wild-type ligases. Thus, the change at position 216 may determine the activation energy and whether D-alanine or D-lactate is attached to D-alanyl acylphosphate, leading to D-Ala–D-Ala and ultimate vancomycin sensitivity or to D-Ala–D-lactate and vancomycin resistance.

To the extent that **1** would be oriented as is **3** in the active site, the role of several residues in catalysis and specificity are implicated and will be subsequently tested by mutagenesis. The structure-based design of novel inhibitors should now be possible for both wild-type DD ligases and modified ligases arising under the pressure of vancomycin usage (*10*, *18*).

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19. The folded structures may differ at three points (see Fig. 2): (i) A 43-residue segment of VanA is inserted near position 52 between helix H2 and strand B3; (ii) an eight-residue insertion exists near 194 between B9 and B10; and (iii) an eight-residue deletion results in the loss of surface helix H7. The first two insertions may appear in VanA as surface segments between rigid secondary structures.
20. Our alignment of the sequences containing the H9 loop, which differs slightly from other alignments (*16*, *21*), is

DdlA	YAYDTKYIDED
DdlB	YDYEAKYLSDE (220)
VanA	QEVEPEKGSEN
VanC	FDFEEKYQLIS

(Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.) While the EKY sequence of VanC could match either the EK of VanA or the KY of DdlA and DdlB, other matches favor the latter. Further, the existence of KY in VanC is compatible with our mechanism for peptide synthesis in DdlB, because the VanC ligase produces a peptide, D-Ala–D-Ser, rather than a depsipeptide (*21*).
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29. The DD-ligase, purified as described (*6*), was co-crystallized with ATP and **2** by the hanging drop method. The drop contained enzyme (10 mg/ml) in the presence of 5 mM ATP and 5 mM **2** in 20% (v/v) ammonium sulfate at pH 7.0 in Hepes buffer with 1 mM $MgCl_2$ and NaN_3. The drop was suspended over 45% (v/v) ammonium sulfate at pH 5.5 in MES buffer. Crystals up to 0.6 by 0.4 mm grew over a few weeks in space group $P2_12_12$, with one molecule in the asymmetric unit. Cell dimensions are a = 99.32 Å, b = 51.44 Å, and c = 51.22 Å. Diffraction data were collected on a Siemens area x-ray detector with a Rigaku rotating anode x-ray generator operating at 38 kV and 180 mA with graphite-monochromatized Cu-Kα radiation. Data reduction was done with the program XENGEN (*22*). Phases were obtained by the method of multiple isomorphous replacement with anomalous scattering by use of the program PHASIT (*23*). With seven derivatives contributing to 3.0 Å resolution, the mean figure-of-merit for 5512 reflections with $F > 0$ was 0.86. Fitting of the map with all 306 residues was done with CHAIN (*24*) and FRODO (*25*). The R factor for a starting model, exclusive of ADP and **3**, was 0.43 to 3 Å resolution. When an initial refinement with X-PLOR (*26*) reached R = 0.24 for 9501 reflections [$F > 3\sigma(F)$] from 10.0 to 2.3 Å resolution, ADP and **3** were fitted into a $2F_o - F_c$ map. Continued refinement with PROLSQ (*27*) with the addition of 292 water molecules produced a final R factor of 0.172 for 9776 $3\sigma(F)$ data from 15.0 to 2.3 Å. Root-mean-square deviations of bonds and angles from ideal values are 0.013 Å and 1.7°, respectively. Coordinates have been deposited in the Protein Data Bank at the Brookhaven National Laboratory, Upton, NY (entry 1DLN).

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c-Fos: A Key Regulator of Osteoclast-Macrophage Lineage Determination and Bone Remodeling

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Mice lacking the proto-oncogene c-*fos* develop the bone disease osteopetrosis. Fos mutant mice were found to have a block in the differentiation of bone-resorbing osteoclasts that was intrinsic to hematopoietic cells. Bone marrow transplantation rescued the osteopetrosis, and ectopic c-*fos* expression overcame this differentiation block. The lack of Fos also caused a lineage shift between osteoclasts and macrophages that resulted in increased numbers of bone marrow macrophages. These results identify Fos as a key regulator of osteoclast-macrophage lineage determination in vivo and provide insights into the molecular mechanisms underlying metabolic bone diseases.

The c-Fos oncoprotein is a component of the AP-1 transcription factor complex and is a member of a multigene family, including the *fos*-related (*fosB*, *fra-1*, *fra-2*) and *jun*-related (c-*jun*, *junB*, *junD*) genes (*1*). The function of Fos is dependent on formation of heterodimers with specific Jun proteins, but also with other leucine zipper–containing molecules, and subsequent binding to specific regulatory sequences (*1*). Because different dimer combinations can confer AP-1–dependent gene transcription in different cell types, it is a challenge to identify the biological processes in which the individual AP-1 family members are essential molecules, as opposed to those in which specific gene function may be redundant.

Overexpression of c-*fos* in transgenic and chimeric mice causes osteosarcomas and chondrosarcomas through mechanisms that involve specific transformation of osteogenic and chondrogenic cells, respectively (*2*, *3*). Mice lacking Fos develop osteopetrosis as a primary pathology and also exhibit altered hematopoiesis (*4*). The hematopoietic defects are secondary to the altered bone environment, because Fos mutant hematopoietic stem cells are capable of reconstituting lethally irradiated wild-type mice (*5*). However, the causal role of Fos in osteopetrosis is not yet known.

Mammalian osteopetroses are disorders of bone metabolism characterized by a reduction in osteoclast function, resulting in a net increase in skeletal mass (*6*). Bone remodeling is a dynamic process whereby osteoclastic bone resorption is tightly coupled to osteoblastic bone formation (*7*, *8*).

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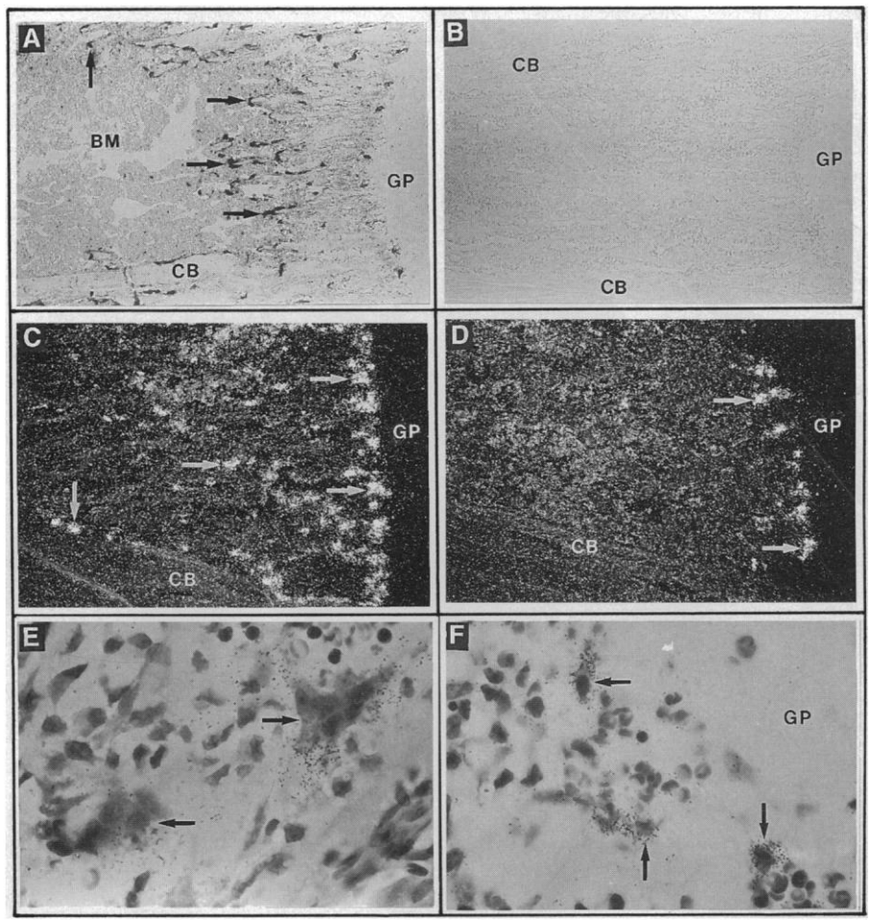

Fig. 1. Identification of osteoclasts by TRAP histochemistry and in situ hybridization for 92-kD type IV collagenase (gelatinase B) in long bones of wild-type mice (**A**, **C**, and **E**) and Fos mutant (**B**, **D**, and **F**) littermates (*24*). TRAP-positive osteoclasts are observed lining the bone surfaces in the proximal tibiae of 10-day-old wild-type mice [arrows in (A)], whereas Fos mutant bones lack TRAP-positive cells (B). Dark-field micrograph showing 92-kD type IV collagenase expression along the bone and growth plate surfaces of proximal tibiae in 2-week-old wild-type [arrows in (C)] and Fos mutant [arrows in (D)] mice. Higher magnification bright-field micrographs showing that type IV collagenase is expressed in multinucleated osteoclasts of wild-type mice [arrows in (E)], but only in mononuclear cells of mutant mice [arrows in (F)]. BM, bone marrow; CB, cortical bone; GP, growth plate. Magnification: (A to D), ×100; (E and F), ×630.

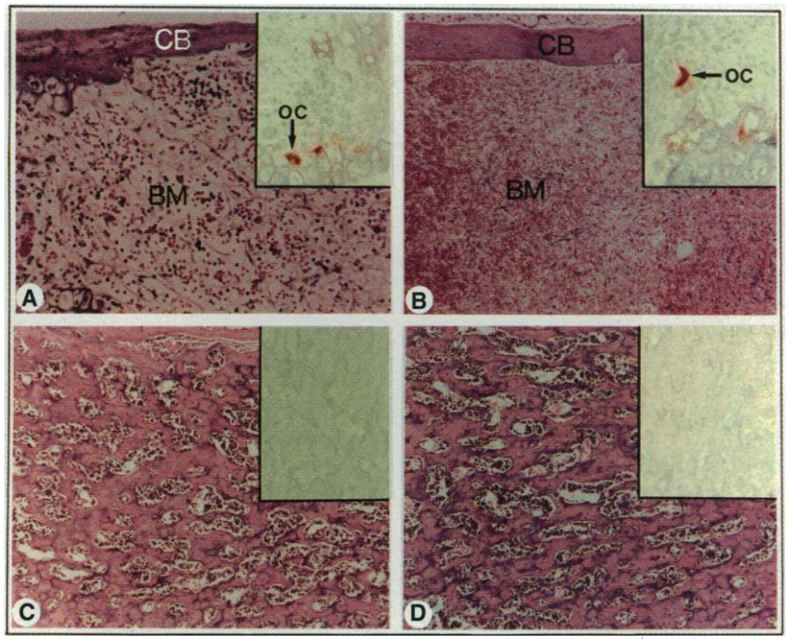

Fig. 2. Bone development after transplantation of embryonic limbs under kidney capsules (*26*). Limb rudiments from wild-type (**A**) and mutant (**B**) mouse embryos (embryonic day 13 to 13.5) transplanted under kidney capsules of wild-type mice develop into normal long bones containing osteoclasts. In contrast, wild-type (**C**) and mutant (**D**) limb rudiments transplanted under kidney capsules of mutant mice develop abnormally into osteopetrotic long bones lacking osteoclasts. Insets to each figure show the presence (A and B) and absence (C and D) of osteoclasts as demonstrated by TRAP histochemistry. CB, cortical bone; BM, bone marrow; OC, osteoclasts. Magnification: (A to D), ×200.

Whereas osteoblasts are derived from mesenchymal progenitor cells and are related to other mesenchymal cell types (*9*), osteoclasts are derived from hematopoietic (myeloid) progenitors that differentiate through committed osteoclast progenitors and postmitotic osteoclast precursors before fusing to form mature multinucleated cells (*8*, *10*, *11*). Although osteoclasts are highly related to mononuclear phagocytes (monocytes and macrophages), the exact lineage from which osteoclast progenitor cells are derived and the molecules responsible for lineage restriction to osteoclasts are not yet defined. Both natural and experimental mammalian osteopetrotic mutants have shown that abnormalities in either osteoclast differentiation and activity or in the bone marrow environment (for example, osteoblasts and stromal cells) can cause osteopetrosis (*6*, *12*). Osteopetrotic Fos mutant mice provide a model system in which to investigate the biological role of this transcription factor in osteoclast development and bone remodeling.

Tartrate-resistant acid phosphatase (TRAP), present in postmitotic osteoclast precursors and in differentiated multinucleated osteoclasts, and 92-kD type IV collagenase (gelatinase B) are specific in vivo markers for the osteoclast lineage (*13*). Fos

mutant long bones are devoid of TRAP-positive cells (Fig. 1B). In contrast, type IV collagenase was present in mutant bones, but in fewer cells (Fig. 1D). Closer examination revealed that type IV collagenase was expressed only on mononuclear cells in mutant mice, in contrast to wild-type mice where both mononuclear cells and multinucleated osteoclasts stained positively (Fig. 1, E and F). These results suggest that early osteoclast progenitors that are not yet expressing TRAP are present in the mutant bones, but differentiated osteoclasts are absent.

We next investigated whether the absence of osteoclasts was due to an intrinsic defect in the osteoclast lineage or to an altered osteoblastic or stromal environment. It is known that transplantation of day 13 to 13.5 embryonic limbs under the kidney capsule of adult hosts mimics a normal sequence of limb development, involving the interaction between mesenchymal cells (including osteoblastic cells) from the donor limbs and hematopoietic cells (including osteoclast progenitor cells) from the vascularized microenvironment of the host kidney capsule. Both wild-type and mutant limbs that were transplanted under kidney capsules of normal mice developed into long bones containing a well-formed bone marrow cavity, cortical surface, cartilagenous growth plate, and TRAP-positive multinucleated osteoclasts (Fig. 2, A and B). In contrast, wild-type and mutant limbs transplanted under kidney capsules of mutant mice did not develop a bone marrow cavity, and no cells were TRAP-positive (Fig. 2, C and D). These results suggest that whereas mutant osteoblasts and stromal cells present in the grafts are fully capable of supporting normal osteoclast development and differentiation, hematopoietic cells lacking Fos are unable to differentiate into functional osteoclasts in vivo.

To verify further that the osteoclast defect lies within the hematopoietic cell compartment and not in the stromal environment, we transplanted normal bone marrow cells into lethally irradiated newborn mutant mice (6, *14*). In two independent experiments, we obtained two homozygous Fos mutants in which the osteopetrosis was rescued. X-ray analysis of a rescued mutant mouse revealed complete eruption of incisors as well as the development of a bone marrow cavity, similarly to a normal transplanted littermate but unlike that of a control Fos osteopetrotic mouse (Fig. 3A). Histological analysis demonstrated that the calcified bone and cartilage present in the bone marrow space of osteopetrotic mice was removed in the rescued mouse, resulting in a normal marrow cavity and a well-developed cortical bone. Thus, it appears that the wild-type bone marrow transplants provide osteoclast progenitors that differentiate into functional osteoclasts and restore bone resorption.

To test whether the defective osteoclast progenitor cells can transfer the bone disease in reconstituted mice, we injected Fos mutant hematopoietic progenitor cells into irradiated newborn wild-type mice. Splenocytes were used as donor cells because it is difficult to obtain hematopoietic cells from osteopetrotic bones and because mutant spleen cells are able to reconstitute lethally irradiated wild-type mice (5). After 3 weeks we observed a defect in bone resorption specifically at anatomical sites undergoing active bone remodeling. Bone accumulated along the metaphyses with a complete absence of the secondary ossification centers, another characteristic trait of osteopetrosis (Fig. 3B). Thus, the induction of bone resorption defects further proves that functional osteoclasts cannot be generated from Fos mutant hematopoietic cells.

Bone-resorbing, multinucleated osteoclasts can differentiate in vitro from hematopoietic progenitor cells present in spleen cell suspensions when cocultured with primary osteoblastic cells (*15*). Calvarial osteo-

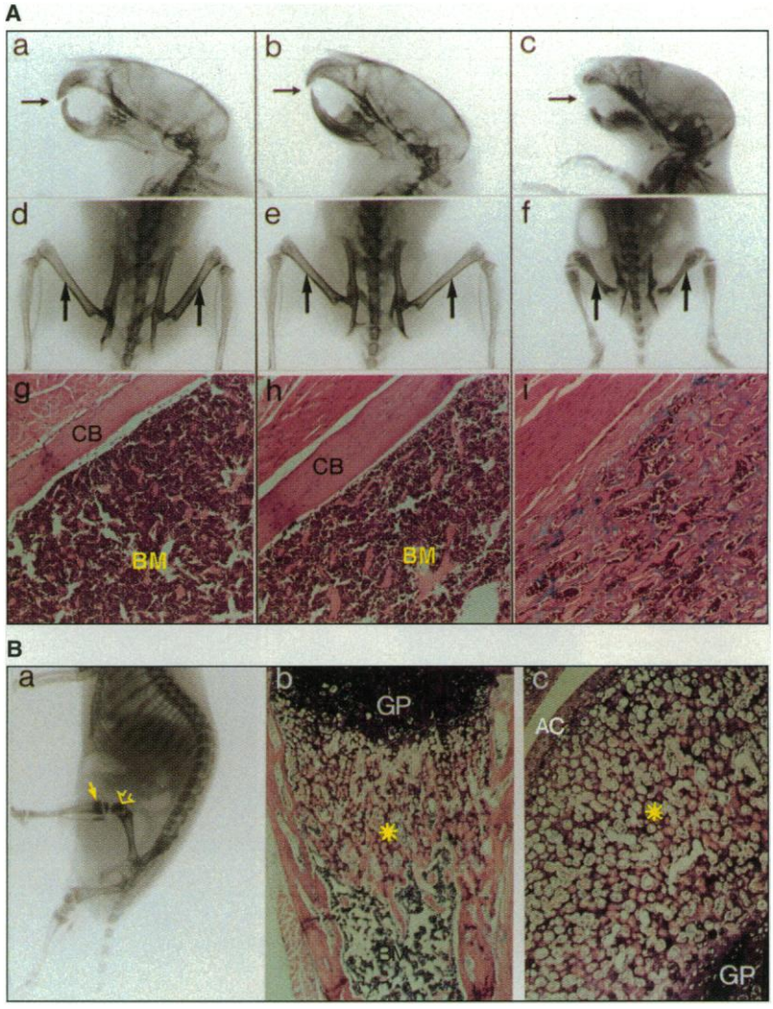

Fig. 3. Bone marrow transplantation into newborn mice. (**A**) X-ray and histological analyses of a 6-week-old Fos mutant mouse (a, d, and g) and a normal control littermate (b, e, and h), both of which were lethally irradiated and received wild-type bone marrow cells at birth (*27*). A control Fos osteopetrotic mouse is also shown (c, f, and i). The rescued mutant mouse exhibits complete tooth eruption and a clear bone marrow cavity [arrows in (a) and (d)], similarly to the normal control littermate [arrows in (b) and (e)] and different from a control osteopetrotic mouse [arrows in (c) and (f)]. Magnification: (a to f), ×3.5; (g to l), ×200. (**B**) X-ray and histological analyses of a wild-type mouse, 3 weeks after lethal irradiation and transplantation with mutant spleen cells at birth. Note the radio-dense lesions in the metaphyses and epiphyses of the long bones [arrows in (a)]. (b) High magnification of the area marked by the closed arrow in (a) demonstrating the accumulation of bone and cartilage trabeculae along the metaphysis of the proximal tibia [asterisk in (b)]. (c) High magnification of the area marked by the open arrow in (a) showing the absence of the secondary ossification center in the distal femur [asterisk in (c)], which normally contains a bone marrow space. CB, cortical bone; BM, bone marrow; GP, growth plate; AC, articular cartilage. Magnification: (a), ×3; (b), ×100; (c), ×200.

blasts derived from both wild-type and mutant mice supported TRAP-positive multinucleated cell formation equally well in cultures with normal spleen cells (Fig. 4A). These TRAP-positive multinucleated cells also expressed calcitonin receptors (*16*), another specific osteoclast marker, and could form resorption pits on ivory slices, suggesting that they were bona fide osteoclasts (Fig. 4A). These results indicate that osteoblasts lacking Fos are not affected in their ability to support osteoclastogenesis. In contrast, no TRAP-positive cells (Fig. 4A) or calcitonin receptor–positive cells (*16*) were detected when spleen cells from Fos mutant mice were cultured with osteoblasts from wild-type or mutant mice, which shows that mutant hematopoietic progenitor cells are unable to differentiate into osteoclasts.

To confirm that the block in osteoclast differentiation was due to the absence of Fos itself, rather than being mediated by other factors present in vivo, we infected mutant spleen cells in vitro with a c-*fos*–expressing retrovirus before culture with osteoblastic cells. In contrast to uninfected mutant spleen cells that do not form TRAP-positive cells, c-*fos*–infected mutant spleen cells were able to form TRAP-positive osteoclasts (Fig. 4B), which could form resorption pits when cultured on ivory slices (*17*). Thus, exogenous c-*fos* expression in mutant hematopoietic progenitor cells can overcome the block in osteoclast differentiation in vitro.

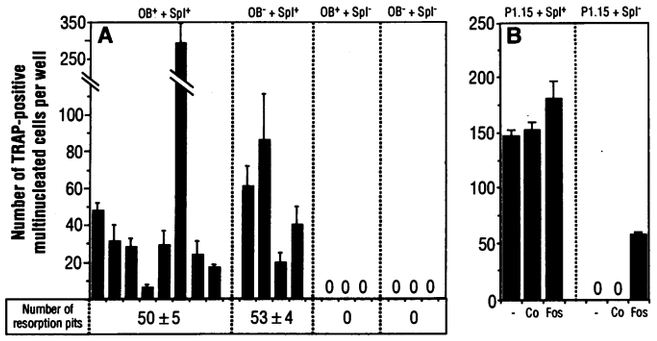

Fig. 4. Formation of TRAP-positive multinucleated cells after coculture of spleen cells with osteoblastic cells from wild-type and Fos mutant mice. (**A**) Primary osteoblasts isolated from newborn wild-type mice (OB^+) or Fos mutant mice (OB^-) were cultured with spleen cells isolated from either wild-type (Spl^+) or mutant (Spl^-) mice (*28*). Each bar represents the mean ± SEM of the number of TRAP-positive multinucleated cells formed in quadruplicate cultures from individual mice. The numbers below each panel show the number of resorption pits formed in ivory slices by osteoclasts generated in each culture. The data represent the mean ± SEM of eight individual ivory slices. (**B**) P1.15 osteoblastic cells (*3*) were cultured with spleen cells that were infected with either no virus (−), a control virus (Co), or a c-*fos*–expressing virus (Fos). The number of TRAP-positive cells was counted after 8 days in culture. The data represent the mean ± SEM of quadruplicate cultures from a representative experiment.

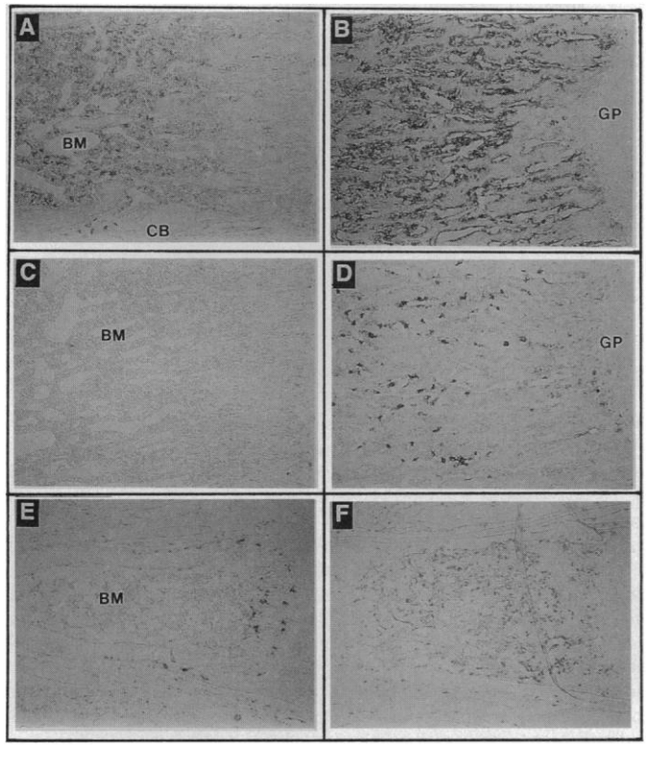

Fig. 5. Fos mutant long bones contain greater numbers of macrophages. Immunohistochemistry and in situ hybridization analysis of expression of macrophage markers in wild-type (**A**, **C**, and **E**) and Fos mutant (**B**, **D**, and **F**) bones (*29*). Immunohistochemical staining for F4/80 antigen (A and B) and Mac-2 antigen (C and D) in distal tibiae from 10-day-old mice. In situ hybridization analysis for c-*fms* (CSF-1R) expression in metatarsals from 10-day-old mutant (E) and control (F) mice. BM, bone marrow; CB, cortical bone; GP, growth plate. Magnification: (A) to (F), ×100.

To determine whether other osteoclast-related cell types, such as macrophages, were affected in Fos mutant bones, we used several macrophage markers, namely, F4/80, Mac-2, and CSF-1 receptors (CSF-1R) (*18*). The number of F4/80- and Mac-2–positive cells was increased in Fos mutant long bones compared to controls (Fig. 5, A to D). Similarly, a high number of mononuclear cells in mutant bones expressed CSF-1R, as judged by expression of the proto-oncogene c-*fms* that encodes CSF-1R (Fig. 5, E and F); radioligand binding experiments indicated that CSF-1 binding paralleled CSF-1R expression (*16*). In contrast to the increased numbers of terminally differentiated macrophages, there were no differences in the number of cells expressing ER-MP58, a marker for CSF-1–responsive macrophage progenitors (*16*, *18*). Finally, no differences were observed in the number of F4/80-positive macrophages in the liver (Kupffer cell density of 795 per square millimeter and 774 per square millimeter in wild-type and Fos mutant livers, respectively). These results indicate that although putative macrophage progenitors are present in normal amounts in Fos mutant bones, the density of tissue macrophages increased in bone marrow, which is not observed in extramedullary tissues. Macrophages isolated from Fos mutant bones that stained positively for F4/80 antigen retained their ability to phagocytose latex beads in culture, indicating that they were functional (*17*).

The transcription factor c-Fos appears to have divergent effects on two cell lineages with a common origin. Mice lacking Fos are devoid of differentiated, multinucleated osteoclasts and, as a consequence, develop the bone disease osteopetrosis. The bone marrow of these mice, however, contained a greater number of tissue macrophages. The osteoclast defect was due to the absence of Fos itself because gene transfer in vitro rescued osteoclast differentiation, and preliminary data show that the expression of a c-*fos* transgene (*3*) can partially rescue the osteopetrosis in ~1-year-old Fos mutant mice (*17*).

The effect of Fos on osteoclast differentiation is unexpected given that c-*fos* overexpression in transgenic and chimeric mice transforms osteoblasts and chondroblasts rather than hematopoietic cells (*2*, *3*). The transformable cells probably express the appropriate cooperating genes that are necessary for transformation and subsequent tumor formation, in contrast to osteoclasts. Thus, the transforming ability of exogenous Fos does not necessarily reflect the true

function of the endogenous c-*fos* gene in vivo. Our data strongly indicate that whereas the putative role of Fos in some specific osteoblastic functions can be substituted by other *fos*- or *jun*-related genes, Fos function in osteoclast progenitors cannot be substituted in vivo by other AP-1 family members.

The precise lineage relationships between osteoclasts and macrophages are poorly characterized, although some in vivo evidence for a common progenitor cell for these cell types has been provided by the *op/op* osteopetrotic mouse mutant in which lack of CSF-1 causes deficiencies in both osteoclasts and tissue macrophages (*8*). Macrophage cell lines and human osteoclasts both express c-*fos* (*19*), which further suggests that Fos may be an additional regulatory factor in these cells, similarly to the zinc finger transcription factor, Egr-1, that is essential for lineage restriction to macrophages (*20*). Several explanations could account for why Fos mutant bones contain more macrophages. First, the abnormal bone marrow environment caused by the osteopetrosis may have indirectly induced macrophage differentiation, perhaps to compensate for the lack of osteoclasts. This is unlikely because other osteopetrotic mutants, such as *mi/mi* and mice lacking c-*src*, do not exhibit specific increases in resident bone marrow macrophages (*16*, *18*). Second, under certain conditions, terminally differentiated macrophages can also form osteoclasts in vitro (*11*); thus, the absence of Fos may block the differentiation of macrophages to osteoclasts, resulting in the accumulation of macrophages. Third, although the latter possibility cannot be excluded, it is likely that Fos affects earlier progenitor cells, that is, committed osteoclast and macrophage progenitors, putative bipotential progenitor cells, or both. Whether the increase in macrophages in Fos mutant bones is a direct result of the absence of Fos or a consequence of the block in osteoclast differentiation is not clear. Nevertheless, the mechanism is likely to involve CSF-1R, which is required for proliferation, differentiation, and survival of cells of the mononuclear phagocyte system, including osteoclasts (*11*, *18*, *21*). We propose that aside from having more mature macrophages in Fos mutant bones, there exists in addition a subpopulation of osteoclast-macrophage progenitor cells that cannot differentiate further in the absence of Fos. The presence of such progenitors is likely in view of the recent evidence that Mac-2 antigen is also expressed in pre-osteoclasts (*22*). Finally, we cannot exclude the possibility that such progenitors may also require Fos for normal proliferation.

The Fos osteopetrosis mutation is different from other mammalian osteopetrotic mutants in that it is the only example in which a cell-intrinsic defect is due to a complete absence of mature osteoclasts and which is accompanied by alterations in the number of tissue macrophages. Therefore, these mice provide an opportunity to dissect the role of Fos as a transcriptional regulator of osteoclast and macrophage differentiation and proliferation. Our results provide a demonstration that a single member of the AP-1 transcription factor complex can determine the differentiation of specific cell types in vivo. In view of the increasing number of molecules that are being shown to dimerize with Fos and other AP-1 proteins, our findings will aid in identifying the Fos-responsive genes involved in osteoclast and macrophage differentiation and will provide new insights into the molecular basis of metabolic bone diseases.

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24. Mice were killed either by cervical dislocation and the bones fixed immediately in fresh 3.7% formaldehyde or by fixation under anesthesia by perfusion with periodate-lysine-2% paraformaldehyde-0.05% glutaraldehyde (PLPG) as described (*18*). Bones were then demineralized, dehydrated, and embedded in paraffin. For identification of osteoclasts, sections were stained for TRAP in the presence of 100 mM sodium tartrate (*23*). For in situ hybridization, we used a 323–base pair Sma I–Eco RI fragment from the murine 92-kD type IV collagenase complementary DNA (cDNA) (*13*). Preparation of sense and antisense riboprobes and hybridization conditions were as described (*4*). Exposure times were 3 to 4 days, and all hybridizations with sense riboprobes did not show any specific signals. All specimens are 5-μm paraffin sections stained with hematoxylin and eosin.
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26. Rudiments of femors and humeri were removed from 13.5-day wild-type and Fos mutant embryos and were dissected free of skin and connective tissue. Wild-type limbs were transplanted under the kidney capsules of 6-week-old syngeneic Fos mutant mice, and mutant limbs were transplanted under the kidney capsules of wild-type mice, similarly to the method of K. Kratochwil *et al.* [*Cell* **57**, 807 (1989)]. Each transplant contained one femur and one humerus grafted together under each kidney capsule. All transplants developed into limbs after 2 to 3 weeks, after which they were analyzed by histology and TRAP histochemistry as described (*24*). All specimens are 5-μm paraffin sections stained with hematoxylin and eosin.
27. Newborn mice obtained from intercrosses of c-*fos*$^{+/-}$ mice were lethally irradiated [9.5 gray (Gy)] and injected intraperitoneally with 0.5×10^8 to 1×10^8 wild-type bone marrow cells in a volume of 50 μl of phosphate-buffered saline. At 10 days of age, mice were genotyped by Southern (DNA) blot analysis of tail DNA (*4*). In two independent experiments, we have obtained two homozygous Fos mutants from 23 mice transplanted, both of which exhibited tooth eruption between 13 and 20 days of age. The delay in tooth eruption (~13 to 20 days versus ~10 days for normal littermates) is probably due to the insufficient time that the osteoclasts derived from transplanted progenitors had to resorb the mandibular and maxillary bone and allow tooth eruption. The low frequency of homozygous Fos mutants obtained from heterozygote intercrosses is expected on the basis of previous observations (*4*). For the induction of osteopetrosis, newborn wild-type mice were lethally irradiated (9.5 Gy) and injected with 0.5×10^8

to 1 × 10^8 spleen cells from Fos mutant mice. Transplanted mice were analyzed radiographically and histologically after 3 weeks, and the presence of donor hematopoietic cells in the irradiated recipients was confirmed by Southern blot analysis of hematopoietic tissues (*4*, *5*). All specimens are 5-μm paraffin sections stained with hematoxylin and eosin.

28. Primary osteoblasts were isolated from calvaria of 3- to 5-day-old mice with a sequential collagenase digestion procedure (*25*). For coculture experiments, primary osteoblasts (10^4 cells per 16-mm well) and spleen cells (5 × 10^5 cells per 16-mm well) from either wild-type or Fos mutant mice were cultured in α–minimum essential medium containing 10% fetal bovine serum and 10^{-8} M 1,25-dihydroxyvitamin D_3 [1,25-$(OH)_2D_3$] (*15*). For virus infection experiments, P1.15 osteoblastic cells (*3*) (10^5 cells per 16-mm well) were cultured with either wild-type or Fos mutant spleen cells (10^6 cells per 16-mm well) that had been infected previously in suspension with either a control virus (pMV7) or a c-*fos*–expressing virus (pMV-c-*fos*) for 6 hours at 37°C. Cells were cultured in the presence of 10^{-8} M 1,25-$(OH)_2D_3$ and 10^{-7} M Dexamethasone. After 8 days in culture the cells were fixed in 3.7% formaldehyde and stained for TRAP with a commercially available kit (Sigma) in the presence of 50 to 100 mM sodium tartrate. TRAP-positive cells containing three or more nuclei were counted as osteoclasts. Osteoclast resorption activity was measured by plating of primary calvarial osteoblasts (5.6 × 10^3 cells per 6-mm well) and spleen cells (7 × 10^4 cells per 6-mm well) from either wild-type or Fos mutant mice, with slices of ivory as a mineral substrate. After 8 days in culture in the presence of 10^{-8} M 1,25-$(OH)_2D_3$, resorption pits were quantified as described previously (*25*).

29. Tissue specimens were prepared as described (*24*). Immunostaining for F4/80 antigen and Mac-2 antigen (clone M3/38, Boehringer Mannheim) was done according to the indirect peroxidase-conjugated streptavidin procedure (*25*). In situ hybridization was done with sense and antisense riboprobes synthesized from a cDNA for murine c-*fms* (*11*). Exposure times were 3 to 4 days, and all hybridizations with sense riboprobes did not show any specific signals.

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Differential Regulation of RNA Polymerases I, II, and III by the TBP-Binding Repressor Dr1

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RNA polymerases I, II, and III each use the TATA-binding protein (TBP). Regulators that target this shared factor may therefore provide a means to coordinate the activities of the three nuclear RNA polymerases. The repressor Dr1 binds to TBP and blocks the interaction of TBP with polymerase II– and polymerase III–specific factors. This enables Dr1 to coordinately regulate transcription by RNA polymerases II and III. Under the same conditions, Dr1 does not inhibit polymerase I transcription. By selectively repressing polymerases II and III, Dr1 may shift the physiological balance of transcriptional output in favor of polymerase I.

Three RNA polymerases (Pols) are responsible for the transcription of nuclear genes. Although much has been learned about how these polymerases are individually controlled, little is known about how they are regulated with respect to each other. Coordination of the three polymerases must be important for cellular metabolism, because unbalanced activity would be wasteful. An obvious way to coordinate the regulation of the polymerases would be to regulate shared components. A strong candidate for such regulation is TBP, which is used by RNA Pols I, II, and III (*1*).

Although TBP is used by all three polymerases, it is assembled into polymerase-specific complexes (*1*). These complexes are called SL1 for the Pol I system (*2*–*4*), TFIID for the Pol II system (*5*), and TFIIIB for the Pol III system (*6*–*11*). TFIID can nucleate transcription complex formation at Pol II promoters (*12*). Recruitment of polymerase to this complex requires TFIIB, which binds directly to both TBP and Pol II (*12*). TFIIIB contains a polypeptide called TFIIB-related factor (BRF; also known as TDS4 or PCF4), which is homologous to TFIIB and binds to TBP and Pol III (*13*). TFIIB and BRF therefore perform analogous functions in directing Pol II or Pol III, respectively, to the appropriate complexes. It is not yet clear whether the Pol I system also contains a TFIIB-like factor.

The repressor Dr1 inhibits Pol II transcription by binding directly to TBP and blocking its interaction with TFIIB (*14*). We tested whether Dr1 could also regulate Pol III activity. Transcription of the $tRNA^{Glu6}$ gene was repressed by Dr1 that had been substantially purified from HeLa cells (hDr1) (*15*) (Fig. 1A). Furthermore, ~90% pure recombinant Dr1 (rDr1) (*15*) also inhibited expression of this gene (Fig. 1B) and that of other Pol III templates, including the VA_I (Fig. 1C) and U6 genes (*16*). This was not a nonspecific response to added protein, because the corresponding control fraction from bacteria that lacked the Dr1 expression vector (*15*) had little effect (Fig. 1C). Dr1 can repress transcription of Pol III templates by as much as 70-fold. Indeed, it inhibited $tRNA^{Glu6}$ transcription as much as it inhibited that of the Pol II template G_6TI (Fig. 1B). α-Amanitin treatment confirmed the polymerases responsible for this transcription (*16*). Repression still occurred if the Pol III factors were preassembled on the VA_I gene (*16*). Clearly,

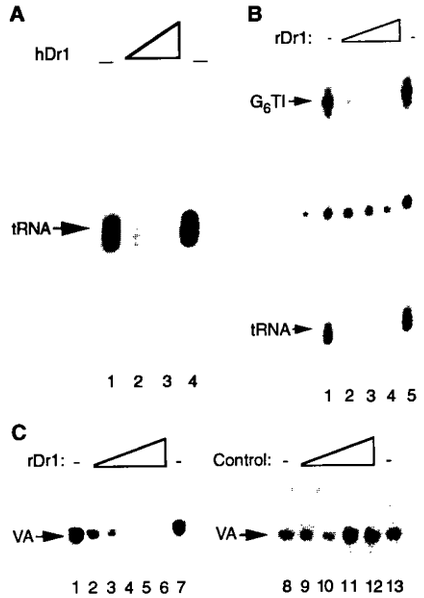

Fig. 1. Dr1 is a potent repressor of Pol III transcription. (**A**) Fractionated factors (*21*) were preincubated without hDr1 (lanes 1 and 4) or with 2 μl (lane 2) or 4 μl (lane 3) of hDr1. pGlu6 (500 ng) (*22*) was added with nucleotides to begin transcription (*23*). (**B**) Nuclear extract (30 μg) (*21*) was preincubated with 100 ng of pG_6TI (*22*) and 100 ng of pGlu6 either without (lanes 1 and 5) or with 1.5 μl (lane 2), 3 μl (lane 3), or 5 μl (lane 4) of rDr1. Nucleotides were added to begin transcription. The asterisk reflects template-independent end-labeling of endogenous small RNAs. (**C**) Fractionated factors (*21*) were preincubated with the following amounts of bacterially expressed protein (*15*): lanes 1, 7, 8, and 13, none; lane 2, 0.5 μl of rDr1; lane 3, 1 μl of rDr1; lane 4, 2 μl of rDr1; lane 5, 4 μl of rDr1; lane 6, 8 μl of rDr1; lane 9, 1 μl of bacterial control; lane 10, 2 μl of control; lane 11, 4 μl of control; and lane 12, 8 μl of control. pVA_I (500 ng) (*22*) was added with nucleotides to begin transcription.

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