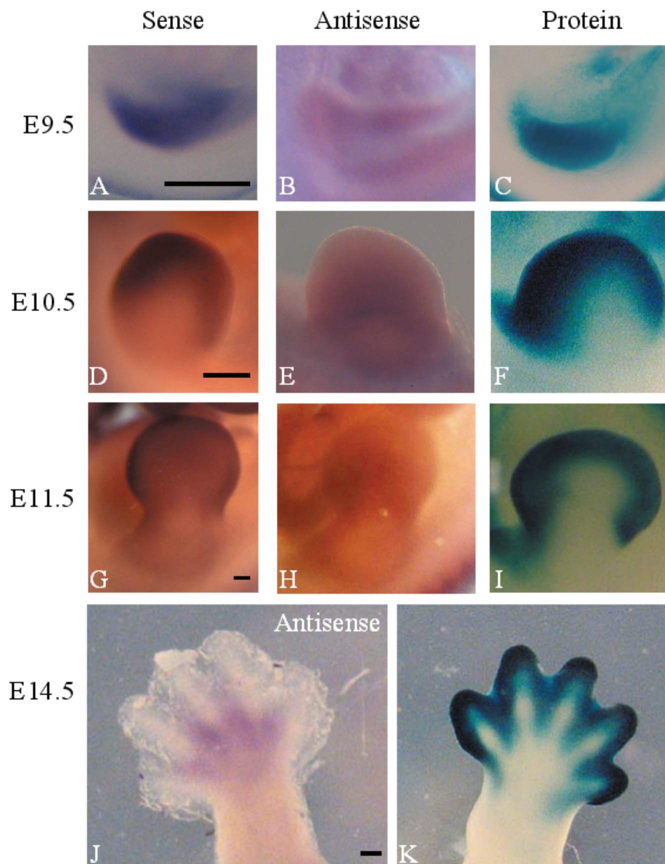

Figure 6. *Msx1* S and AS transcripts detection and Msx1 protein expression from E9.5 to E14.5 in mouse anterior limb bud. Both transcripts of *Msx1* were detected by whole mount *in situ* hybridization in wild-type mouse embryos. Msx1 protein expression was detected by the β-galactosidase activity in *Msx1-nLacZ* heterozygote mouse embryos. The *Msx1* AS transcript was detected in limb bud at E10.5 and E11.5 but not at E9.5 (**B**, **E** and **H**). Its detections territories edged the *Msx1* S transcript and protein detection territories (**A**, **C**, **D**, **F**, **G** and **I**). At E14.5, *Msx1* AS transcript and protein show complementary expression pattern (**J** and **K**). The bar scale represent 200 μm.

In the past, *Msx1* gene expression has been documented in numerous developing organs and systems, notably, heart (18,50), brain (51), limb (50), mammary glands (52), hair follicles (43,50) and teeth (22,28,33). Due to the detailed analysis of the role of *Msx1* during odontogenesis, experimental studies of *Msx1* AS transcript were carried out in this developmental model system. In addition, Msx1 protein expression was compared with the AS transcript throughout tooth morphogenesis stages and showed a continuous expression until late bell stage (E16.5). The results suggest that, during tooth development, *Msx1* AS transcript may not only be involved in cell terminal differentiation control as proposed previously (12), but also in tooth initiation and subsequent morphogenesis through the control of Msx1 homeoprotein expression and function.

The most important result obtained here was that both S and AS *Msx1* transcripts were present during embryonic development. Indeed, it appeared that during development, when an organ expressed *Msx1* S transcript and protein, it also expressed the AS transcript. This observation was not only verified in tooth but also in bone (12), hair follicles (data not shown), neural tube (data not shown) and limb bud (Figure 6).

Msx1 AS transcript and tooth initiation

Msx1 (S transcript) has been proposed to be an important factor in the odontogenic homeobox code proposed to define the different tooth fields at E10.5 in mouse prior to the morphological appearance of tooth formation (25–27). This homeobox code is based on combinations of homeobox gene expression patterning territories in the first branchial arch neural crest derived mesenchyme (37,53–56). In this homeobox code, *Msx1* may define the incisor region (28) whereas *Barx1* in combination with *Dlx2* specifies molar region (27,57,58). This assertion is exemplified in *Msx1* knock out mice which exhibit no initiation of incisor development in contrast to molars where development is blocked later at the bud stages (32). To understand further the means of spatially restricting the expression of the Msx1 homeoprotein, the role of *Msx*1 AS transcript in the control of protein expression was considered. Our data showed that at E9.5 *Msx1* AS transcript was not expressed in the mandibular arch in contrast to the S transcript, whereas at E10.5 *Msx1* AS transcript was detected and presented an expression pattern complementary to the S transcript and protein. Such a result suggests that *Msx1* AS transcript could participate to Msx1 homeoprotein expression restriction within the distal region of the mandible. At this stage, some homeobox containing gene expression patterns in the mesenchyme are driven by epithelial signaling (28,31,33,34,37,54,59,60). Two major signaling molecules implicated are growth factors BMP4 and FGF8. They are secreted by the distal and proximal parts of the mandibular oral epithelium, respectively. Effects of these diffusible growth factors on *Msx1*, *Barx1* and *Dlx2* expression have been established by application of protein soaked beads in mandibular explant cultures. These experiments show that mesenchymal expression of *Barx1* and *Dlx2* is induced by FGF8 while inhibited by BMP4 (34,53,61). In contrast, *Msx1* S transcript appears to be induced around both FGF8 and BMP4 soaked beads (28,33,37,53) raising the question of *Msx1* S transcript expression control (inhibition) in the mandible proximal parts *in vivo*. Interestingly, at E10.5, *Msx1* AS transcript showed an expression pattern in the mesenchyme of the mandible similar to those of *Barx1* and *Dlx2* (Figure 4A, B and D) and in opposition to *Msx1* S transcript and homeoprotein expression pattern (Figure 4C). The presently reported expression of *Msx1* AS transcript lead to propose a hypothesis: the AS RNA would inhibit S RNA and protein expression in the proximal parts of the mandible by a mechanism that still remains to be elucidated but that could occur at transcriptional level according to the absence of S transcript detection. The present soak-beads experiments provided evidence that the mesenchymal expression of *Msx1* AS transcript at E10.5 is indeed responsive to FGF8 and not BMP4 (Figure 4F, I and K). Interestingly, the *Msx1* AS transcript activation by FGF8 at E10.5 appears to be correlated to an absence of *Msx1* S transcript response to this growth factor, while beads experiments suggest that *Msx1* S transcript should be activated (Figure 4G). *In vitro* overexpression experiments in mesenchymal MD10-H1 dental cells (39) show that *Msx1* AS transcript expression may be regulated by Dlx2 but not by Barx1 (Figure 4L), suggesting that Dlx-2 could act as an intermediary in the pathway between FGF8 and *Msx1* AS. A schematic representation of FGF8 and BMP4 differential regulation of both *Msx1*

transcripts mesenchymal expression in the E10.5 mandible is presented in Figure 4M.

Finally, *Msx1* AS transcript appeared to be inserted inside the homeobox code at E10.5 through its joint expression in the proximal region with *Barx1* and *Dlx2*. At this developmental stage, the obtained data suggest that *Msx1* AS transcript would spatially limit *Msx1* S transcript and protein expression areas. The exclusive *Msx1* AS transcript expression might result in the complete inhibition of *Msx1* S transcript expression by a mechanism that remains to be elucidated.

Msx1 AS transcript and tooth morphogenesis

During tooth morphogenesis, *Msx1* (S transcript) was shown to be expressed in the mesenchyme with a progressive restriction, from E11.5 to E12.5, to the dental mesenchyme surrounding both incisor and molar buds (22,62,63) (Figure 5). The importance of epithelial signaling, in particular BMP4 expression, in such a restriction has been shown and corresponds to a crucial step in the tooth morphogenesis process (31,33). Moreover, the importance of Msx1 homeoprotein expression in dental mesenchyme is evidenced by the observation of molar development arrest at the bud stage (E13.5) in *Msx1* null mutant mice (32). Recombination experiments between wild-type and *Msx1* null mutant dental tissues showed that *Msx1* mesenchymal expression is transiently necessary at the early bud stage (33) mainly to induce BMP4 expression in the dental mesenchyme as validated by BMP4 complementation experiment using transgenic animals (64,65). Indeed, the important function of Msx1 homeoprotein in tooth morphogenesis is its implication in epithelial–mesenchymal interactions and the question of the AS transcript part in this function control is raised from this point of view. Our results showed that *Msx1* AS transcript was continuously expressed during tooth morphogenesis with a stage-specific pattern. At the dental lamina stage (E11.5), the AS transcript was detected jointly with the S transcript in the mesenchyme and by E12.5, its expression was progressively restricted to the mesenchyme area surrounding the epithelial invagination, as reported previously for the S transcript. At the following stages (cap and bell stages), the *Msx1* S and AS transcripts expression patterns diverge showing a complementary in the mesenchyme. The S transcript was expressed in the dental cells which will differentiate into odontoblasts and pulp cells whereas the AS transcript was expressed in cells which will give rise to the follicular sac and alveolar bone. However, the function associated to such a distribution of the two transcripts remains unclear. Concerning *Msx1* S and AS transcript expression in dental epithelium (detected only with certain probes), the situation is even more complex and discussed below. Indeed, according to our data whatever be the stage of tooth morphogenesis Msx1 homeoprotein expression was restricted to the mesenchyme. Finally, our result establishes that during tooth morphogenesis, *Msx1* AS transcript showed a dynamic expression pattern relative to the S transcript and the protein suggesting its implication in the protein localization and expression level.

In conclusion, regarding *Msx1* transcripts and relative protein expression during tooth morphogenesis, five distinct situations were identified depending on the developmental stages, areas and tissues: no expression (at E11.5–E12.5 in the epithelium), exclusive expression of S transcript (at E14.5 and E16.5 in dental mesenchyme), exclusive expression of AS transcript (at E16.5 in epithelial regions such as stellate reticulum and stratum intermedium), coexpression of S and AS (i) with all probes (E11.5–E12.5) or (ii) with certain probes (at E13.5, E16.5 and D0). The differences observed with the set of probes used could be explained by the complementarities of S and AS exon 2 sequences. Indeed, the exclusive results obtained with S and AS specific probes suggest that at E13.5, E16.5 and D0 the overlapping sequence from both transcripts are not accessible to exon two probes. So, we hypothesized that S and AS transcripts could physically interfere and impair riboprobe hybridization in this area. Functionally, the five situations should be considered through the narrow window of AS RNA knowledge. Several transcriptional and post-transcriptional events are candidates for the inhibition of Msx1 homeoprotein. These events have been recently reviewed and classified in four categories (5). Transcriptional interference and S gene silencing ends with exclusive expression of AS RNA. Posttranscriptional mechanisms (RNA masking and degradation) are those which involve S–AS coexpression and finely tuned ratios of S/AS transcripts or modifications in RNAs overlapping sequences. According to coexpression or distinct expression patterns of both *Msx1* transcripts, the bidirectional convergent and overlapping transcription system would function differently. In the case of distinct expression pattern of S and AS transcripts, transcriptional interference or gene silencing could be implied, whereas, in the case of coexpression, RNA masking and degradation could be involved (5,66,67). Hence two types of ratio threshold could be defined. A macrothresholding when only one kind of *Msx1* transcript is expressed (E9.5–E10.5) and a microthresholding (E13.5–E16.5-D0) when the two transcripts are coexpressed. Further complementary studies at the cellular level would be necessary to clearly establish the presumably distinct mechanisms.

Natural antisense transcripts: a growing list

The *Msx1* AS transcript is not an isolated case of large natural AS RNA. Bidirectional convergent and overlapping transcription is an emerging field opening a previously unsuspected level of complexity in protein expression regulation (5). AS transcripts have been associated with different processes during development including parental imprinting (68), developmental clock control (7) and transcriptional regulation (5). The precise molecular modalities of such a control are not clarified. However, *in vitro* experiments have established the importance of the relative ratio of the two transcripts. This has been shown previously for *Msx1* by *in vivo* observations of the two transcripts distribution during osteoblast differentiation process (12).

Interestingly, *Msx1* is not the only gene implicated in maxillofacial and limb development showing a bidirectional convergent and overlapping transcription. Thus, homeobox containing genes belonging to the *Dlx* gene family have also AS transcripts. This is the case for *Dlx1* (69) and *Dlx6* (67) genes. AS transcripts were also described for other divergent homeobox genes as *Otx2* (70) and members of the Hox clusters such as *HoxA11* (11) and *HoxD3* (71), raising the question of the general importance of bidirectional convergent

and overlapping transcription during development. *In vitro* studies have established that (i) Msx and Dlx homeoproteins can form heterodimers through their homeodomains and consequently inhibit each other's transcriptional activity (21) (F. Lezot, unpublished data) and (ii) that Dlx5 (12) and Dlx2 (this study) homeoproteins can differentially modulated *Msx1* AS transcript expression, and thus Msx1 homeoprotein expression. These *in vitro* observations strongly suggest that understanding AS transcript integration in developmental signaling pathways will be a difficult, but necessary challenge for the next decade. Interestingly, 9 out of 248 genes shown to be involved in tooth development (indexed on http://bite-it.helsinki.fi) have so far been shown to have AS transcripts. Moreover, computer analysis of available human and mouse genome sequences have shown that 15 and 20% of the mouse and human genes can have AS transcript, respectively (4,9). Finally it appears that the importance of AS transcripts is largely underestimated concerning development and more precisely during tooth development. 'Making sense with antisense' may be operative for the understanding of morphogenetic gradients in developmental biology.

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