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Invited Review

Cellular and molecular strategies for studying the regulation of bone resorption using the toothless (osteopetrotic) mutation in the rat

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Summary. The division of labor among cells of the skeleton is distinct and diverse and the regulation of these cells is interdependent. Osteoclasts are the cellular source of bone resorption and signals for their development and activation come, at least in part, from bone and other cells in the local environment. Studies of isolated cells have identified some factors in the developmental cascade of osteoclasts but there is little understanding of the sequence and local concentrations, not to mention other factors, needed for both the development of competent osteoclasts and for coordinated bone resorption. We review the skeletal biology of one osteopetrotic mutation in the rat, toothless, in which bone resorption is severely reduced because of a failure in the development and function of osteoclasts. Furthermore, we review the advantages and limitations of a relatively new method, differential display of mRNA (DD), that identifies differences in gene expression in two or more populations of cells. We present a strategy and preliminary data for the application of DD to this mutation. We propose that application of this method to these and other skeletal diseases, with the appropriate controls and confirmations, will provide data about pathogenetic pathways and has a high probability for identifying new regulators of skeletal development and turnover.

Key words: Osteoclast, Molecular biology, Bone resorption, Regulation, Rat

A. Bone cells and the regulation of bone resorption

The skeleton is largely a mineralized connective tissue permeated and covered by cells which regulate its formation, mineralization and turnover in response to a variety of factors whose precise mechanisms and even identities remain unknown (Marks and Hermey, 1996). These bone cells and their environment in the skeleton are illustrated in Fig. 1.

Osteoblasts, the progeny of local mesenchymal osteoprogenitor cells, produce the extracellular matrix of bone and regulate its mineralization. Some osteoblasts are surrounded by matrix to become osteocytes. Bone lining cells are related to osteoblasts and cover inactive bone surfaces. Osteoclasts are large multinucleated cells that arise from the fusion of specific circulating mononuclear cells of CFU-GM lineage. The production of osteoclasts is dependent upon a number of factors, including the local production of colony-stimulating factor- 1 (CSF-1), a cytokine produced by osteoblasts and other mensenchymal cells (Felix et al., 1996). CSF-1 is also required for the production of some but not all tissue-specific macrophages (Cecchini et al., 1994), and this may be the case for site-specific osteoclasts as well (Sundquist et al., 1995a). Osteoclasts resorb bone in discrete sites and are directed to these sites by factors in the local and systemic environments (for review see: Marks, 1983; Marks and Popoff, 1988; Athanasou, 1996; Marks and Hermey, 1996).

The activities of osteoblasts (bone formation) and osteoclasts (bone resorption) are coupled by a variety of mechanisms (Fig. 2) and their relative activities are variable. During skeletal development the activity of osteoblasts is much greater than that of osteoclasts in order to produce a skeleton. During the decades of skeletal maintenance these activities are equal and during episodes of skeletal pathology they become unbalanced (Marks and Hermey, 1996). While the activities of resorption and formation are interdependent there is little general agreement on the specific mechanisms and mediators.

Bone is resorbed by osteoclasts, again by mechanisms that are incompletely understood. Active osteoclasts are large cells (Fig. 3) that have ultra-

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structural features related to their function. These include the clear zone, a circumferential tight seal against bone surfaces mediated by integrins, and a ruffled border, under which the bone surface is degraded. The resorption of bone involves a host of enzymes delivered to the confined extracellular space below the ruffled border and acidification on of this space by proton pumps located on ruffled border membranes (Marks and Popoff, 1988; Zaidi et al., 1993; Nordström et al., 1995; Hall and Chambers, 1996). Enzymes include a tartrate resistant acid phosphatase, cathepsins and other lysosomal hydrolases. The products of resorption are endocytosed and further degraded in secondary lysosomes (the active endosomal/lysosomal pathway is seen as vacuoles in Fig. 3). Thus, the origin, differentiation and activation of osteoclasts is under complex regulation and our present understanding is extremely limited *in vivo* (Kukita and Kukita, 1996).

B. Osteopetrosis as a defect in bone resorption

The osteopetroses are a heterogeneous group of metabolic bone disease characterized by a sclerotic skeleton (Fig. 4a,b) due to reduced bone resorption, the result of impaired differentiation and/or function of osteoclasts (Marks, 1989; Seifert et al., 1993; Popoff and Marks, 1995). Among these mutations is the toothless *(tl)* mutation in the rat.

C. The toothless *(tl)* mutation in the rat

The toothless mutation is characterized by reduced numbers of osteoclasts, macrophages and monocytes (Fig. 5a,b). The fact that this mutation is not cured by bone marrow transplantation (Marks, 1977) has been interpreted to mean that the problem is not in the osteoclast stem cell pool *per se*, but in local factors that support the differentiation and function of these cells. CSF-1 is such a factor and CSF-1 injections have been shown to reduce skeletal sclerosis in *tl* rats (Marks et al., 1992) but do not cure the disease (Marks et al., 1993). Skeletal sclerosis persists in some sites in *tl* rats after CSF-1 treatment (Fig. 4c) and this treatment does not fully restore the number (Marks et al., 1993) or cytochemical staining of osteoclasts for characteristic enzymes (Fig. 5c). Thus, something other than CSF- 1 appears to be missing in *tl* rats.

There is evidence that osteoblasts themselves are abnormal and reduced in number in this mutation (Seifert et al., 1988; Shalhoub et al., 1991; Sundquist et al., 1995b). Because the development and activation of osteoclasts is strongly influenced by osteoblasts (Fig. 2),

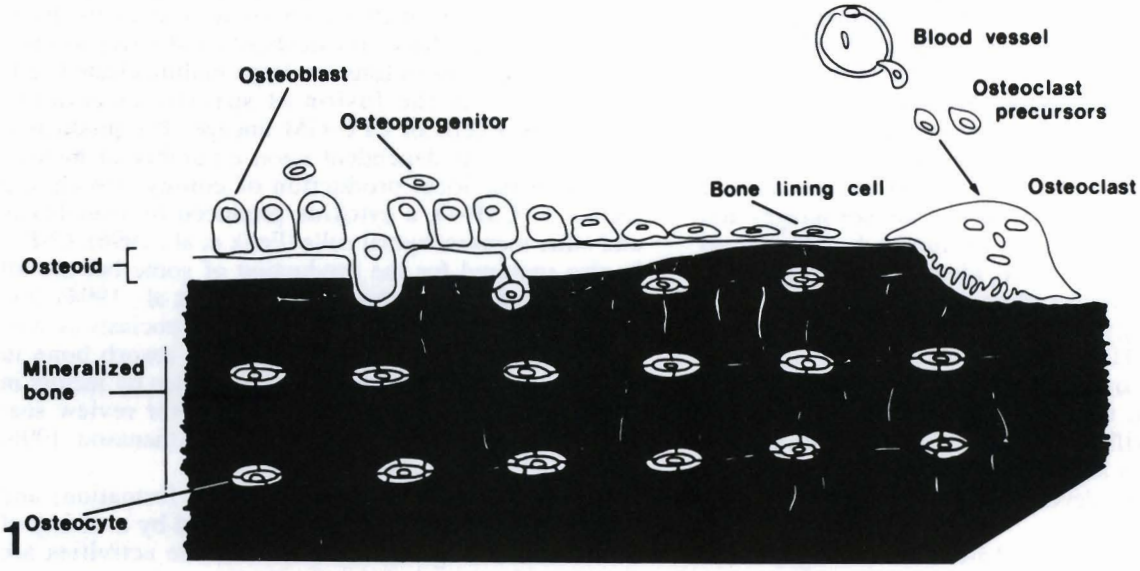

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Fig. 1. The origins and locations of bone cells. Taken from Marks and Popoff (1988). (Reprinted by permission of John Wiley and Sons, Inc.)

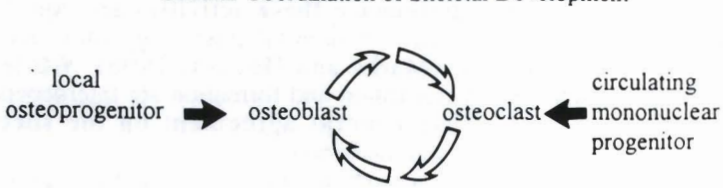

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Fig. 2. Cellular coordination of skeletal development. Schematic of the divergent origin and interrelated function of the principal bone cells. Taken from Marks and Hermey (1996). (Reprinted by permission of Academic Press.)

we have pursued the hypothesis that the reduction in bone resorption in the *tl* rat is the result of an osteoblast rather than an osteoclast abnormality. This was first suggested by the work of (Seifert et al., 1988) who described the presence of osteoblasts on the youngest bone surface in *tl* rats and the absence of these cells on older bone surfaces. Further work on gene expression in mutant osteoblasts *in vivo* (Shalhoub et al., 1991) demonstrated abnormalities in mRNA levels for type I collagen, fibronectin, alkaline phosphatase, osteocalcin

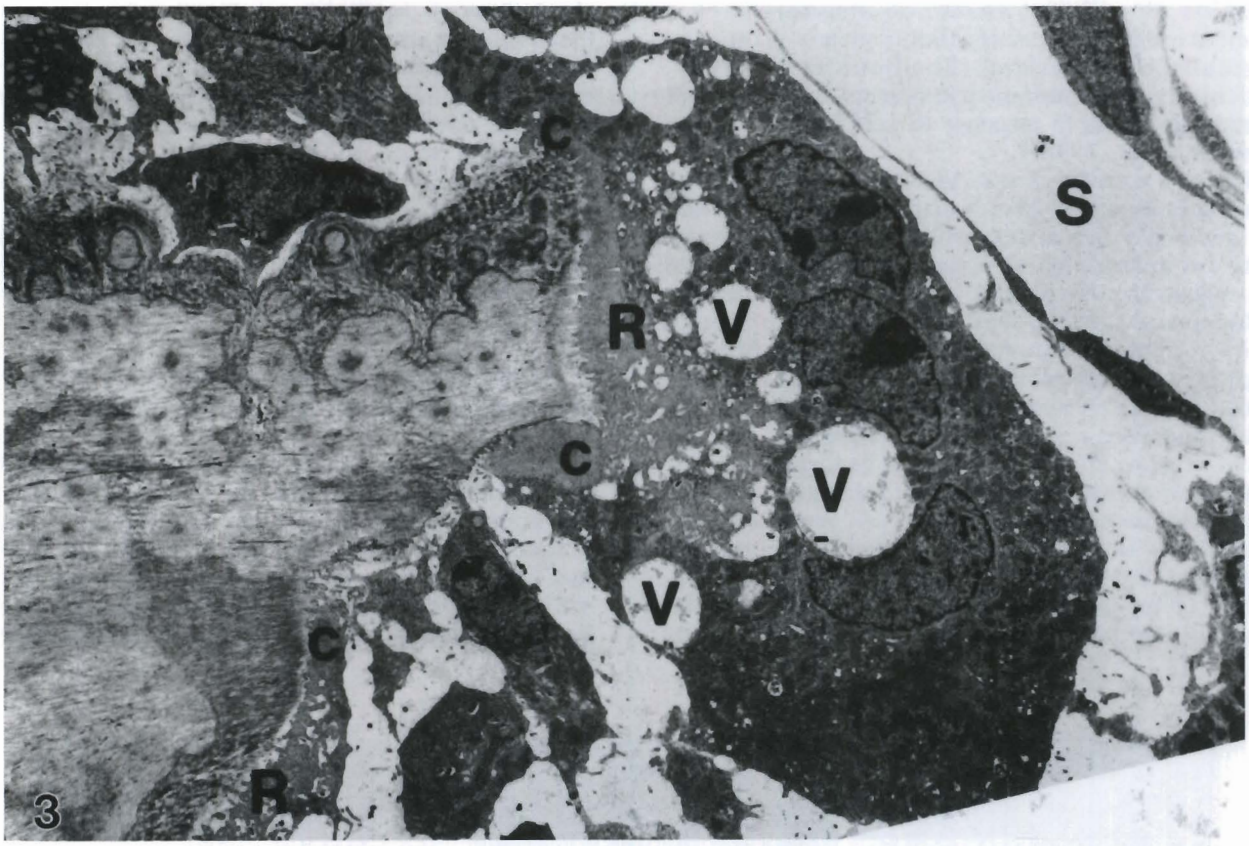

Fig. 3. Transmission electron micrograph of parts of two osteoclasts. These multinucleated cells attach to bones at clear zones (C), which create a three-dimensional seal around the ruffled border (R) working area. Active cells are highly vacuolated (V) in the cytoplasm next to the ruffled border. S: vascular sinus. Taken from Marks and Hermey (1966). (Reprinted by permission of Academic Press). x 2,240

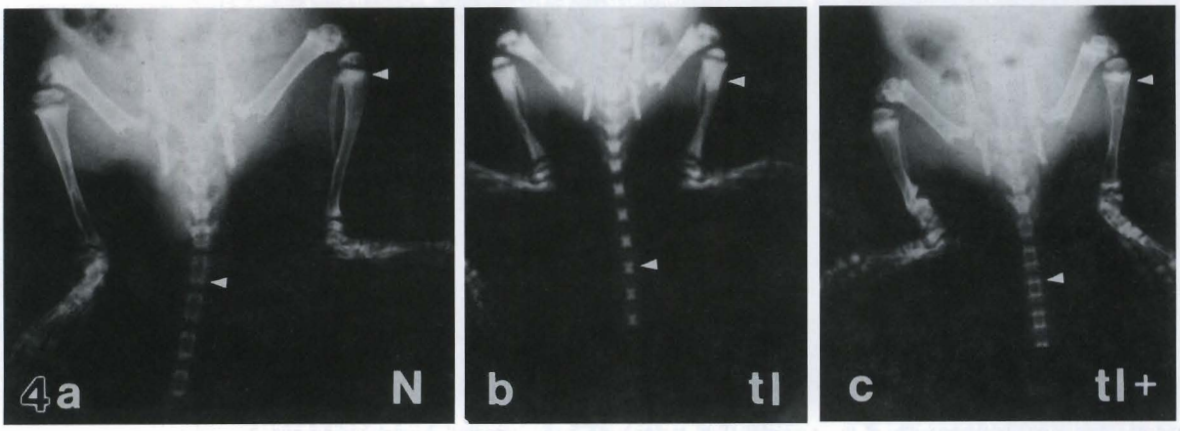

Fig. 4. Radiographs of 2-week-old normal (N) and osteopetrotic (*tl*) rats untreated or treated (+CSF-1) with 10^6 units of CSF-1 every other day from birth. Marrow spaces and the small metaphyseal regions in the long bones and caudal vertebrae (arrows) of the normal untreated rat (A) contrast with the sclerotic skeleton of the untreated mutant (B). CSF-1 treatment of mutants (C) produces marrow cavities but metaphyseal areas (arrowheads) remain more sclerotic than in normal littermates (A).

and histone H4 in the mutant skeleton. However, subsequent work showed that treatment of mutants with CSF-1 (which improves but does not cure the skeletal sclerosis) normalized the changes in osteoblast but not osteoclast gene expression in *tl* rats (Wisner-Lynch et al., 1995). In light of these results it is unlikely that this mutation is a direct result of abnormalities or deficiencies in these extracellular matrix proteins as originally hypothesized (Shalhoub et al., 1991), particularly since mutant osteoblasts fail to activate normal osteoclasts in response to 1,25 (OH)2D *in vitro* (Sundquist et al., 1995b).

We have recently shown (Watanabe et al., 1997) that osteoblasts in *tl* rats have a disorganized cytoskeleton and specifically lack a well developed stress fiber system in the cytoplasm next to bone surfaces *in vivo*. These data suggest that the paucity of osteoblasts in older parts of the mutant skeleton may be related to the impaired ability of mutant osteoblasts to form stress fibers for attachment to extracellular matrices and that their early demise may be related to this lack of attachment. The most compelling argument for an osteoblast defect in this mutation of reduced bone resorption is the demonstration that *tl* osteoblasts are not able to activate co-cultured osteoclasts to form pits on bone slices (bone resorption) in response to a known activator of resorption, 1,25(OH)2 vitamin D (Sundquist et al., 1995b). Other recent provocative and unexplained data include: 1) the promotion of angiogenesis in *tl* rats after treatment with CSF-1 (Aharinejad et al., 1995); 2) a reduction of platelets (Thiede et al., 1996) and a subset of monocytes expressing the growth hormone receptor (Symons et al., 1966) in untreated *tl* rats; and 3) their restoration after treatment with CSF-1.

Taken together these data illustrate directly the aberrations of metabolism in the skeleton and related tissues in one osteopetrotic mutation and indirectly the complexities of normal skeletal development and maintenance. Unraveling either situation requires more than the usual practice of testing whether newly discovered genes or their products are abnormally expressed in this and other mutations. We believe that a relatively new method, differential display of mRNA, has the potential to uncover both the basic defect in the *tl* mutation and its effect on other genes. We present below a rationale and strategy for its application to this mutation.

D. Differential display of mRNA as a tool for identifying differences in gene expression

In 1992, Liang and Pardee introduced the technique of differential display (DD) as a means to detect and characterize mRNA species whose expression is altered in different conditions (Liang and Pardee, 1992). Since that time, they and other investigators have applied this method to a wide variety of developmental and disease-related questions and have made technical changes to

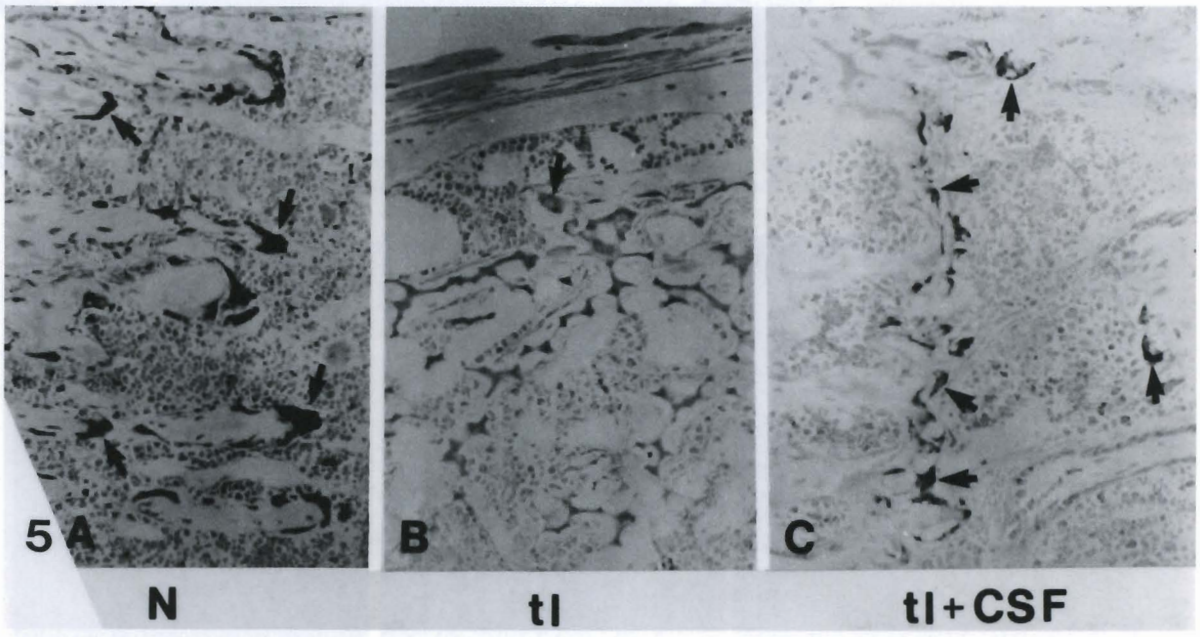

Fig. 5. Representative photomicrographs of the proximal tibial metaphysis from untreated 2-week-old normal (N) and mutant (*tl*) rats or mutant rats treated from birth with 10^6 units CSF-1 every 48h. Sections are stained histochemically for TrATPase and lightly counterstained. Osteoclasts (arrows) in normal rats (A) are large and heavily stained for this enzyme. The untreated mutant (B) exhibits a single osteoclast (arrow) which is small and does not stain for TrATPase. CSF-1 treatment of *tl* rats (C) produces numerous osteoclasts which are smaller in size and stain less intensely for TrATPase than those observed for untreated normal littermates. Taken from Wisner-Lynch et al. (1995). (Reprinted by permission of Elsevier Science Inc.). x 160

improve its performance (reviewed in Liang and Pardee, 1995; McClelland et al., 1995). We have developed a strategy for the investigation of the *tl* mutation which uses DD as the starting point. Preliminary results (see below) indicate that this approach promises to increase our knowledge of the molecular events which underlie the *tl* mutation and the normal communication and interaction between cells responsible for bone resorption.

The basic strategy of DD is to obtain RNA from cells or tissue representing two different states (in the present case, from skeletal tissues of *tl* rats and their normal littermates) as the starting material. Next, one primes reverse transcription of mRNA with oligo-dT-containing anchoring primers, which generates antisense strands of cDNA molecules from subsets of the mRNA. Then, using both the original anchoring (3') primer in combination with various, arbitrary 5' primers, the polymerase chain reaction (PCR) is performed in the presence of radiolabeled nucleotides. The resulting PCR products obtained from the test and control tissues are separated on denaturing polyacrylamide sequencing gels and autoradiographed. Bands which are differentially expressed are identified and excised. After further PCR amplification and subcloning, these bands can be used for 1) confirming positives by Southern and northern analysis; 2) sequencing; 3) probing genomic or cDNA libraries by hybridization; or 4) as templates for PCR extensions using other primer sets.

Several previous problems with DD have been addressed by technical improvements over the past few years. The single most important difficulty with DD (as is true for many screening methods) is the generation of false positives, i.e., spurious bands which do not represent genuine differentially expressed mRNAs. Several sources of these false positives can be reduced or eliminated by technical improvements (McCelland et al., 1995), and others minimized by means of careful study design. First, the use of anchoring primers which contain a single 3' anchoring nucleotide after the poly-(T), rather than pairs of nucleotides, not only reduces false positives, but also requires fewer reactions per analysis (Liang et al., 1994). Also, the inclusion of restriction sites at the 5' end of the primers greatly facilitates the subsequent cloning of bands of interest. These and other improvements are incorporated into commercially available kits, and we have found them to perform well.

E. Strategies for the use of DD to identify disease related genes in toothless (osteopetrotic) rats

DD is well-suited to the investigation of the *tl* mutation. First, *tl* is a recessive mutation, and mutant animals are unambiguously identified by postnatal X-ray. Thus, their normal littermates serve as the source of control RNA. This helps to minimize the number of uninformative bands which appear through allelic variability. Also, the partial cure of the phenotype which is obtained by CSF-1 treatment (Marks et al., 1993) provides a further means to reduce the number of bands to be investigated. Of course, a major strength of DD is that it requires far less source RNA than does subtractive hybridization, for example. This makes re-testing feasible and saves sufficient material for northern blot confirmation, both of which are key to the identification of genuine positive bands.

Table 1 shows the approach we have developed to pursue these investigations. The starting materials are pooled calvarial RNA from 2-week-old mutants and from their normal littermates. Once bands of interest have been identified (and confirmed by repeating the experiment), it is important to distinguish between genuine disease related gene expression and simple variations in the developmental timing of events in the two groups of animals. To this end, northern blots, "reverse" northern blots (Mou et al., 1994), and/or RNase protection are performed on samples from 2, 4, and 6 week-old animals. As a final step in filtering out uninformative PCR bands, RNA from mutant and normal animals is tested following treatment with CSF-1. There is residual skeletal sclerosis and deficient marrow space following treatment (Marks et al., 1993). We therefore are focusing our investigations initially on

Table 1. Strategy to identify disease-related gene expression in *toothless* (osteopetrotic) rats by mRNA differential dysplay (DD)

1. Initial identification of differentially expressed genes
2. Minimization of false-positives by repetition of DD
3. Distinguishing disease- and skeletal developmental-related genes - by Northern analysis of 2, 4 and 6 week RNA
4. Distingushing disease-related genes from those normalized by CSF-1 treatment - by DD of RNA from *toothless* rats ± CSF-1
5. Clone and sequence the remaining differentially expressed cDNAs

Table 2. Hypothetical detection of disease-related genes in *toothless* rats by mRNA differential display.

BANDS	INITIAL DD	CONFIRM BY REPETITION	TEST-AGE-RELATED EFFECTS	TEST-CSF-1 EFFECTS	MUTATION-RELATED GENE
Toothless					
A	-	-	+		
B	-	-	-	+	
C	-	-	-	-	-
Normal littermate					
A	+	+	+		
B	+	+	+	+	
C	+	+	+	+	+

mRNA species whose differential expression escapes correction by treatment with this cytokine.

The chart in Table 2 presents the deductive scheme we follow to identify bands of the greatest potential interest. There are three hypothetical bands, designated A, B and C, which are absent in *tl* mutants and present in their normal littermates' RNA. All three are confirmed in repeat experiments. When samples of RNA from different ages are tested, band A is shown to be age-related, and so is not pursued further. Likewise, band B is found to be normalized upon administration of CSF-1; therefore, while of potential interest, it is not pursued further at this time. Finally, we see that the differential expression of band C persists after CSF-1 treatment. This represents the type of band whose identity and function we intend to pursue fully.

We have begun the process of identifying differentially expressed genes in the *tl* mutation. Fig. 6 shows a typical autoradiograph obtained from the first rounds of screening. The three pairs of lanes represent side-by-side comparisons of mutant and normal animals. Each pair represents the DD products obtained with one anchoring and arbitrary primer pair. The four possible classes of results are indicated in the center lane pair, i.e., bands present only in normal, overexpressed in normal, present only in mutant, or overexpressed in mutant.

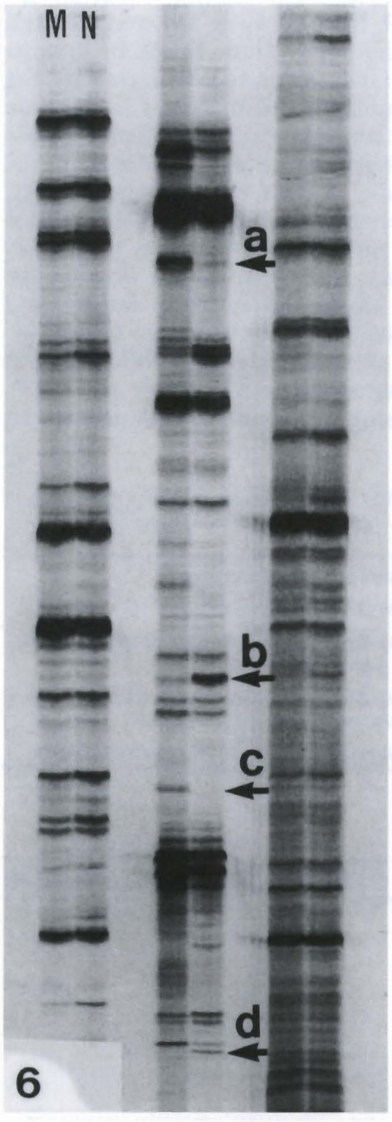

Fig. 6. Representative autoradiographs of mRNA differential display comparing *tl* mutants (M: left of each lane pair) with normal littermates (N: right of each lane pair). Each pair of lanes was generated using a different combination of anchoring and upstream PCR primers. For all three lane pairs, the anchoring primer = 5'-$HT_{11}C$-3', "H" being a HinD III restriction site. For the left lane pair, the upstream primer = 5'AAGCTTGCTGCTC-3'; for the middle pair, the upstream primer was 5'AAGCTTCAGCAGC-3"(; for the right lane pair, it was 5'AAGCTTCAGGGCA-3'. Arrows in the middle pair denote examples of differentially expressed mRNA species in *tl* vs. normals: a) overexpressed in tl; b) overexpressed in normal; c) absent from normal; and d) absent from *tl*.

In summary, investigations of the *tl* mutation have revealed a great deal about potential regulatory mechanisms which underlie normal skeletal growth and development. The defect results in a nearly complete ablation of osteoclasts (Cotton and Gaines, 1974) and in the abnormal expression of a variety of bone-specific genes (Shalhoub et al., 1991; Wisner-Lynch et al., 1995), with resulting skeletal malformation and pathology (Seifert et al., 1988). The partial rescue of the phenotype by CSF-1 treatment (Marks et al., 1993) clearly implicates this or a closely related cytokine signaling pathway in the pathogenesis of the *tl* mutant phenotype; furthermore, the failure of *tl* osteoblasts to activate normal, exogenous osteoclasts *in vitro* (Sundquist et al., 1995b) strongly implies an osteoblast signaling defect in the CSF-1 gene itself, or in the gene for a closely related cytokine. Elements in the osteoblast signaling pathway upstream of the production and secretion of CSF-1 are also possible sites for the action of this lethal mutation, including cytoplasmic signal transducers, cell surface receptors, and gene transcriptional activators. We anticipate that the DD approach described here will continue to deepen our understanding of the *tl* mutation, and in so doing will shed new light on the normal regulation of skeletal growth and maintenance.

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