Pax9 and Msx1 encode transcription factors that are known to be essential for the switch in odontogenic potential from the epithelium to the mesenchyme. Multiple lines of evidence suggest that these molecules play an important role in the maintenance of mesenchymal Bmp4 expression, which ultimately drives morphogenesis of the dental organ. Here we demonstrate that Pax9 is able to directly regulate Msx1 expression and interact with Msx1 at the protein level to enhance its ability to transactivate Msx1 and Bmp4 expression during tooth development. In addition, we tested a missense mutation (T62C) in the paired domain of PAX9 that is responsible for human tooth agenesis (1) affects its functions. Our data indicate that although the mutant Pax9 protein (L21P) can bind to the Msx1 protein, it fails to transactivate the Msx1 and Bmp4 promoter, presumably because of its inability to bind cognate paired domain recognition sequences. In addition, synergistic transcriptional activation of the Bmp4 promoter was lost with coexpression of mutant Pax9 and wild-type Msx1. This suggests that Pax9 is critical for the regulation of Bmp4 expression through its paired domain rather than Msx1. Our findings demonstrate the partnership of Pax9 and Msx1 in a signaling pathway that involves Bmp4. Furthermore, the regulation of Bmp4 expression by the interaction of Pax9 with Msx1 at the level of transcription and through formation of a protein complex determines the fate of the transition from bud to cap stage during tooth development.

The formation of mammalian dentition is a remarkable developmental process that provides a valuable model for studying genes that control three-dimensional patterning and morphogenesis. The genetic control of tooth development is underscored by the findings that mutations in genes that encode transcription factors involved in tooth signaling may influence the background responsible for the phenotype. To date, several reports have described the association of dominant mutations in MSX1 and PAX9, two transcription factors that are expressed in dental mesenchyme (1, 3–11). More recently, AXIN2, a Wnt-signaling receptor was identified as responsible for a nonsyndromic form of tooth agenesis (12, 13). When compared with a fairly mixed pattern of tooth agenesis seen in individuals with a nonsense mutation in AXIN2, the phenotypes reported in MSX1 and PAX9 affected families are more restricted to posterior dentition. Although mutations in MSX1 contribute to a pattern that mainly involves premolars and more rarely molars, PAX9 mutations show a strong association with molar agenesis and sometimes premolars. In family members with PAX9 mutations the congenital absence of premolars in addition to molars may reflect a secondary down-regulation in MSX1 expression. This implies that PAX9 shares an upstream genetic epistasis with MSX1.

Studies of tooth development in mice also indicate a molecular relationship between Pax9 and Msx1. Both genes are coexpressed in dental mesenchyme and appear critical for tooth morphogenesis, because in Msx1 and Pax9 homozygous null mutants, tooth organs arrest at the bud stage (14, 15). However, there is little known about the interactions of Pax9 with Msx1 at the level of gene regulation and function. It is possible that Pax9 could interact directly with Msx1 or activate a regulator of Msx1. Alternatively, the molecular relationship shared by Pax9 and Msx1 could involve functional interactions on the post-transcriptional or protein level.

Clues about a potential downstream effector gene of the Pax9-Msx1 pathway come from the observation that in Msx1 and Pax9 single homozygous mutant mice, Bmp4 expression is markedly reduced in dental mesenchyme (15, 16). This suggests that both genes may be required for the modulation of Bmp4 in dental mesenchyme. As an effector molecule, Bmp4 is known to be involved in downstream signaling events that result in the induction of the enamel knot, a transient signaling center within dental epithelium (17). Subsequent changes in the enamel organ result in the progress of cuspal morphogenesis. Initial molecular studies of the BMP4 promoter identified a region between −1100 and −45 as important for basal transcription (18). Although a variant of the Bmp4 promoter has been shown to be a target for regulation by a number of transcription factors (16, 19, 20), there are no data available on the modulation of the Bmp4 promoter by either Pax9 or Msx1, or both.

Here we report that Pax9 interacts with Msx1 at both the gene and protein levels and that the interaction enhances the ability of Pax9 to
Interactions of Pax9 and Msx1 Genes in Tooth Development

transactivate Msx1 and Bmp4 expression during tooth development. In addition, we tested how a missense mutation (T62C) in the paired domain of PAX9 that is responsible for human tooth agenesis (1) affects its functions. Our data indicate that although the mutant Pax9 protein (L21P) can bind to both wild-type Msx1 and Pax9 proteins, it fails to transactivate either the Msx1 or the Bmp4 promoter. Furthermore, synergistic transcriptional activation of the Bmp4 promoter was lost with coexpression of mutant Pax9 and wild-type Msx1. These data suggest that Pax9 is critical for the regulation of Msx1. These data suggest that Pax9 is critical for the regulation of Msx1 expression through its paired domain rather than Msx1.

Together, these findings demonstrate a signaling pathway involving Pax9, Msx1, and Bmp4 that is critical for the progress of tooth morphogenesis from the bud to cap stage.

MATERIALS AND METHODS

Pax9-deficient Mice and in Situ Hybridization—The generation of Pax9 knock-out alleles in mice and the preparation of paraffin sections of mutant tooth organs have been described previously (15). Conditions for in situ hybridizations using a 32P-labeled riboprobe specific for murine Msx1 were followed as described earlier (21).

Plasmid Constructs and Site-directed Mutagenesis—The mammalian expression vector pcMV-Pax9 with c-Myc epitope tag was used as previously described (22). Expression plasmids containing the cytomegalovirus promoter linked to the full coding sequence of Msx1 were constructed in pcMV-Tag2b (Stratagene, CA). A murine Msx1 cDNA clone comprising the full-length coding sequence was kindly provided by Dr. John Rubenstein (University of California at San Francisco). The FLAG epitope is in frame with the amino terminus of Msx1. Three fragments of the Msx1 promoter (−3.5 kb/+106 bp, −1282/106 bp, and −165/+106 bp) containing the luciferase gene and the 5′ region of the Bmp4 promoter (−2372/+258 bp) within the luciferase vector were described previously (23–25).

To construct pcMV-L21PPax9, in vitro site-directed mutagenesis was performed using the QuikChange mutagenesis kit (Stratagene). The specific primer sets used for the generation of mutant Pax9 cDNA were as follows: L21P, forward, 5′-CGGAAGGCCCAGCCGC-CCCAACGCGCAT-3′; and reverse, 5′-ATGGCGTGGGGCGGGCGGCTCCTGA-3′. The mutated construct was sequenced entirely to confirm the point mutation. The Myc epitope was in frame with the amino terminus of mutant Pax9.

Electrophoretic Mobility Shift Assay—Oligonucleotides corresponding to e5 and CD19-2(A-ins) were synthesized (Sigma/Genosys), and gel retardation assays were performed as previously described (22). The oligonucleotide probes used are two previously described (22). The oligonucleotide probes used are two previously described (22). The oligonucleotide probes used are two previously described (22).

Immunolocalization of the Mutant Proteins in Mammalian Cells—To demonstrate the in vivo expression of wild-type and mutant Pax9, the cells were harvested 48 h post-transfection. Transfections of COS7 cells were carried out using FuGENE 6 (Roche Applied Science). Concentrations were measured by the BCA protein assay kit (Pierce), and equal amounts of proteins were analyzed by Western blotting using 1:200 dilution mouse α-FLAG M2 monoclonal antibody (Santa Cruz Biotechnology) and the ECL kit (Amer sham Biosciences). Transfected cells were fixed and permeabilized with methanol prior to incubation with the c-Myc antibody (1:100), then washed, and incubated with a fluorescein isothiocyanate-conjugated goat anti-mouse antibody (Santa Cruz Biotechnology). Immunostaining was visualized with an Olympus BH-2 microscope.

RESULTS

Pax9 Is Needed for the Expression of Msx1 in Mesenchyme during Tooth Morphogenesis—To determine whether Pax9 is involved in the regulation of Msx1, the expression of Msx1 was analyzed in tooth primordia of Pax9-deficient mice. At E12.5, no change in expression of Msx1 was observed in the Pax9 mutant embryo (Fig. 1, A and B). At E13.5, Msx1 expression appeared drastically reduced in the mesenchyme of Pax9 mutant molar organs (Fig. 1, C and D). Although Msx1 transcripts were present at E14.5 in wild-type tooth organs, no expression was detectable in mutant tooth organs (Fig. 1, E and F).

Pax9 Can Transactivate Msx1 Promoter—Our in situ hybridization analysis of Msx1 expression in Pax9-deficient dental mesenchyme showed that both genes act within the same signaling pathway and that Pax9 shares an upstream epistasis with Msx1. To test whether Pax9 directly regulates Msx1 expression, we performed reporter assays using different Msx1 promoter constructs. Three Msx1 promoter constructs (23, 24) with the luciferase gene were each cotransfected with Pax9 into COS7 cells. These cells do not express either Pax9 or Msx1. The p3.5Msx1-Luc promoter contains 3500 bp of sequence upstream of the translation initiation site and comprises upstream and downstream promoter elements. It contains five putative paired-domain-binding sites, which are located in the highly conserved region within 3.5 kb of the human and murine Msx1 promoter (Fig. 2A). When Pax9 expression plasmids were cotransfected, a dose-dependent increase in reporter gene activity was observed with increasing lengths of the Msx1 promoter (Fig. 2B).

3 The abbreviation used is: En, embryonic day n.
Combinatorial Effects of Pax9 and Msx1 on the Msx1 Promoter—To begin to address the mechanism underlying the potential actions and interactions of Pax9 and Msx1, we first asked whether the proteins can physically associate in vivo. Briefly, we tested for a physical interaction between Pax9 and Msx1 by immunoprecipitating epitope-tagged proteins expressed in cultured COS7 cells. Our coimmunoprecipitation analysis demonstrates that both proteins interact stably within cells (Fig. 3A). We next tested whether the transcriptional activities of Pax9 and Msx1 are competitive or complementary using transient transfection assays on the Msx1 promoter element in COS7 cells. Transfection of the Pax9 expression plasmid alone results in significant activation of the p3.5Msx1-Luc reporter plasmid, whereas transfection of Msx1 expression plasmid alone showed that Msx1 can slightly repress this transactivation when compared with the basal level. We found that introduction of 250 ng of Msx1 expression vector in this assay enhanced Pax9-mediated activation of the reporter construct almost 4-fold. However, doubling the concentration of Msx1 expression plasmid resulted in a 2-fold activation of the Msx1-Luc reporter construct (Fig. 3B).

Combinatorial Effects of Pax9 and Msx1 on the Bmp4 Promoter—To test the direct effect of Pax9 and Msx1 on Bmp4 regulation, we performed reporter assays using a Bmp4 promoter construct (p2.4Bmp4-Luc) containing 2372 bp of sequence upstream of the translation initiation site. We further tested whether the expression of Bmp4 is regulated via Pax9-Msx1 protein interactions. Transfection of the Pax9 expression plasmid alone results in significant activation of the p2.4Bmp4-Luc reporter plasmid, whereas transfection of Msx1 expression plasmid alone failed to regulate this promoter. Cotransfections with Pax9 and Msx1 expression plasmids showed that doubling the concentration of Msx1 while keeping the concentration of Pax9 the same resulted in a decrease in activation from almost 14-fold to nearly 5-fold (Fig. 3C). Western blotting demonstrated that although doubling the amount of Msx1 reduced Pax9-mediated transcriptional activity, no changes were observed in Pax9 expression with increasing levels of Msx1 (Fig. 3D). This suggests that increasing the concentration of Msx1 does not affect the level of Pax9 protein.

L21PPax9 Mutant Can Bind to Msx1 but Is Unable to Up-regulate either Msx1 or Bmp4 Expression Because of Defects in DNA Binding—Using site-directed mutagenesis, we generated a plasmid encoding the nucleotide change and obtained mutant proteins from transfected COS7 cells. Immuno-
Localization studies performed with c-Myc antibody showed that L21PPax9 protein is stable in mammalian cells and localized in the nucleus (Fig. 4, A and B). These data validated the need to assess whether the natural mutant Pax9 protein could interact with wild-type Msx1. Coimmunoprecipitation analysis performed on cells coexpressing combinations of wild-type and mutant Pax9 and Msx1 proteins demonstrate that the L21PPax9 protein interacts with the wild-type Msx1 to the same extent as wild-type Pax9 protein (Fig. 4 C).

To assess the functional consequences of the paired domain substitution mutation in Pax9, we analyzed whether the point mutation would have effects on DNA binding mediated by the paired domain. The mutant protein was unable to form a complex with either of the two cognate paired domain recognition sequences tested (Fig. 5). Next, transcriptional activation was assessed by transient transfection assays using a 3.5-kb Msx1 promoter or 2.4-kb Bmp4 promoter upstream of a luciferase reporter. Transcriptional activity of wild-type Pax9 was compared with the L21PPax9. Overexpression of wild-type Pax9 led to activation of both Msx1 and Bmp4 promoters. However, L21PPax9 demonstrated no observable change on these activations when compared with basal levels of transcription (Fig. 6, A and B). Coexpression of wild-type Pax9 and Msx1 resulted in complementary activation at the Bmp4 promoter, whereas synergistic transcriptional activation was lost with cotransfection of L21PPax9 and Msx1 (Fig. 6 C).

**DISCUSSION**

Despite the wealth of information available on the molecular signals required for tooth morphogenesis, there is little known about how the independent and coordinated activities of key dental mesenchymal transcription factors like Msx1 and Pax9 affect the progress of the tooth organ from the bud to cap stage of development. The down-regulation of Msx1 expression in Pax9-deficient mice and decreased levels of Bmp4 expression in Pax9 and Msx1 homozygous null mice indicate that the three genes act within the same signaling pathway. In these studies, we provide the first experimental evidence in support of a direct molecular
relationship between Pax9 and Msx1 on the transcriptional level and their interactions as proteins. We report that Pax9 forms a heterodimeric complex with Msx1 and that the interaction enhances the ability of Pax9 to transactivate Msx1 and Bmp4 expression during tooth development. Our functional studies of the protein product of a naturally occurring paired domain mutation (Pax9 L21P) demonstrate that although it physically associates with Msx1, the mutant protein is unable to transcriptionally activate both the Msx1 and Bmp4 promoters. Furthermore, we found that coexpression of the Pax9 paired domain mutant and wild-type Msx1 results in a loss of synergistic transcriptional activation of the Bmp4 promoter. These data suggest that the regulation of Bmp4 expression is mediated by the paired domain of Pax9 rather than through its interactions with Msx1. Together, these findings demonstrate that the regulation of Bmp4 expression by the interaction of Pax9 with Msx1 determines the fate of the transition from bud stage to cap stage during tooth development (Fig. 7).

Although mutations in MSX1 contribute to a phenotype that primarily involves premolars and rarely molars, PAX9 mutations show a strong association with molar agenesis and sometimes premolars. In family members with PAX9 mutations, the congenital absence of premolars in addition to molars may reflect a secondary down-regulation in MSX1 expression. This theory is supported by two lines of evidence. First, our in situ hybridization analysis shows that although Msx1 expression is not altered at E12, the expression of Msx1 is down-regulated at E13.5 and E14.5. This suggests that Pax9 is not a transcriptional regulator of Msx1 at the time of tooth initiation but that its presence is necessary for advancing tooth morphogenesis. Second, our analysis of the Msx1 promoter revealed several putative paired domain-binding sites with wild-type Pax9 or L21P Pax9 reveals abolished DNA binding activity of the mutant. 219InsG, a previously described Pax9 insertion mutation, is used as a negative control. Specificity of complex formation was confirmed by supershift using Pax9 and Myc antibodies. Lanes a, protein, oligonucleotide probe; lanes b, protein, oligonucleotide probe, a-Pax9; lanes c, protein, oligonucleotide probe, a-Myc).
nificantly affect the expression of either molecule. However, cotransfection of a low concentration of Msx1 with Pax9 enhances the Pax9-mediated activation of Msx1 and Bmp4 promoters, whereas equal amounts of Pax9 and Msx1 reduce this activity. This dose-dependent effect is likely analogous to the modulation of Msx1 expression, depending on the stage of tooth development. Additionally, we show a synergistic functional relationship between Pax9 and Msx1 in the regulation of Bmp4 expression, which is consistent with the observation that overexpression of Bmp4 rescues the tooth arrest shown in Pax9/Msx1 double (H11001/H11002) mice. This is in contrast to previous reports of protein-protein interactions between Msx1 and other transcription factors that result in repression of cooperative DNA binding and transcriptional activities (29, 30). A possible explanation of how this occurs comes from a recent study showing that Msx1 interacts with another nuclear protein, H1b, to confer a repressed chromatin structure that inhibits terminal differentiation in myogenesis (31). In the context of tooth morphogenesis, it is possible that the presence of a transcriptional activator like Pax9 can remove Msx1-mediated repression and allow for the transcription of a downstream target gene, Bmp4. Bmp4 can subsequently initiate signaling events that would lead to advancement of tooth development from the bud to cap stage. Thus, we propose that the synergistic regulation of Bmp4 expression by the interaction of Pax9 with Msx1 at both the transcriptional and protein level is essential at a critical stage of tooth morphogenesis.

Although recent studies of human PAX9 mutations have enabled structure-function correlations, the precise molecular mechanisms that contribute to tooth agenesis are poorly understood. Our functional studies of a previously described mutation (L21P) in the amino-terminal subdomain of the paired domain (1) demonstrate impaired transcriptional activation of the Msx1 and Bmp4 promoters. It is likely that the proline cyclic side chain blocks the main chain nitrogen atom and chemically prevents it from forming a hydrogen bond. Hence, we speculate that the mutation of Leu to Pro eliminates this hydrogen bonding interaction and therefore precludes the binding and subsequent transcription of a promoter-reporter construct. Additionally, evidence has shown that the paired domain of Pax3 and the homeodomain of Msx1 are essential for the interaction between the two proteins. The results of our coimmunoprecipitation analysis indicate that the L21PPax9 mutation has no effect on its ability to interact with Msx1, suggesting that the amino-terminal subdomain of the paired domain is not involved in protein-protein interactions. Thus, it is likely that either the carboxyl-terminal subdomain of the paired domain of Pax9 is critical for this interaction or the mutation does not alter the structure of the paired domain to an extent that would disrupt heterodimerization with Msx1. Although not as likely, another explanation is that protein-protein interactions...
Interactions of Pax9 and Msx1 Genes in Tooth Development

interactions may also occur through the octapeptide motif of Pax9, which is unaffected by the mutation studied (32). We also demonstrated that synergistic transcriptional activation is reduced between L21P Pax9 and Msx1 at the Bmp4 promoter. Taken together with the observation that mutant Pax9 interacts with Msx1, our results suggest that defects in DNA binding, rather than protein-protein interactions, are responsible for the pathogenesis of tooth agenesis for this mutation.

In summary, our biochemical data presented here implicate direct interactions between Pax9 and Msx1 on two levels. The first level is that of transcriptional regulation. The second possibility is that Pax9 and Msx1 interact as proteins, i.e. via protein-protein interactions. Furthermore, the integration of human genetics data indicates that the regulation of Bmp4 expression by the interaction of Pax9 with Msx1 through the paired domain of Pax9 determines the fate of the transition from the bud to cap stage of tooth development. Our studies raise the possibility that Pax9 and Msx1 may control aspects of important early developmental processes by modulating the expression of Bmp4 during tooth morphogenesis. Such data are important for advancing our understanding of normal and abnormal tooth development. In addition, these studies offer valuable insights into the pathogenesis of human tooth agenesis.

Acknowledgments—The advice and insights provided by Drs. Brad Amendt and Peter Bialek are appreciated, as is the technical assistance of Adriana Cavender.

REFERENCES

1. Das, P., Hai, M., Elcock, C., Leal, S. M., Brown, D. T., Brook, A. H., and Patel, P. I. (2003) Am. J. Med. Genet. 118A, 35–42
2. Vastardis, H. (2000) Am. J. Orthod. Dentofacial. Orthop. 117, 650–656
3. Mostowska, A., Kobiela, A., Biedrzycki, B., and Trzezciak, W. H. (2003) Eur. J. Oral. Sci. 11, 272–276
4. Vieira, A. R. (2003) J. Dent. Res. 82, 162–165
5. Vastardis, H., Karimbas, N., Guthua, S. W., Seidman, J. G., and Seidman, C. E. (1996) Nat. Genet. 13, 417–421
6. Stockton, D. W., Das, P., Goldenberg, M. D., D’Souza, R. N., and Patel, P. I. (2000) Nat. Genet. 24, 18–19
7. Nieminen, P., Arte, S., Tanner, D., Paulin, L., Alahussa, S., Thesleff, I., and Pirinen, S. (2001) Euro. J. Hum. Genet. 9, 743–746
8. Klein, M. L., Nieminen, P., Lamm, L., Niebuhr, E., and Kreiborg, S. (2005) J. Dent. Res. 84, 43–47
9. Mostowska, A., Kobiela, A., and Trzezciak, W. H. (2003) Eur. J. Oral. Sci. 111, 365–370
10. Vieira, A. R., Meira, R., Modesto, A., and Murray, J. C. (2004) J. Dent. Res. 83, 723–727
11. Frazier-Bowers, S. A., Guo, D. C., Cavender, A., Xue, L., Evans, B., King, T., Milewicz, D., and D’Souza, R. N. (2002) J. Dent. Res. 81, 129–133
12. Lamm, L., Halonen, K., Pirinen, S., Thesleff, I., Arte, S., and Nieminen, P. (2003) Eur. J. Hum. Genet. 11, 866–871
13. Lamm, L., Arte, S., Somer, M., Jarvinen, H., Laheremo, P., Thesleff, I., Pirinen, S., and Nieminen, P. (2004) Am. J. Hum. Genet. 74, 1043–1050
14. Satokata, I., and Maas, R. (1994) Nat. Genet. 6, 348–356
15. Peters, H., Neubüser, A., and Balling, R. (1998) Eur. J. Oral Sci. 106, 38–43
16. Chen, Y., Bei, M., Woo, I., Satokata, I., and Maas, R. (1996) Development 122, 3035–3044
17. Laurikkala, J., Kassai, Y., Pakkasjarvi, L., Thesleff, I., and Itoh, N. (2003) Dev. Biol. 264, 91–105
18. Kawai, S., and Sugiura, T. (2001) Bone 29, 54–61
19. Bei, M., and Maas, R. (1998) Development 125, 4325–4333
20. Kettunen, P., and Thesleff, I. (1998) Dev. Dyn. 211, 256–269
21. Peters, H., Doll, U., and Niessing, J. (1995) Dev. Dyn. 203, 1–16
22. Mensah, J. K., Ogawa, T., Kapadia, H., Cavender, A. C., and D’Souza, R. N. (2004) J. Biol. Chem. 279, 5924–5933
23. Takahashi, T., Guron, C., Shetty, S., Matsu, H., and Raghov, R. (1997) J. Biol. Chem. 272, 22667–22678
24. Shetty, S., Takahashi, T., Matsu, H., Ayengar, R., and Raghov, R. (1999) Biochem. J. 339, 751–758
25. Zhang, Z., Song, Y., Zhao, X., Zhang, X., Fermin, C., and Chen, Y. (2002) Development 129, 4135–4146
26. Goulding, M. D., Chalepakis, G., Deutsch, U., Erselius, J. R., and Gruss, P. (1991) EMBO J. 10, 1135–1147
27. Neubüser, A., Koseki, H., and Balling, R. (1995) Dev. Biol. 170, 701–716
28. Czerney, T., Schaffner, G., and Busslinger, M. (1993) Genes Dev. 7, 2048–2061
29. Zhang, H., Hu, G., Wang, H., Sciavolino, P., Iler, N., Shen, M. M., and Abate-Shen, C. (1997) Mol. Cell. Biol. 17, 2920–2932
30. Bendall, A. J., Rincon-Limas, D. E., Botas, J., and Abate-Shen, C. (1998) Differentiation 63, 151–157
31. Lee, H., Habas, R., and Abate-Shen, C. (2004) Science 304, 1675–1678
32. Eberhard, D., Jimenez, G., Heavey, B., and Busslinger, M. (2000) EMBO J. 19, 2292–2303