An Extensive 3′ Regulatory Region Controls Expression of *Bmp5* in Specific Anatomical Structures of the Mouse Embryo

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ABSTRACT

Bone morphogenetic proteins (BMPs) are secreted signaling molecules that control important developmental events in many different organisms. Previous studies have shown that BMPs are expressed at the earliest stages of skeletal development, and are required for formation of specific skeletal features, strongly suggesting that they are endogenous signals used to control formation of skeletal tissue. Despite the importance of BMP signaling in normal development, very little is known about the mechanisms that control the synthesis and distribution of BMP signals in vertebrates. Here, we identify a large array of *cis*-acting control sequences that lay out expression of the mouse *Bmp5* gene in specific skeletal structures and soft tissues. Some of these elements show striking specificity for particular anatomical features within the skeleton, rather than for cartilage and bone in general. These data suggest that the vertebrate skeleton is built from the sum of many independent domains of BMP expression, each of which may be controlled by separate regulatory elements driving expression at specific anatomical locations. Surprisingly, some of the regulatory sequences in the *Bmp5* gene map over 270 kb from the *Bmp5* promoter, making them among the most distant elements yet identified in studies of eukaryotic gene expression.

THE bone morphogenetic proteins (BMPs) comprise a large subset of the transforming growth factor-β family of secreted signaling molecules (Kingsley 1994a). Studies in multiple organisms have shown that the BMPs are used to control a wide variety of events during embryonic development, including dorsal-ventral and left-right axis formation, mesenchymal-epithelial interactions, and differentiation of many specific tissues including lung, gut, kidney, hair, teeth, cartilage, and bone (Kingsley 1994a; Hogan 1996). Some of these functions, such as control of dorsal-ventral axis formation in both flies and vertebrates, appear to be ancient functions conserved in many metazoans (Holley *et al.* 1996). Other functions may be specific to particular classes of animals: for example, the role of BMPs in controlling wing vein development in insects, or cartilage and bone formation in vertebrates.

Studies of the Drosophila *decapentaplegic* (*dpp*) gene suggest that BMPs have been recruited into distinct developmental roles by the addition of separate *cis*-acting regulatory elements that control expression at specific times and locations. For example, the *dpp* gene is normally expressed in the dorsal half of the early embryo, in the constrictions of the midgut, and in stripes along the anterior-posterior border in many developing imaginal discs (St. Johnston and Gelbart 1987; Posakony *et al.* 1990). Separate elements have been identified that control expression at each of these locations, and many have been shown to interact with different transcription factors that positively or negatively affect expression (Huang *et al.* 1993; Capovilla *et al.* 1994; Manak *et al.* 1994; Sanicola *et al.* 1995).

Much less is known about the mechanisms that control expression of BMP genes in vertebrates. Some of these mechanisms may be very similar to those observed in invertebrate systems. This seems particularly likely for expression in structures that may have already been present in the common ancestors of vertebrates and invertebrates, including the heart, gut, and dorsal-ventral axis. It is more difficult to predict the mechanisms that control expression in tissues and structures that are unique to the vertebrate lineage, such as cartilage and bone. A large number of studies suggest that BMPs are endogenous signals used by vertebrate embryos to induce the formation of skeletal tissue (Kingsley 1994b). BMPs can trigger the entire process of cartilage and bone formation when implanted at ectopic sites in adult animals (Urist 1965; Reddi and Huggins 1972) and are normally expressed in and around early cartilage and bone precursors during embryonic development (Lyons *et al.* 1989; Jones *et al.* 1991; King *et al.* 1994; King *et al.* 1996). Moreover, mutations in different BMP genes block the formation of particular skeletal features, showing that BMPs are also required for the normal formation of skeletal tissue (Kingsley *et*

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al. 1992; Storm *et al.* 1994; Luo *et al.* 1995; Thomas *et al.* 1996).

How has an ancient class of signaling molecules been recruited into controlling the formation of skeletal structures in vertebrates? New roles for the BMPs must have involved changes in downstream responses; for example, the formation of a link between BMP signaling and cartilage and bone differentiation. Once this link was established in the vertebrate lineage, gain and loss of regulatory elements in individual BMP family members could provide a simple mechanism for inducing new domains of BMP expression and new cartilage and bone elements at particular locations during embryonic development. Based on the specific skeletal defects seen in mice missing different BMPs, and the different expression patterns of BMPs in developing skeletal structures, we have previously proposed that different members of the BMP family may have accumulated unique sets of control elements that induce the formation of specific anatomical structures in the skeleton (Kingsley 1994b). According to this model, the large family of different vertebrate BMP genes may exist in part because gene duplication and regulatory changes within the BMP family provide a useful mechanism for independently controlling BMP signaling events in different body regions.

To begin to test this model, we have initiated a detailed characterization of the molecular mechanisms that drive expression of BMP genes in mammals. The mouse *Bmp5* gene is a particularly favorable place to begin these studies. Null mutations in this gene reduce or eliminate many specific skeletal elements and produce an easily observable external trait, short ears (Lynch 1921; Green 1951; Kingsley *et al.* 1992). Mice carrying the original *short ear* (*se*) mutation have been used for extensive specific-locus mutagenesis experiments (Russell 1951). As a result of these experiments, over two dozen viable radiation- and chemically-induced alleles have been isolated at the *Bmp5* locus (Russell 1971; Russell *et al.* 1989; Kingsley *et al.* 1992; Marker *et al.* 1997). Here, we use this large collection of *short ear* alleles to identify *cis*-acting regulatory sequences that control where and when the *Bmp5* gene is expressed during development. Regulatory mutations and transgenic reporter studies show that *Bmp5* expression is regulated by a large number of separate DNA elements that control expression at specific anatomical locations in the skeleton and other tissues. Many of these elements are located at unusually large distances from the *Bmp5* promoter, suggesting that important *cis*-acting control elements can act over distances of hundreds of kilobases in the mammalian genome.

MATERIALS AND METHODS

Mice: The $se^{30DThWb}$, se^{sv}, and se^{4CHLd} alleles were generated in specific locus mutagenesis tests with 101/Rl and C3H/Rl as the parental background strains (Russell 1971), using x-rays, neutrons, or chlorambucil (Russell *et al.* 1989) in spermatogonia, spermatogonia, or male meiosis, respectively. The se^{2OZb} mutation, x-ray induced in oocytes, is a null allele that deletes the entire *Bmp5* coding region (Kingsley *et al.* 1992). All mutations are maintained on outbred genetic backgrounds and are currently being backcrossed to inbred strains for detailed studies of skeletal phenotypes (Green 1957).

Inversion breakpoint mapping: DNA samples from control and mutant strains were prepared for conventional and pulsed-field gel analysis as previously described (Marker *et al.* 1997). A probe at position 84 to 87 kb of the *short ear* chromosome walk was used to look for alterations in a large 725-kb *Bss* H2 restriction fragment that spans all *Bmp5* coding exons and the 3′ flanking genomic region (Kingsley *et al.* 1992). Novel-sized fragments were detected in the $se^{30DThWb}$, se^{sv}, and se^{4CHLd} mutations. Probes near the 3′ end of this fragment (position 709 kb) detected different novel *Bss*H2 fragments in each mutation, suggesting a chromosome inversion or translocation between the two probes. Sequential hybridizations with probes located between positions 84 and 440 kb indicated that, for all three alleles, the transition point in the altered *Bss*H2 restriction fragment maps 3′ of the *Bmp5* coding exons. Conventional restriction mapping and hybridizations with probes near the predicted transition points confirmed the location of each mutation (see Figure 1C). Probe 1 in Figure 1 is the last 1.0 kb of the full-length *Bmp5* cDNA. Probe 2 is an end fragment from phage VII. The phage clones located between positions 325 and 430 were generated from a YAC4 (Kingsley *et al.* 1992) subgenomic library (Avraham *et al.* 1995). cDNA clones for 3′ end mapping were isolated from a E8.5 library (provided by B. Hogan) and by RACE-PCR from adult lung RNA samples. Genomic sequencing showed that alternate ends were encoded by sequences contained within the last *Bmp5* coding exon.

Reporter gene constructs: For reporter constructs II–VIII, phage insert DNA was isolated from the lambda-Dash vector by digestion with *Not*I. For constructs III and IV, the ends were filled in with T4 DNA polymerase, and the insert was ligated into the *Sma*I site of the pKS(*Sal-Sal*)+ hsp68*lacZ*pA vector (Kothary *et al.* 1989) (a gift from A. Joyner). For constructs II, and V–VIII the same hsp68*lacZ*pA cassette was cut out of the pKS(*Sal-Sal*) and subcloned into the *Bam*HI site of a standard pKS vector such that the *Not*I site is located 5′ of the hsp (*Not*5′hsp*lacZ*). The inserts from phage II and V–VIII were ligated into *Not*I-digested *Not*5′hsp*lacZ* vector. The III and IV constructs were linearized with *Not*I and the II, V–VIII constructs with *Sal*I. All constructs were ethanol-precipitated and resuspended in microinjection buffer (10 mM Tris, pH 7.4; 0.15 mM EDTA, pH 8.0). After two rounds of buffer-exchange with Centricon-30 concentrators (Amicon, Beverly, MA), the DNA concentration was adjusted to 2 ng/μL and filtered through a Centrex MF-1.5 (0.2 μM) Centrifugal Microfilter (Schleicher & Schuell, Keene, NH).

Transgenic production and typing: The DNA was injected into the pronuclei of fertilized mouse eggs derived from FVB/N or C57BL/6 × CBA F1 mice (Hogan *et al.* 1994). The injected oocytes were transferred into oviducts of pseudopregnant CD-1 mice and transgenic embryos were identified at E14-E15.5 by typing yolk sac DNA with primers from the *lacZ* gene (forward primer, 5′-TTTCCATGTTGCCACTCGC 3′; reverse primer, 5′-AACGGCTTGCCGTTCAGCA-3′).

***lacZ* detection and cryosectioning of embryos:** Embryos were dissected in cold PBS and a 27-gauge needle was used to make holes in the torso and head cavities. The embryos were fixed for 60–75 min at 4° in 4% paraformaldehyde, cut in half sagitally, fixed for another 10–15 min and washed 3 × 20 min in wash buffer (0.1 m sodium phosphate buffer (pH 7.3), 2 mm

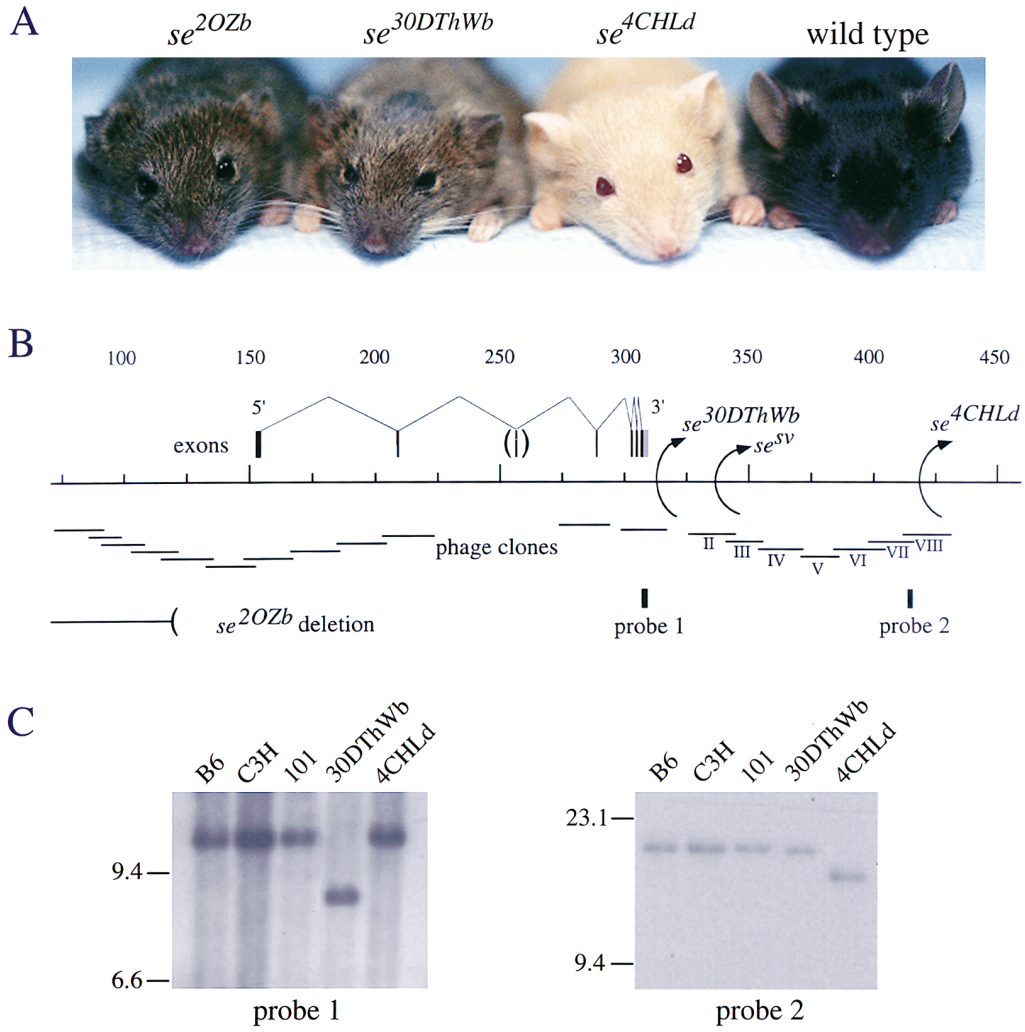

Figure 1.—Chromosome rearrangements 3′ of *Bmp5* exons produce a *short ear* phenotype. (A) Mice homozygous for $se^{30DThWb}$, se^{4CHLd}, or a null mutation at the *Bmp5* locus (se^{2OZb}) are shown next to wild-type (C57BL/6J) mice. The reduction in ear size is more pronounced in the $se^{30DThWb}$ than in the se^{4CHLd} mutant. (B) Map of the *short ear* chromosome region, showing DNA clones and coordinates from the previous chromosome walk (Kingsley *et al.* 1992) (including phage clones II–VIII used in reporter constructs), the location of *Bmp5* exons, and the genomic breakpoints of four different *se* alleles. se^{2OZb} is a 550-kb deletion that includes all of the *Bmp5* exons (Kingsley *et al.* 1992). The $se^{30DThWb}$, se^{sv}, and se^{4CHLd} mutations are all chromosome rearrangements that break in a large genomic region 3′ of the last *Bmp5* coding exon. Two alternative 3′ ends are encoded by the final exon; the gray sequence is contained only in the larger transcript form. The exact position of exon 3 (in parentheses) is not known. (C) Genomic Southern analysis confirming the position of the $se^{30DThWb}$ and se^{4CHLd} breakpoints near probes labeled 1 (*Eco*RV digest) and 2 (*Bam*HI digest) in Figure 1B. DNA samples from parental control strains (C3H and 101) are shown for comparison.

MgCl, 0.01% deoxycholate, 0.02% Nonidet P-40). *lacZ* staining was done in wash buffer supplemented with 1 mg/mL X-gal (GIBCO-BRL, Gaithersburg, MD), 4 mm $K_3Fe(CN)_6$, 4 mm $K_4Fe(CN)_6 \cdot 3\ H_2O$, and 0.1 m Tris (pH 7.4) for 40–48 hr at room temperature. Embryos were then rinsed 3 × 30 min in wash buffer and cleared with 75% glycerol. For sectioning, whole-mount *lacZ*-stained embryos saturated in 50% sucrose were embedded in gelatin/sucrose solution (15% sucrose, 7.5% gelatin in water) and cryosectioned at −25° with thicknesses between 20–30 μm. Sections were dried overnight, rinsed in 1× PBS, counterstained with 0.01% neutral red for 4′, and directly mounted in Aquamount (Lerner Laboratories, Pittsburgh, PA).

***In situ* hybridization:** The *Bmp5* probe was a 1.03-kb probe derived from a PCR product of the pro-region of the mouse cDNA (bases 671–1702) (King *et al.* 1994) cloned into pCRII (Invitrogen, San Diego, CA). Digoxigenin-labeled RNA probes were transcribed using the Genius™ labeling system (Boehringer Mannheim, Indianapolis, IN). Timed matings were performed and noon of the day of the vaginal plug was designated as embryonic day 0.5. *In situ* hybridizations were done as described (Storm and Kingsley 1996) with the following modifications: sections were 14 μm and the hybridization temperature was raised to 64°.

RESULTS

***se* mutations with chromosome rearrangments are potential regulatory alleles of the *Bmp5* gene:** Molecular studies have shown that most of the spontaneous, radiation-, and chemically-induced *se* alleles have lesions that disrupt the BMP5 protein (Kingsley *et al.* 1992; King *et al.* 1994; Marker *et al.* 1997). However, 7 of 27 mutations analyzed do not show any sequence changes in the *Bmp5* open reading frame (Marker *et al.* 1997), suggesting that they may be regulatory mutations. Four of these mutations ($se^{30DThWb}$, se^{sv}, se^{4CHLd}, se^{1FrThc}) were induced with radiation or chemicals known to cause chromosome rearrangements (Russell 1971; Russell *et al.* 1989). Probes throughout the 710-kb *short ear* chromosome walk (Kingsley *et al.* 1992) detect cross-

hybridizing fragments in DNA samples from these four mutations, ruling out any large DNA deletions in the four alleles (data not shown). However, pulsed-field gel mapping with numerous probes located between positions 84 and 430 kb on the chromosome walk suggested the presence of chromosome inversions or translocations in three of the mutations near the sites indicated in Figure 1B (see materials and methods). Probes at positions 310, 335, and 415 detected altered restriction fragments in DNA from the $se^{30DThWb}$, se^{sv}, and se^{4CHLd} alleles, respectively, confirming breakpoints at the predicted locations (Avraham *et al.* 1995) (Figure 1C). The breakpoint in the se^{sv} mutation has been cloned and sequenced, and consists of a 2-cM chromosome inversion with loss of only 106 bp at the inversion junction (Avraham *et al.* 1995).

All three breakpoints map 3′ of previously reported *Bmp5* coding exons, consistent with the possibility that the mutations represent regulatory alleles of the *se* gene. cDNA cloning, 3′ RACE, and genomic sequencing analyses were carried out to test whether additional *Bmp5* exons map farther 3′ from those previously identified (data not shown). These studies identified two alternative 3′ ends for *Bmp5* cDNAs that account for the major transcripts seen in many different tissues (King *et al.* 1994) (Figure 1B). Both ends are encoded by genomic sequences located immediately adjacent to the *Bmp5* open reading frame; the most 3′ cDNA sequence for *Bmp5* thus maps to position 310 of the chromosome walk (Figure 1B). The three mutant breakpoints are located approximately 6 kb, 18 kb, and 105 kb past all known *Bmp5* exons, confirming that the mutations do not affect *Bmp5* exon sequences (Figure 1B).

The $se^{30DThWb}$ and se^{4CHLd} mutations produce an apparent gradient of effects on the size of the external ear. The more distant mutation (se^{4CHLd}) has the mildest effect on ear length. Other rare alleles of this type have previously been classified as intermediate short ear (Russell 1971). The $se^{30DThWb}$ allele has a more pronounced effect, but does not produce ears as short as those seen in mice completely missing the *Bmp5* gene (se^{2OZb}) (Figure 1A). Although these mutations have not yet been analyzed on a uniform genetic background, the different ear phenotypes and the 3′ position of the breakpoints suggest a possible analogy with previous studies of the Drosophila BMP-related gene *decapentaplegic* (*dpp*). Genetic and molecular studies of *dpp* have identified a large 3′ regulatory region that contains multiple modular enhancers which drive expression in different subdomains of imaginal discs (Masucci *et al.* 1990; St. Johnston *et al.* 1990; Blackman *et al.* 1991). Mutations that break at increasing distances from the *dpp* gene disrupt fewer regulatory elements, and lead to milder truncations of legs and other appendages. This analogy suggests that important *Bmp5* regulatory elements might be located in the genomic region between the $se^{30DThWb}$ and se^{4CHLd} mutations.

Reporter analysis identifies numerous enhancers located 3′ of the *Bmp5* exons: To test whether the 3′ region contains important *Bmp5* regulatory sequences, we surveyed 106 kb of 3′ genomic DNA for the ability to drive expression of a reporter gene in *Bmp5* expression domains. Seven constructs (Figure 1B; clones II–VIII), each covering 14 to 20 kb, were fused to a minimal promoter driving a *lacZ* reporter (Kothary *et al.* 1989) and microinjected into fertilized mouse eggs. Transgenic founder embryos were collected at embryonic day 15 (E15) and assayed for *lacZ* staining patterns. Several constructs gave reproducible patterns in different regions of the mouse embryo, including the upper part of the sternum (rostral manubrium), the genital tubercle, thyroid cartilage, and lung mesenchyme (Figure 2, A, D, G, J; see figure legend for number of transgenic embryos analyzed). Histological sections of stained and sectioned embryos show that these patterns closely resemble subsets of the normal expression pattern of the endogenous *Bmp5* gene (Figure 2, B, C, E, F, H, I, K, L). Moreover, several sites correspond to specific anatomical structures that are disrupted in *short ear* mice (sternum, thyroid cartilage, and lung; see Figure 2M) (Green 1951, 1968).

The 3′ mutations disrupt specific parts of the complete *Bmp5* expression pattern: To confirm that DNA sequences in the 3′ region are necessary for normal expression of *Bmp5*, we used *in situ* hybridization analysis to examine the expression pattern of the endogenous *Bmp5* gene in embryos homozygous for the $se^{30DThWb}$ or se^{4CHLd} mutations. In $se^{30DThWb}$ embryos, expression of *Bmp5* RNA is greatly reduced in the thyroid cartilage, genital tubercle, and lung mesenchyme (Figure 3, E, K, N). In contrast, the expression of *Bmp5* is either not affected, or is more mildly affected at these locations in embryos homozygous for the se^{4CHLd} mutation (Figure 3, F, L, O). These data are consistent with the placement of the genital tubercle, thyroid cartilage, and lung enhancers between the two breakpoints (Figure 4). The appearance of *Bmp5* transcripts in the lung epithelium of $se^{30DThWb}$ embryos is unexpected, since little expression occurs in the epithelium of wild-type lungs (Figure 3, M and N). The chromosome rearrangement in $se^{30DThWb}$ could remove a silencer that normally masks expression at this site or introduce a new regulatory element; or, the ectopic expression may result from altered signaling between mesenchyme lacking *Bmp5* and the lung epithelium.

The effects of the $se^{30DThWb}$ and se^{4CHLd} mutations are unlikely to be caused by general silencing of the *Bmp5* locus. Both mutations disrupt very specific subsets of the complete *Bmp5* expression pattern. For example, expression in the pinna of the external ear is more strongly affected than in the underlying middle ear, inner ear, or temporal bone elements (Figure 3, A–C; pinna *vs.* Tb). In the $se^{30DThWb}$ embryos, expression around the sternum is more affected than expression

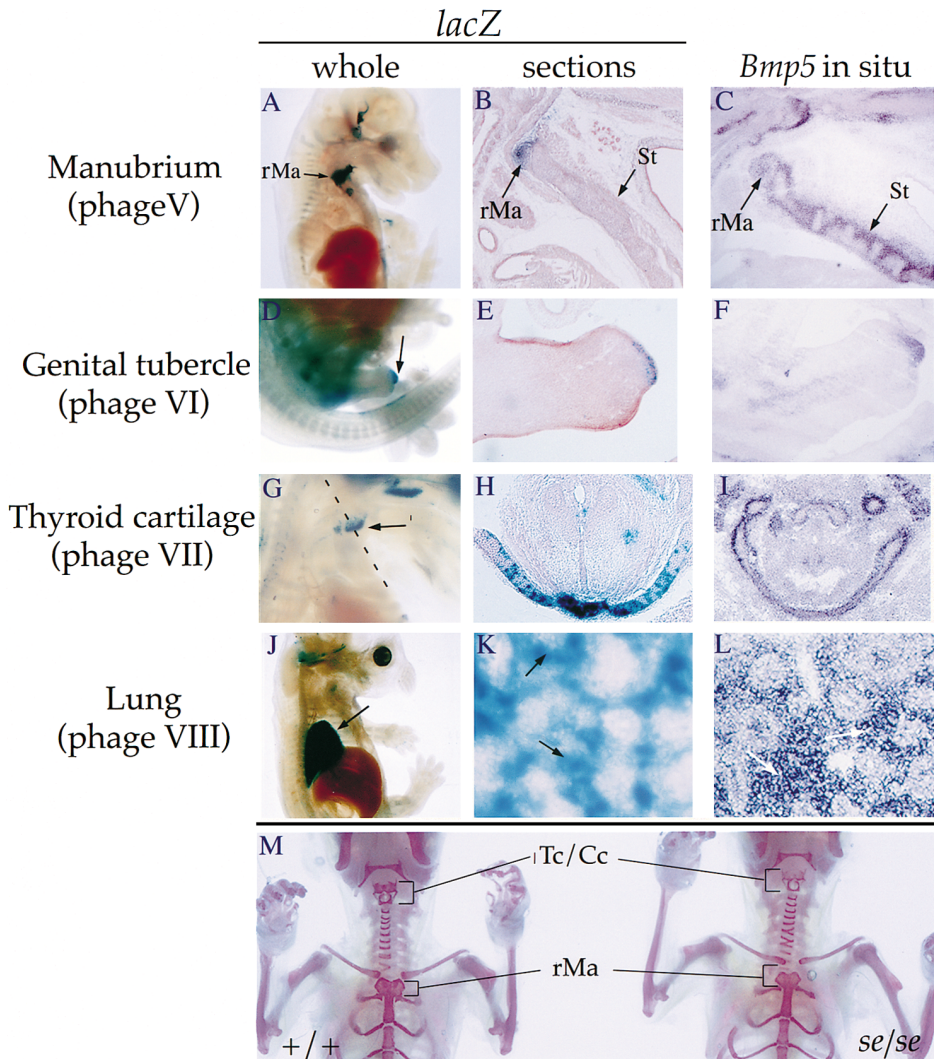

Figure 2.—Numerous regulatory elements in the large 3′ genomic region drive *lacZ* expression in different subsets of the normal *Bmp5* expression pattern. Transgenic mice carrying different DNA inserts attached to a minimal promoter *lacZ* cassette (Kothary *et al.* 1989) show consistent expression of the reporter gene in either: the rostral manubrium (rMa) of the sternum (A, B); the tip of genital tubercle (D, E); thyroid cartilage (G, H); or lung mesenchyme (J, K). These sites closely resemble patterns of transcription of the endogenous *Bmp5* gene (C, F, I, L), as well as sites of skeletal phenotypes in the rostral manubrium (rMa) and thyroid cartilage/cricoid cartilage (Tc/Cc) in *short ear* null mutant mice (M). All *lacZ* and *Bmp5* expression data are shown in embryos at 14.5–15.5 days of gestation (E14.5-15.5). The patterns shown were included in the *lacZ* staining domains of 6/6, 5/5, 5/6, and 8/8 transgenic embryos analyzed for constructs V, VI, VII, and VIII, respectively.

around the ribs (Figure 3 G–I; St *vs.* Ribs). Neither mutation has a strong effect on expression in the thyroid gland (Figure 3, D–F; Tg), the intestine (Figure 3, P–R), the nasal cartilage, or around the tips of digits (data not shown).

DISCUSSION

Here, we report a large array of 3′ regulatory sequences that drive expression of *Bmp5* in specific skeletal elements or soft tissues. These control sequences are sufficient to drive expression of an exogenous reporter gene in subsets of the normal *Bmp5* expression pattern. They are also essential for normal expression of *Bmp5* as shown by the specific alterations in *Bmp5* mRNA expression patterns seen in mice carrying different mutant breakpoints in the 3′ region. These results suggest that the overall expression pattern of the *Bmp5* gene is controlled by a large number of separate regulatory elements, many of them located in the 3′ flanking region.

Remarkably, many of the elements appear to be specific for anatomical locations, rather than for particular types of differentiating tissue. For example, most skeletal structures arise from mesenchyme through a sequence that includes condensation, cartilage formation, cartilage hypertrophy, and ossification (Fell 1925). Enhancers, such as that from the collagen II gene, have previously been described that will drive the expression of genes in most differentiating cartilage structures throughout the body (Yamada *et al.* 1990; Niederreither *et al.* 1992). In contrast, the control elements in the *Bmp5* gene drive expression at specific anatomical locations in the skeleton. For example, enhancers have been found that drive reporter gene expression only in the upper region of a single bone found at the top of the sternum (the manubrium). Moreover, the regulatory mutations in the 3′ region disrupt expression in specific subsets of skeletal structures, rather than in all the skeletal elements in which *Bmp5* is normally expressed. Even expression patterns that appear to be continuous, such as *Bmp5* expression in the perichondrium of ribs and sternal bands, may be composite patterns that are built from multiple independently-con-

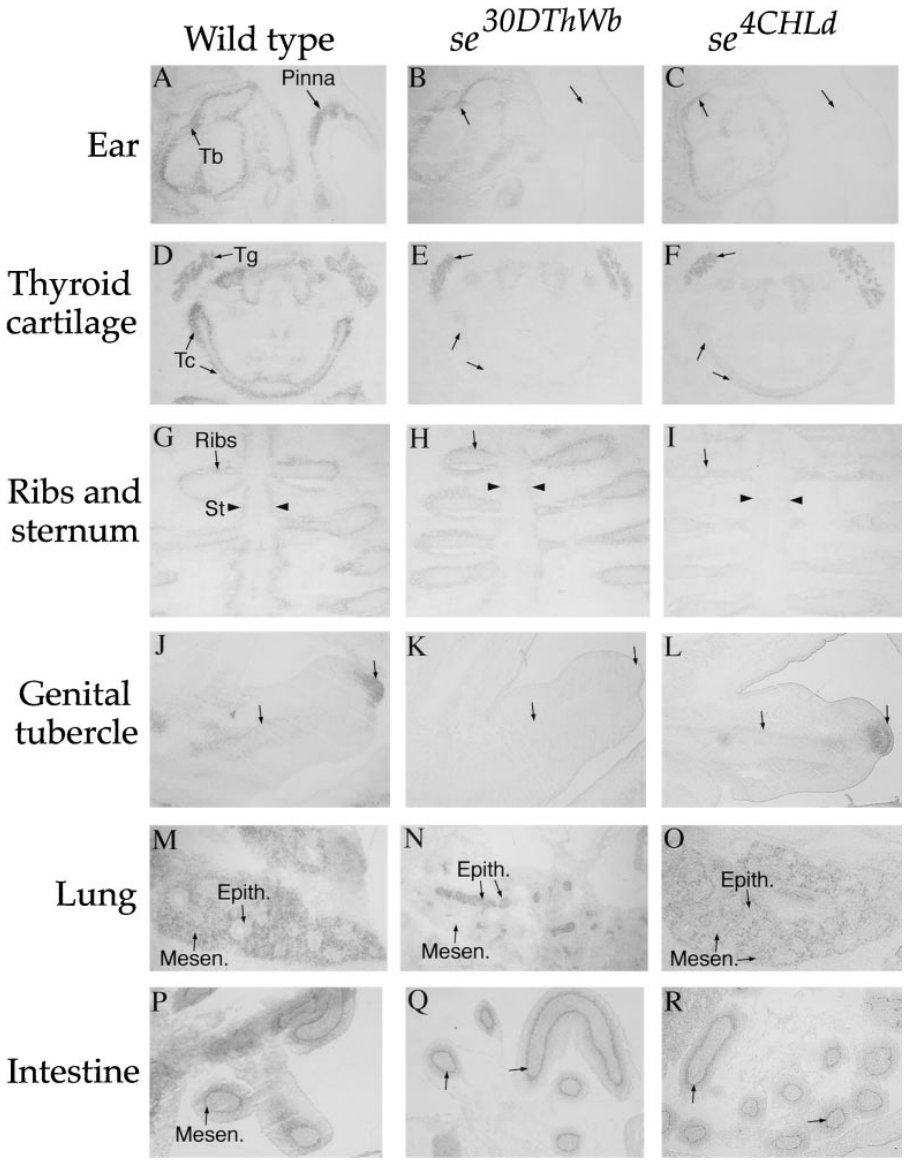

Figure 3.—Embryos homozygous for 3′ regulatory alleles show altered *Bmp5* expression at specific anatomical sites. (A–C) The $se^{30DThWb}$ and se^{4CHLd} mutations cause a dramatic reduction in *Bmp5* RNA expression in the external ear pinna but not in the petrous part of the temporal bone (Tb) surrounding inner ear structures. (D–F) Expression of *Bmp5* in the thyroid cartilage (Tc) is reduced in both the $se^{30DThWb}$ and se^{4CHLd} mutants whereas wild-type levels are seen in the thyroid gland (Tg). (G–I) The $se^{30DThWb}$ mutation reduces *Bmp5* expression in the sternum perichondrium (St, arrowheads) whereas levels remain normal around the ribs (arrows). The se^{4CHLd} allele reduces expression in both structures. (J–L) The $se^{30DThWb}$ mutant eliminates all expression in the genital tubercle; the se^{4CHLd} allele has no effect. (M–O) Expression in wild-type lung mesenchyme is greatly reduced by the $se^{30DThWb}$ mutation, but not the se^{4CHLd} mutation. $se^{30DThWb}$ also causes ectopic expression in epithelial cells. (P–R) Neither mutation disrupts the normal expression of the *Bmp5* gene in the mesenchyme surrounding the epithelium of the gut. Panels O and R are from E15.0 embryos while all other panels are from E14.5.

trolled units. In combination with previous studies showing that particular BMPs are expressed at different levels in different skeletal structures (Lyons *et al.* 1995; King *et al.* 1996), and are required for formation of specific anatomical traits (Kingsley *et al.* 1992; Storm *et al.* 1994; Luo *et al.* 1995; Thomas *et al.* 1996), these results suggest that different BMP genes have accumulated highly specific control elements that induce expression in the outlines of particular skeletal features.

Bmp5 expression in soft tissues also appears to be regulated by more elements than apparent from simple RNA expression patterns. For example, *Bmp5* expression has previously been observed in mesenchyme immediately underlying epithelium in the lungs, the intestine, the ureter, and the bladder (King *et al.* 1994). *Sonic hedgehog* is normally expressed in the overlying epithelium at some of these sites, and has previously been proposed as a possible signal that may induce BMP expression in both the lung and the intestine (Bitgood and McMahon 1995; Roberts *et al.* 1995; Bellusci *et al.* 1996). Although this still may be the case, current studies have identified DNA sequences that drive reporter genes in lung but not intestinal mesenchyme, and regulatory mutations that disrupt expression in lung mesenchyme but not intestines. Thus, the similar expression of *Bmp5* in mesenchyme underlying epithelium in lungs and intestines appears to be controlled by distinct *cis*-acting regulatory sequences.

Previous studies in flies have shown that *dpp* expression in the wing imaginal discs is also controlled by multiple modular regulatory elements that drive expression in subsets of what otherwise appear to be continuous stripes (Masucci *et al.* 1990; Blackman *et al.* 1991). As in the present studies, many of these elements were originally suggested by regulatory alleles that disrupt subsets of *dpp* expression (Masucci *et al.* 1990; St. Johnston *et al.* 1990). It is not clear if the regulatory elements present in these distantly related BMP

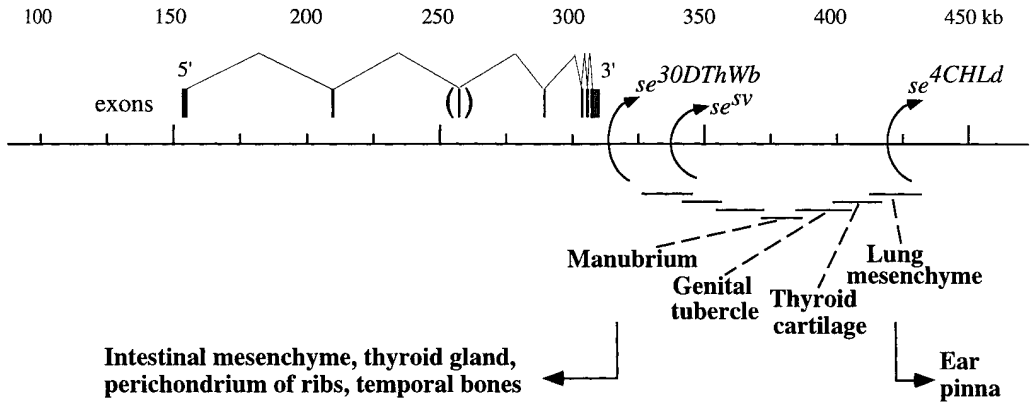

Figure 4.—Summary of multiple regulatory elements in the *Bmp5* locus. The elements identified by reporter gene studies are indicated with dotted lines. Anatomical sites with normal expression in $se^{30DThWb}$ and se^{4CHLd} mutant mice are placed 5′ of the $se^{30DThWb}$ breakpoint. The ear phenotype and expression changes in se^{4CHLd} mutant mice suggest that additional ear regulatory elements may be located distal to the se^{4CHLd} breakpoint.

genes will be similar to each other. Many surprising examples of conservation of molecular pathways have been found (Awgulewitsch and Jacobs 1992). On the other hand, *dpp* and *Bmp5* belong to distinct subgroups within the BMP family (Kingsley 1994a), so any shared regulatory elements would have to predate the divergence of the different BMP subgroups. Moreover, many of the elements found in the *dpp* and *Bmp5* genes drive expression in structures that probably have evolved since the divergence of invertebrates and vertebrates (wing veins, thyroid cartilage, manubrium). We have previously proposed that gene duplication in the BMP family, followed by gain and loss of specific control elements, could provide a general mechanism for altering the size, shape, or number of skeletal features in higher animals. (Kingsley 1994b). Further study of the anatomy-specific regulatory elements present in the *Bmp5* locus should help determine some of the molecular mechanisms used in higher animals to control the size and shape of specific skeletal structures.

Many of the control elements identified in this study are located at a surprisingly large distance from the *Bmp5* promoter. The lung mesenchyme regulatory sequences, for example, are located more than 270 kb from the *Bmp5* transcription initiation site, one of the longest distances yet reported in studies of eukaryotic gene expression. Other elements may map even farther away, as suggested by the ear phenotype and changes in skeletal expression associated with the se^{4CHLd} mutation. Although there are other published examples of distant control regions (Duncan 1987; Grosveld *et al.* 1987; Masucci *et al.* 1990; Blackman *et al.* 1991; Goldhamer *et al.* 1992) it is not clear how common such arrangements will be. In the case of *Bmp5*, such distant elements might not have been found without the extensive genetic and molecular resources available at the *short ear* locus. Several other mutations have been described in mice and humans that disrupt the function of genes located at large distances from the mutant lesion (Ton *et al.* 1991; Vortkamp *et al.* 1991; Foster *et al.* 1994; Belloni *et al.* 1996; Kluppel *et al.* 1997). Although some of these "position effect" alleles could be due to non-specific effects of newly apposed chromatin, it is also possible that long-range regulatory elements are disrupted by the mutations (Bedell *et al.* 1996). Given the long distances over which *Bmp5* elements act, we think it is likely that many genes in higher animals will turn out to be controlled by regulatory sequences that act at much greater distances from the gene than is currently recognized.

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