Osteoblasts Are Target Cells for Transformation in c-*fos* Transgenic Mice

Agamemnon E. Grigoriadis, Karl Schellander, Zhao-Qi Wang, and Erwin F. Wagner

Research Institute of Molecular Pathology, Dr. Bohr-Gasse 7, A-1030, Vienna, Austria

Abstract. We have generated transgenic mice expressing the proto-oncogene c-*fos* from an H-2K^b class I MHC promoter as a tool to identify and isolate cell populations which are sensitive to altered levels of Fos protein. All homozygous H2-c-*fos*LTR mice develop osteosarcomas with a short latency period. This phenotype is specific for c-*fos* as transgenic mice expressing the *fos*- and *jun*-related genes, *fos*B and c-*jun*, from the same regulatory elements do not develop any pathology despite high expression in bone tissues. The c-*fos* transgene is not expressed during embryogenesis but is expressed after birth in bone tissues before the onset of tumor formation, specifically in putative preosteoblasts, bone-forming osteoblasts, osteocytes, as well as in osteoblastic cells present within the tumors.

Primary and clonal cell lines established from c-*fos*–induced tumors expressed high levels of exogenous c-*fos* as well as the bone cell marker genes, type I collagen, alkaline phosphatase, and osteopontin/2ar. In contrast, osteocalcin/BGP expression was either low or absent. All cell lines were tumorigenic in vivo, some of which gave rise to osteosarcomas, expressing exogenous c-*fos* mRNA, and Fos protein in osteoblastic cells. Detailed analysis of one osteogenic cell line, Pl, and several Pl-derived clonal cell lines indicated that bone-forming osteoblastic cells were transformed by Fos. The regulation of osteocalcin/BGP and alkaline phosphatase gene expression by 1,25-dihydroxyvitamin D_3 was abrogated in Pl–derived clonal cells, whereas glucocorticoid responsiveness was unaltered. These results suggest that high levels of Fos perturb the normal growth control of osteoblastic cells and exert specific effects on the expression of the osteoblast phenotype.

THE proto-oncogene c-*fos* is the cellular homologue of the v-*fos* oncogene which was first detected as the transforming gene of the FBJ- and FBR-murine sarcomas viruses (MSVs)[1] (Finkel et al., 1966, 1975). As a member of the AP-1 transcription factor complex the Fos oncoprotein has been implicated as a key molecule in cell proliferation and signal transduction as well as in regulating gene transcription (for review see Curran 1988; Bravo, 1990; Angel and Karin, 1991). Transcriptional regulation by Fos and *fos*-related genes (*fos*B, *fra*-1, and *fra*-2) involves formation of heterodimeric complexes with members of the *jun* family of proto-oncogenes (c-*jun*, *jun*B, *jun*D) and subsequent binding to AP-1 consensus sequences in the regulatory regions of target genes (Lee et al., 1987; Chiu et al., 1988; Sassone-Corsi et al., 1988; Kouzarides and Ziff, 1989; Ransone and Verma, 1990; Angel and Karin, 1991).

The regulation of c-*fos* expression is quite complex as many factors have been shown to cause both transient activation and repression of c-*fos* transcription (Treisman, 1985; Shaw et al., 1989; Lucibello et al., 1989; see also Ovitt and Rüther, 1990 for review). Despite this apparent complexity, stable expression of endogenous c-*fos* has been documented in vivo in different mouse tissues. For example, expression in the adult animal has been observed in haematopoietic cells, germ cells, in the central nervous system, and in bone (Müller et al., 1984; Morgan et al., 1987; Pelto-Huikko et al., 1991; Smeyne et al., 1992; Cohen et al., 1993). During embryogenesis c-*fos* is expressed during mid to late gestation, specifically in the central nervous system and in the growth regions of fetal bones, cartilage, and developing teeth (Dony and Gruss, 1987; De Togni et al., 1988; Sandberg et al., 1988; Caubet and Bernaudin, 1988; Caubet et al., 1989). The association between *fos* expression and bone-forming cells is of specific interest as evidenced by the observations that c-*fos* is expressed at high levels in murine (Schön et al., 1986) and human osteosarcomas (Wu et al., 1990). Moreover, bone formation in in vitro organ culture systems and during fracture healing is preceded by c-*fos* expression (Closs et al., 1990; Birek et al., 1991; Ohta et al., 1991). In addition, v-*fos* induces chondro-osseous neoplasms in

1. *Abbreviations used in this paper*: ALP, alkaline phosphatase; Dex, dexamethasone; ES, embryonic stem; GRE, glucocorticoid response element; MSV, murine sarcoma virus; OC, osteocalcin/BGP; 1,25-$(OH)_2D_3$, 1,25-dihydroxy-vitamin D_3; OP, osteopontin/2ar; VDRE, vitamin D_3 responsive element.

neonatal mice (Ward and Young, 1976) and transforms osteogenic cells in tissues undergoing osteogenesis in vitro (Schmidt et al., 1986). Although these data suggest that Fos protein may have a specific function in osteogenic cells, its specific role in normal bone formation and its causal role in bone neoplasia cannot be established by these studies.

Despite the numerous studies investigating the regulation of bone cell function, the molecular basis of osteoblast commitment and differentiation, and the factors which regulate osteoblast gene expression are not fully understood. The use of primary, transformed, and immortalized osteoblastic cell populations has indicated that the regulation of the osteoblast phenotype by systemic hormones and local factors is very complex (Rodan and Rodan, 1984; Nijweide et al., 1986; Heath et al., 1989; Guenther et al., 1989; Kellermann et al., 1990; Heersche and Aubin, 1990; Aubin et al., 1992). Osteoblasts comprise a heterogeneous group of cells and definitive markers for different stages of osteoblast differentiation are not yet known. Nevertheless, cells in the osteoblastic lineage express at high levels a number of structural genes, for example, type I collagen, alkaline phosphatase, osteopontin/2ar, bone sialoprotein, osteocalcin/BGP, and osteonectin/SPARC, some of which have been proposed recently to be expressed sequentially during osteoblast differentiation in vitro (Aronow et al., 1990; Turksen and Aubin, 1991; Aubin et al., 1992). How these genes are regulated at the molecular level is not entirely clear, although the AP-1 transcription factor complex has received much attention in this regard. The AP-1 complex is considered to be a general transcription factor, but it may have a specific role in bone cell differentiation and osteoblastic gene expression as evidenced by the observations that the expression of some genes which are expressed by osteoblastic cells appear to be regulated by AP-1 in vitro (Kerr et al., 1988; Schönthal et al., 1988; Schüle et al., 1990; Lian et al., 1991). Thus, the identification of such regulatory factors is important not only for investigating normal bone development but also for understanding the cellular and molecular mechanisms underlying the perturbation of cell growth and differentiation in metabolic bone disease and in bone neoplasia.

Attempts to define the biological role of c-*fos* have involved both in vitro and in vivo approaches. Gene transfer studies in vitro have indicated that c-*fos* may play a role in cell differentiation, although its effects may vary in different cell types (Müller and Wagner, 1984; Rüther et al., 1985; Mitchell et al., 1986; Distel et al., 1987; Rahm et al., 1989; Lassar et al., 1989; Field et al., 1992). With respect to in vivo studies, we have previously used "gain-of-function" and "loss-of-function" approaches to address the biological function of c-*fos* (for review see Wagner and Keller, 1992; Grigoriadis et al., 1993). Transgenic mice over-expressing c-*fos* under the control of the human metallothionine promoter (MT-c-*fos*LTR mice) develop specific lesions in the long bones as early as 2–3 wk after birth (Rüther et al., 1987), and a low frequency of the mice (~15%) developed osteosarcomas after a 9–10-mo latency period (Rüther et al., 1989). By comparison with the transgenic mice, MT-c-*fos*LTR chimeric mice generated using embryonic stem (ES) cells selected for high exogenous c-*fos* expression develop chondrosarcomas, which are caused by a specific effect of Fos on chondrogenic cells (Wang et al., 1991, 1993). Finally, we and others have recently reported that mice lacking functional Fos protein develop osteopetrosis (Wang et al., 1992; Johnson et al., 1992), demonstrating unequivocally that Fos is a critical molecule for the normal development and/or differentiation of skeletal tissues. Taken together, these data suggest that bone and cartilage tissues are sensitive to altered levels of Fos protein.

In contrast to c-*fos* chimeric mice in which chondrogenic cells are specifically transformed (Wang et al., 1991), there is no direct evidence from the MT-c-*fos*LTR transgenic mice indicating which specific cell types are affected. A preliminary characterization of primary cell populations from MT-c-*fos*LTR–induced tumors suggested that osteoblastic cells may indeed be affected (Goralczyk et al., 1990). To better define the causal role of c-*fos* in osteosarcoma formation and to identify the affected cell populations, we aimed to establish a c-*fos* transgenic family in which osteosarcoma formation was more prevalent. To this end, we have generated transgenic mice with the c-*fos* gene fused to the H-2K^b class I MHC promoter which should lead to a broader expression pattern and higher expression levels in transgenic mice (Morello et al., 1986). To assess the specificity of c-*fos* in comparison with other AP-1 proteins we also generated transgenic mice expressing *fos*B and c-*jun* in several tissues including bone, where these genes are normally not expressed (Wilkinson et al., 1989; Redemann-Fibi et al., 1991). In this report we characterize in detail one c-*fos* transgenic family which develops osteosarcomas with 100% penetrance. This phenotype is specific for the Fos oncoprotein and a developmental analysis of transgene expression and characterization of primary and clonal cell populations has demonstrated that osteoblastic cells are principal targets for c-*fos*–induced oncogenesis.

Materials and Methods

Construction of DNA Vectors and Generation of Transgenic Mice

The DNA vector used in this study (H2-c-*fos*LTR) contains the murine genomic c-*fos* gene fused to the MHC class I H2-K^b promoter (H2; Morello et al., 1986). In addition, the 3′-unstranslated region including the polyadenylation site of c-*fos* was replaced with the intact 3′ LTR of the FBJ-MSV. This vector (pl28/1) was constructed from the MT-c-*fos*LTR vector (p76/21) described previously (Rüther et al., 1985). Briefly, the 3′ SalI-EcoRI fragment of p76/21 containing the 3′ LTR of the FBJ-MSV virus was exchanged with the 3′ SalI-EcoRI fragment of c-*fos* in the H2-c-*fos* vector described earlier (Rüther et al., 1988). The FBJ-derived fragment also contains the coding region for the viral envelope protein p15E (Van Beveren et al., 1983). For microinjection, a 7-kb HindIII fragment was isolated without any vector sequences and microinjected into fertilized mouse eggs (C57BL/6 × CBA × Him OFl) as described by Hogan et al. (1986).

The H2-c-*jun*LTR DNA vector was constructed via blunt-end ligation by replacing the SalI fragment of pl28/1 containing c-*fos* with a 1.7 kb *Eco*RI-*Pst*I fragment containing the murine c-*jun* gene (NheI-PstI fragment). A 6.1-kb HindIII fragment without vector sequences was microinjected into fertilized MF-1 oocytes. For construction of the H2-*fos*BLTR vector (constructed by M. Schuermann and R. Müller, IMT, Marburg, Germany), a 1.6-kb SalI fragment containing the murine *fos*B cDNA replaced the SalI fragment of pl26/3 (H2-c-*fos*; see Rüther et al., 1988) containing c-*fos*. A 1.5-kb SalI-EcoRI fragment from p76/21 containing the 3′ FBJ-LTR was then cloned into the SalI-EcoRI site of pl26/3 to yield H2-*fos*BLTR. A 6.5-kb BglI fragment without vector sequences was microinjected into fertilized (Him OFl × ICR) oocytes.

Histological Analysis

Transgenic mice were killed by cervical dislocation and tissues were fixed

immediately in 3.7% paraformaldehyde in PBS at room temperature for 16–24 h. Mineralized tissues were demineralized in 0.5 M EDTA, pH 7.6, for 2–4 d at 4°C. After fixation and demineralization, tissues were dehydrated through RNase-free graded alcohols and toluene and infiltrated with paraffin (Histowax; Reichert-Jung, Vienna) at 58°C overnight under vacuum. Sections (4–6 μm) were cut, attached to 3-aminopropyltriethoxysilane-treated slides (TESPA; Sigma Immunochemicals, St. Louis, MO) and stained by Haematoxylin and Eosin (H+E; Sigma Immunochemicals). Radiographs were taken on anesthetized mice.

In Situ Hybridization and Immunohistochemical Analyses

Preparation of tissue sections were performed as described above. The probe used for RNA in situ hybridization experiments, designated pB15 (Fig. 1), is a 480-bp PCR fragment derived from the FBJ-MSV portion of p76/21 using the following PCR primers: primer 1, 5′ AAGACGAGCCAAATATAAAAAG 3′; primer 2, 3′ CCAGGGGACTATTAGGAGAAC 5′. This fragment, was subcloned into a pBluescript KS vector (Stratagene, La Jolla, CA) and sense and antisense cRNA probes were generated by in vitro transcription in the presence of [^{35}S]-labeled rUTP. The radiolabeled transcripts were degraded by controlled hydrolysis in 100 mM bicarbonate buffer (pH 9.4, 65°C, 1 h) to 50–100 bp. In situ hybridizations were performed as described by Aguzzi et al. (1990).

For immunohistochemical staining for Fos protein we used a polyclonal rabbit antiserum specific for c-Fos protein (a gift from Dr. M. Nicklin, IMP, Vienna). An ultrasensitive Avidin-Biotinylated Enzyme Complex (ABC) staining kit (Pierce Chemical Co., Rockford, IL) was used on paraffin sections according to the details specified by the supplier. Positive staining was visualized by incubation with DAB. Control sections were performed using PBS instead of Fos antibody.

Northern Blot Analyses

Northern analyses were performed on poly(A)$^+$ RNA isolated from both cell and tissue samples. RNA isolation from cells was performed as described previously (Wang et al., 1991) using ~5 × 10^7 cells, harvested at confluence and stored at −80°C until further analysis. RNA isolation from tissues was performed using a modification of the methods of Chomczynski and Sacchi (1987) and electrophoresis was performed according to the formaldehyde/agarose method described by Maniatis et al. (1982). The following probes were used: for c-*fos* expression we used either a fragment from v-*fos*/Fox (Van Beveren et al., 1983) or a 8-kb HindIII fragment from p76/21 (MT-c-*fos*LTR). Both probes recognize Fox, an abundant class of RNA present in mouse tissue at loci not related to c-*fos*. Probes for c-*jun* (560-bp fragment; Ryseck et al., 1988), *jun*B (1.8-kb fragment), *jun*D (1.0-kb fragment), and *fra*-1 (1.4-kb fragment) were a gift from Dr. R. Bravo (Bristol-Myers Squibb, Princeton, NJ). Other probes used were: *fos*B (1.6-kb fragment; Dr. R. Müller, IMT, Marburg, FRG), α1(I) collagen (1.5-kb fragment; Dr. K. Kratochwil, University of Salzburg, Austria), alkaline phosphatase (2-kb fragment; Dr. J. Schmidt, GSF, Munich, FRG), osteopontin/2ar (1-kb fragment; Dr. B. Hogan, Vanderbilt University, Nashville, TN), osteocalcin/BGP (450-bp fragment) and BMP-2 (250-bp fragment; Dr. J. Wozney, Genetics Institute, Boston, MA), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH, 1.0-kb fragment). For specific hybridization to the c-*jun* transgene (see Fig. 2 *C*), we used pB15 as a probe (see above and Fig. 1). All probes were labeled with ^{32}P-labeled dCTP using an oligolabeling kit (Pharmacia Fine Chemicals, Piscataway, NJ) to a final specific activity of 4 × 10^8 cpm/μg DNA.

Cell Culture, Cloning, and Tumorigenicity Assay

For the isolation of cell lines from *fos* transgenic mice, tumors from different locations were dissected aseptically, cleaned of adherent connective tissue and washed with PBS containing antibiotics. Tissues were minced with scissors and tissue fragments were either explanted into 35-mm culture dishes, or digested three times (each 20 min) with 0.1% hyaluronidase/0.2% collagenase (Sigma Immunochemicals). After each digest, the cells were centrifuged and resuspended in standard culture medium. All isolated cells were cultured in DME containing 10% FCS and antibiotics at 37°C in a humidified atmosphere of 5% CO_2 in air, propagated to high cell numbers and frozen at −80°C. Cells were subcultured routinely at confluence by a 1:5 split ratio using 0.05% trypsin-EDTA in modified Puck's saline A (GIBCO-BRL, Gaithersburg, MD). Tumor-derived cell lines were designated R3, C1, C2, C3, P1, K6, K7, K11, and K14, isolated from tumors originating from the ribs (R), calvaria (C), pelvis (P), and long bones (K), respectively. One cell line derived from newborn calvaria (NC) of transgenic mice was isolated. The clonal rat osteosarcoma cell line (ROS 17/2.8) which expresses osteoblastic characteristics was obtained from Dr. G. A. Rodan (Merck, Sharpe and Dohme, West Point, PA; see also Rodan and Rodan, 1984) and cultured as described above. For clonal analysis, single-cell suspensions of P1 cells were plated at limiting dilutions of 0.5 and 1.0 cell/6 mm microtitre well. Colonies arising from single cells were identified after several days. 16 clones were isolated (P1.1 to P1.16) and expanded for further study.

For analysis of tumorigenicity, primary cell lines and clones were injected subcutaneously (~1 × 10^6 cells in 50 μl PBS) into the dorsal regions of 4- to 6-wk-old nude mice. Upon macroscopic identification of the tumors, animals were killed, and the tumors were dissected and analyzed histologically as described above.

Treatment with Steroid Hormones

For gene regulation studies by steroid hormones, we treated P1.9 and P1.15 clonal cells with either 1,25-dihydroxyvitamin D_3 (1,25-$[OH]_2D_3$; a gift from Dr. U. Fischer, Hoffmann-La Roche, Basel), or dexamethasone (Dex; Sigma Immunochemicals). Stock concentrations of each hormone were prepared in absolute ethanol and stored at −20°C. Cells were plated in standard media, then incubated with either 10^{-7} M 1,25-$(OH)_2D_3$, 10^{-7} M dexamethasone or 0.1% ethanol vehicle for 72 h in media containing 1% FCS. Cells were then harvested and poly(A)$^+$ RNA was isolated as described above.

Results

Bone Tumors Develop with 100% Efficiency in H2-c-fosLTR Transgenic Mice

The c-*fos* construct used to generate transgenic mice (H2-c-*fos*LTR) contains the murine genomic c-*fos* gene fused to the murine H-2K^b class I MHC promoter (H2) (Fig. 1). In addition, the 3′ mRNA destabilizing sequences and polyadenylation (polyA) site of c-*fos* have been replaced with a 3′ LTR from the FBJ-MSV to ensure stability of the c-*fos* mRNA (Rüther et al., 1985). Using this vector, two exogenous c-*fos* transcripts are predicted: a 3.0-kb transcript terminating at the polyA site present in the FBJ-LTR and a shorter 2.0-kb transcript which terminates at a cryptic polyA site present in the FBJ-derived sequence. For the generation of transgenic mice, the purified H2-c-*fos*LTR fragment without vector sequences was microinjected into fertilized eggs. A total of four independent founder animals were obtained and all developed noticeable swellings in the long bones, pelvis, and along the vertebral column at 2–3 mo of age (see also Rüther

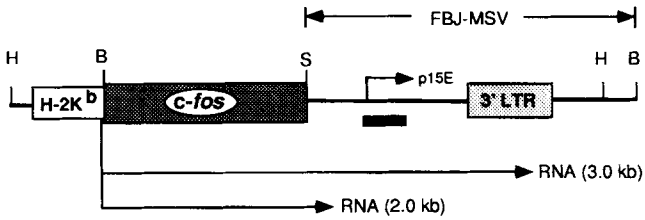


Figure 1. The DNA construct (H2-c-*fos*LTR) used for generation of c-*fos* transgenic mice. The murine genomic c-*fos* gene is fused to the MHC class I H2-K^b promoter. The sequence 3′ to the SalI site is derived from the FBJ-MSV and contains the 3′ FBJ long terminal repeat (3′ LTR) as a polyadenylation signal as well as the coding sequence from the retroviral envelope protein p15E. Two c-*fos* mRNA transcripts are synthesized as indicated. The solid bar represents the transgene-specific probe (pB15) used for in situ hybridization experiments as described in the Materials and Methods. H, HindIII; B, BamHI; S, SalI.

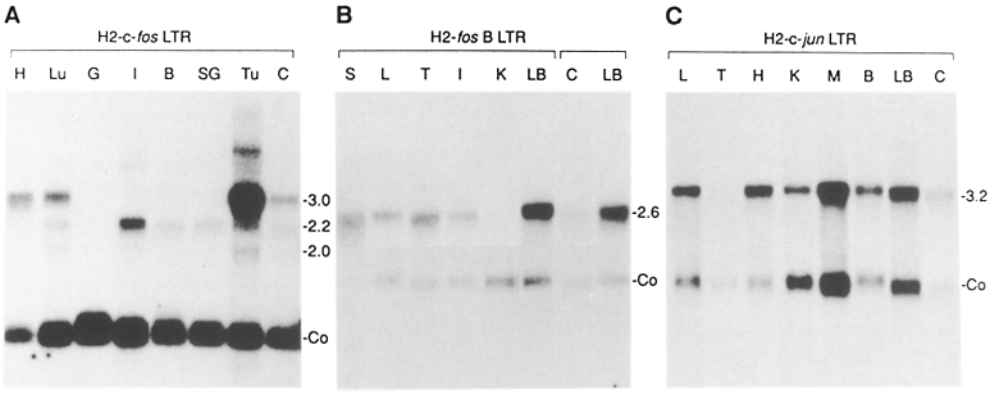


Figure 2. Northern blot analysis of c-*fos*, *fos*B, and c-*jun* transgene expression in different tissues of transgenic mice. (*A*) c-*fos* expression in tissues of a 7-mo-old male homozygous H2-c-*fos*LTR transgenic mouse. The filter was hybridized with a c-*fos* probe which detects both exogenous (3.0 and 2.0 kb) and endogenous (2.2 kb) c-*fos* transcripts. A clear signal for exogenous c-*fos* is also detected in brain and salivary gland tissues upon longer exposure. (*B*) *fos*B expression in tissues of two 2-mo-old male heterozygous H2-*fos*B LTR transgenic mice. The filter was hybridized with a *fos*B probe and shows the exogenous *fos*B transcript (2.6 kb). (*C*) c-*jun* expression in tissues of a 3-mo-old male heterozygous H2-c-*jun*LTR transgenic mouse. The filter was hybridized with a probe which detects only the exogenous c-*jun* transcript (3.2 kb; see Materials and Methods). The endogenous Fox gene (0.6 kb) was used as a control (*Co*) for RNA loading in (*A*) (see Van Beveren et al., 1983) while GAPDH (1.4 kb) was used for *B* and *C*. RNA was isolated and filters were probed as described in the Materials and Methods. 5 μg of poly(A)$^+$ RNA were loaded per lane. Tissues analyzed were: *S*, spleen; *L*, liver; *T*, thymus; *H*, heart; *Lu*, lungs; *G*, gonads; *I*, intestine; *K*, kidneys; *M*, muscle; *B*, brain; *SG*, salivary gland; *LB*, long bone; *C*, calvaria containing tumor; *Tu*, tumor only (osteosarcoma).

et al., 1989). We were not able to obtain offspring from three of the founder animals and breeding was difficult probably due to the size and severity of the lesions. However, one founder animal transmitted the transgene to its offspring and a family was established (designated No. 131) which was bred to homozygosity. All mice developed skeletal tumors: whereas mice heterozygous for the transgene developed palpable tumors after ~6 mo of age, homozygous offspring developed palpable tumors earlier, between 10 and 12 wk of age suggesting that the levels of c-*fos* expression can affect the onset of tumor formation. Examination of the mice at autopsy confirmed that only the bones were affected as no macroscopic abnormalities were observed in any other tissues. All subsequent studies were performed on homozygous mice.

To investigate the relationship between tumor formation and exogenous c-*fos* expression we performed Northern blot analysis on tumor tissues as well as on unaffected tissues of adult transgenic mice. Exogenous c-*fos* was expressed at highest levels in the tumor tissues, however, expression was not confined to the tumors, as the transgene was also expressed at high levels in heart, lung, brain, and salivary gland (Fig. 2 *A*). Unaffected bones, thymus and skeletal muscle also expressed the transgene (data not shown). All mice analyzed showed a similar expression pattern and have maintained this pattern in subsequent generations for over 3 1/2 y (i.e., over 10 generations) (data not shown).

H2-fosBLTR and H2-c-junLTR Transgenic Mice Exhibit No Bone Pathology

Independent transgenic mouse strains harboring *fos*B and c-*jun* transgenes in the context of the identical regulatory elements were established to assess the oncogene specificity of bone tumor formation. In H2-*fos*BLTR transgenic mice, exogenous *fos*B mRNA (and FosB protein; data not shown) was expressed in several tissues, for example, in spleen, liver, thymus, intestine, and in bone (Fig. 2 *B*). These mice have been bred to homozygosity and no abnormalities have been observed in mice older than 1.5 y of age, even though the identical DNA construct can transform fibroblasts in vitro. Similarly in H2-c-*jun*LTR transgenic mice the c-*jun*

Table I. Expression of Different AP-1 Gene Constructs in Transgenic Mice

Construct	No.*	S	L	T	H	Lu	G	I	K	M	B	SG	Bone LB	Bone C	Tumor
H2-c-*fos*LTR	1‡	−	−	+	+++	++	−	−	−	±	+	−	++	++	++++
H2-c-*fos*LTR	2§	+	±	+	+++	+	−	ND	++	++	+++	+	++	ND	+++
H2-*fos*B LTR	5	+++	++	+++	−	−	ND	+++	ND	ND	−	−	+++	+	0
H2-c-*jun*LTR	2	++	++	++	++	++	++	++	++	++	+	+	++	++	0

A summary of ectopic AP-1 gene expression in different tissues of transgenic mice overexpressing c-*fos*, *fos*B, and c-*jun*. In all DNA constructs, the transgene was fused to the murine MHC class I H-2K^b promoter (H2), and contained the 3′ LTR from the FBJ-MSV as a polyadenylation signal (see Materials and Methods). Shown are the relative abundancies of exogenous c-*fos* RNA in adult mouse tissues: *S*, spleen; *L*, liver; *T*, thymus; *H*, heart; *Lg*, lungs; *G*, gonads; *I*, intestine; *K*, kidneys; *M*, skeletal muscle; *B*, brain; *SG*, salivary gland; *LB*, long bone; *C*, calvaria; *Tumor*, osteosarcoma; *ND*, not determined; ±, signal detected after >3-d exposure.

* Number of independent mouse lines (i.e. different integration sites). Data shown for each construct represent a summary of expression from all mouse lines, with the exception of H2-c-*jun*LTR which shows data from one family.

‡ Data represent the expression of exogenous c-*fos* RNA in tissues from transgenic family No. 131 described in this study.

§ Data represent the expression of exogenous c-*fos* RNA in tissues from two founder animals which developed osteosarcomas but could not be bred (see also Rüther and Wagner, 1989).

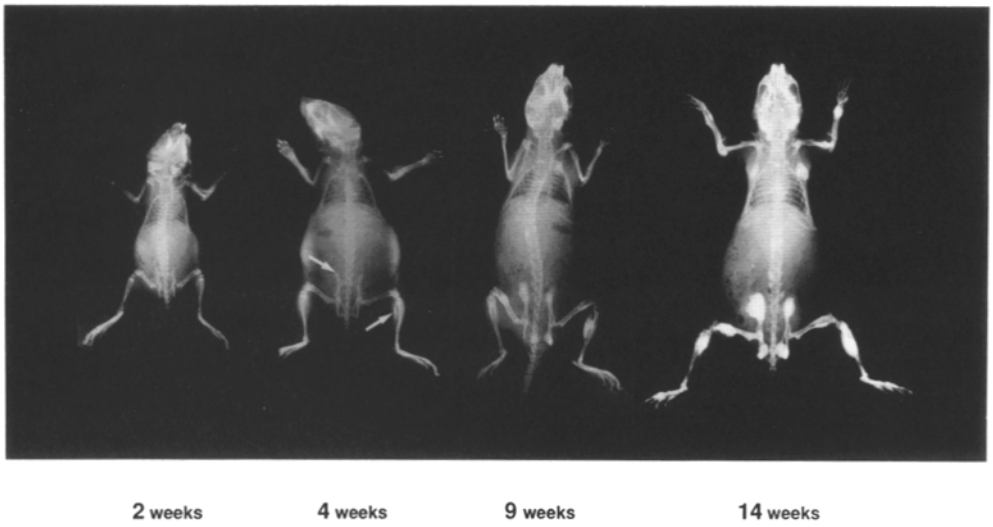


Figure 3. Radiographic analysis showing the development of osteosarcomas in H2-c*fos*LTR transgenic mice. X-rays of a single male homozygous mouse taken at 2, 4, 9, and 14 wk of age illustrate that the bone lesions are detectable at the radiographic level as early as 4 wk of age (*arrows*) and develop into large calcified tumors.

transgene was expressed in almost all tissues including bone (Fig. 2 *C*), and no pathology has been observed to date in heterozygotes over 1.5 y of age. We are currently establishing a line of homozygous H2-c-*jun*LTR mice. Table I summarizes the expression patterns of c-*fos*, *fos*B, and c-*jun* transgenes in several transgenic lines. These results suggest that Fos is the important component of the AP-1 transcription factor complex which specifically alters the phenotype of bone cells.

Osteosarcoma Development in H2-c-fosLTR Mice

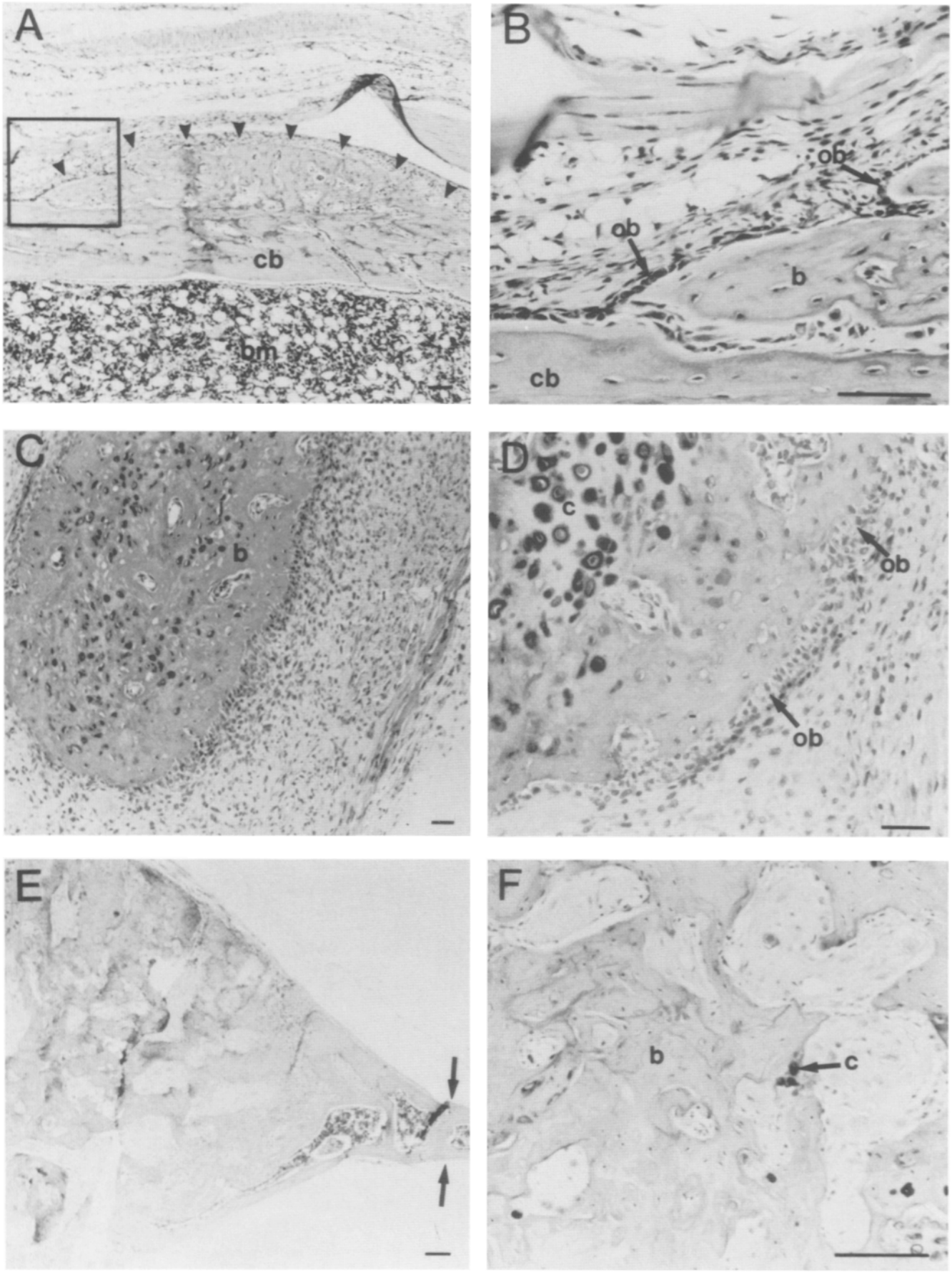
Since all homozygous H2-c-*fos*LTR mice developed bone tumors, we next investigated the time course of tumor development in these mice. Developmental X-ray analysis demonstrated that radio-dense lesions could easily be detected as early as 4 wk of age, typically starting in the long bones in the regions of the proximal tibiae or fibulae. These lesions grew rapidly in size and developed by ~14 wk into large calcified tumors present in virtually all bones of the body, including the long bones, vertebrae, pelvis, ribs, and calvaria (Fig. 3).

Histological analysis before overt tumor formation revealed the presence of small nodules of bone which were situated extracortically in the metaphyseal and diaphyseal regions of the long bones (Fig. 4 *A*). These lesions contained high osteoblastic activity as evidenced by the presence of cuboidal osteoblastic cells lining these areas of new bone (Fig. 4 *B*). In older mice, examination of individual tumors from different animals revealed histopathological features typical of chondroblastic osteosarcoma: all tumors were highly mineralized, containing large areas of bone lined by numerous cuboidal osteoblastic cells (Fig. 4, *C* and *D*) which expressed high levels of alkaline phosphatase activity as judged histochemically (data not shown). These areas of bone formation frequently contained numerous chondrocytes (Fig. 4 *D*). Tumors also developed in the calvaria but only in a specific location, namely, bilaterally at the parieto-occipital sutures, and also contained abundant neoplastic bone and some cartilage (Fig. 4, *E* and *F*). Interestingly, cartilage-containing tissues (e.g., articular joint surfaces, epiphyseal growth plates) did not appear to be affected (data not shown). All transgenic tumors were vascularized, highly malignant, and invasive as evidenced by the high numbers of mitotic figures and by the presence of ectopic bone formation in the bone marrow spaces and in the surrounding tissues.

To investigate the timing of expression of exogenous c-*fos* and its correlation with the onset of osteosarcoma formation, we analyzed by Northern blots the expression of the transgene during embryonic and postnatal development of homozygous mice. The c-*fos* transgene is not expressed during embryonic development although endogenous c-*fos* is expressed in the embryo between d 12.5 and 14.5 of gestation (Fig. 5). Newborn mice also do not express the transgene, but exhibit a dramatic burst of endogenous c-*fos* expression, consistent with previous observations (Kasik et al., 1987). Moreover, stable endogenous c-*fos* expression was observed after birth, in lung, intestine, and in bone tissues. The c-*fos* transgene is expressed specifically after birth in a tissue-specific manner. For example, expression in the lungs is detectable 5 d after birth, while expression in the heart is detectable at 2 wk of age. With respect to bone tissue, exogenous c-*fos* expression occurs between 2 and 3 wk of age in calvaria, long bones, and spine (Fig. 5). Thus, these results clearly indicate that expression of the transgene in the affected bone tissues occurs post-natally, and most importantly, occurs before the initial appearance of the bone lesions (at 4 wk, Fig. 3).

Expression of c-fos in Osteoblastic Cells

Since bone tissues expressed both endogenous and exogenous c-*fos* mRNAs, it was of interest to identify the specific cell types within bone which expressed c-*fos*. To this end we analyzed c-*fos* expression in developing bones and in osteosarcomas using in situ techniques. Immunocytochemical analysis for Fos protein in the limbs of 5-d-old transgenic mice indicated that Fos is expressed in osteoblasts lining the bone surfaces, as well as in articular and growth plate chondrocytes (Fig. 6, *A* and *B*). Since the transgene is not expressed at this young age, the observed staining pattern represents expression of the endogenous gene. With respect to exogenous c-*fos* expression, RNA in situ hybridization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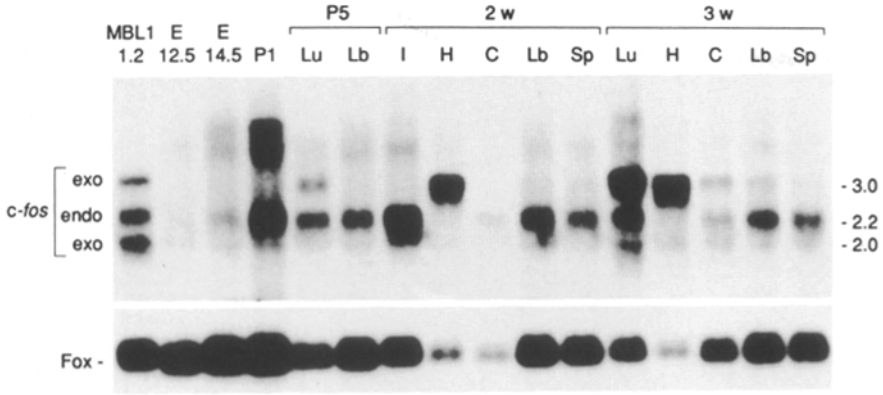

Figure 5. Time course of c-*fos* expression during development of a homozygous H2-c*fos*LTR transgenic mice. Endogenous c-*fos* mRNA (*endo*) is expressed between embryonic days (*E*) 12.5 and 14.5, whereas the exogenous c-*fos* transcripts (*exo*) are expressed after birth in a tissue-specific pattern. While expression in lung and heart tissues occurs shortly after birth, the onset of expression in bone tissue (calvaria, long bones, spine) occurs between 2–3 wk of age. *Pl*, 1-d-old mice torso; *P5*, 5-d-old mice; *Lu*, lungs; *I*, intestine; *H*, heart; *C*, calvaria; *Lb*, long bones; *Sp*, spine; *2w* and *3w*, 2 and 3-wk-old mice, respectively. *MBL 1–1.2*, control embryonic stem (*ES*) cells expressing endogenous and exogenous c-*fos* transcripts. Exogenous c-*fos* expression in 3w spine is clearly evident upon longer exposure. The endogenous Fox gene serves as a control for RNA loading. The data represent 3–6 μg of poly(A)$^+$ RNA loaded per lane, isolated as described in the Materials and Methods. The high molecular weight bands present in some lanes represent the unspliced c-*fos* transcripts (see also Van Beveren et al., 1983). Transcript sizes are indicated in kilobases.

analysis using a riboprobe specific for the transgene demonstrated that c-*fos* is expressed in bone cells before overt tumor formation. Specifically, a strong hybridization signal was observed in osteoblastic cells lining both the long bones and trabeculae, as well as in some periosteal cells containing putative preosteoblasts, and in osteocytes embedded within the bone matrix (Fig. 6, *C–F*). Interestingly, no exogenous c-*fos* expression was detected either in articular chondrocytes on joint surfaces (Fig. 6 *E*) or in proliferative and hypertrophic chondrocytes of the epiphyseal growth plates (data not shown). Within the tumors, exogenous c-*fos* expression was detected in osteoblast-like cells lining the neoplastic bone surfaces (Fig. 6 *G*), and immunocytochemical analysis confirmed the presence and nuclear localization of Fos protein (Fig. 6 *H*). Although the antibody we used detects both endogenous and exogenous Fos proteins, the fact that the tumors expressed little or no endogenous c-*fos* RNA suggests that the signal detected by immunohistochemistry is due to the introduced gene. Occasionally, some chondrocytes within the tumors also expressed the transgene (data not shown). Finally, many fibroblastic cells also expressed high levels of exogenous c-*fos*; whether these cells represent bona fide fibroblasts, or are perhaps, early osteoprogenitors is not known at this time. Nevertheless, the in situ data suggest that the transgene is expressed predominantly in cells of the osteoblastic lineage.

Gene Expression in Primary Osteosarcomas

To characterize the c-*fos*–induced tumors at the molecular level we analyzed the expression of genes which are members of the AP-1 transcription factor complex as well as osteoblast-associated marker genes. The results of Northern blot analyses of four primary osteosarcomas are summarized in Table II. All tumors expressed high levels of exogenous c-*fos* and no endogenous c-*fos*. The *fos*-related gene *fra*-1 was also expressed at high levels, while *fos*B expression was not detectable, supporting further the conclusions from *fos*B transgenic mice that this oncogene may not affect bone cell activity. The proto-oncogene c-*jun* was only moderately expressed in the tumors while the other *jun*-related genes, junB and junD were expressed ubiquitously in all tumors and at relatively higher levels. With respect to the expression of osteoblastic markers, alkaline phosphatase (ALP), osteopontin/2ar (OP), and osteocalcin/BGP (OC) mRNAs were high in all tumors whereas type I collagen expression was slightly lower (Table II). In addition, expression of bone morphogenetic protein-2 (BMP-2) was either very low or absent in all tumors. Finally, some tumors also expressed very low levels of cartilage-specific type II collagen mRNA (data not shown), probably due to a variation in the amount of chondrocytes present within the tumors. Thus, most of the genes tested were expressed, however, since all the tumors were heterogeneous, the information gained from gene expression in primary osteosarcomas is limiting with respect to the specific cell types which are responsible for the expression of these markers.

Isolation and Characterization of Osteoblastic Cell Lines

To characterize further the cell types which are affected by

Figure 4. Histological analysis of an early bone lesion and of the osteosarcomas which develop in H2-c-*fos*LTR transgenic mice. (*A*) Low magnification of a pre-neoplastic lesion (*arrowheads*) present on the surface of the cortical bone (*cb*) of the distal tibia of a 6-week old homozygous mouse. (*B*) High magnification of the area marked in *A* showing that this early lesion consists of new bone (*b*) formed by increased osteoblastic activity (*ob*). (*C* and *D*) Low and high magnifications of a typical chondroblastic osteosarcoma isolated from the pelvis of a 7-mo-old transgenic mouse. Tumors contain abundant bone (*b*) lined by cuboidal osteoblastic cells (*ob*) as well as cartilage (*c*). (*E* and *F*) Low and high magnifications of a calvarial tumor isolated from a 8-mo-old transgenic mouse containing neoplastic bone (*b*) and cartilage (*c*). The arrows in (*E*) delineate the normal thickness of the calvaria. All sections are 4–6 μm paraffin sections, processed and stained with H + E as described in the Materials and Methods. *cb*, cortical bone; *b*, bone; *bm*, bone marrow; *ob*, osteoblastic cells; *c*, chondrocytes. Bars, 25 μm.

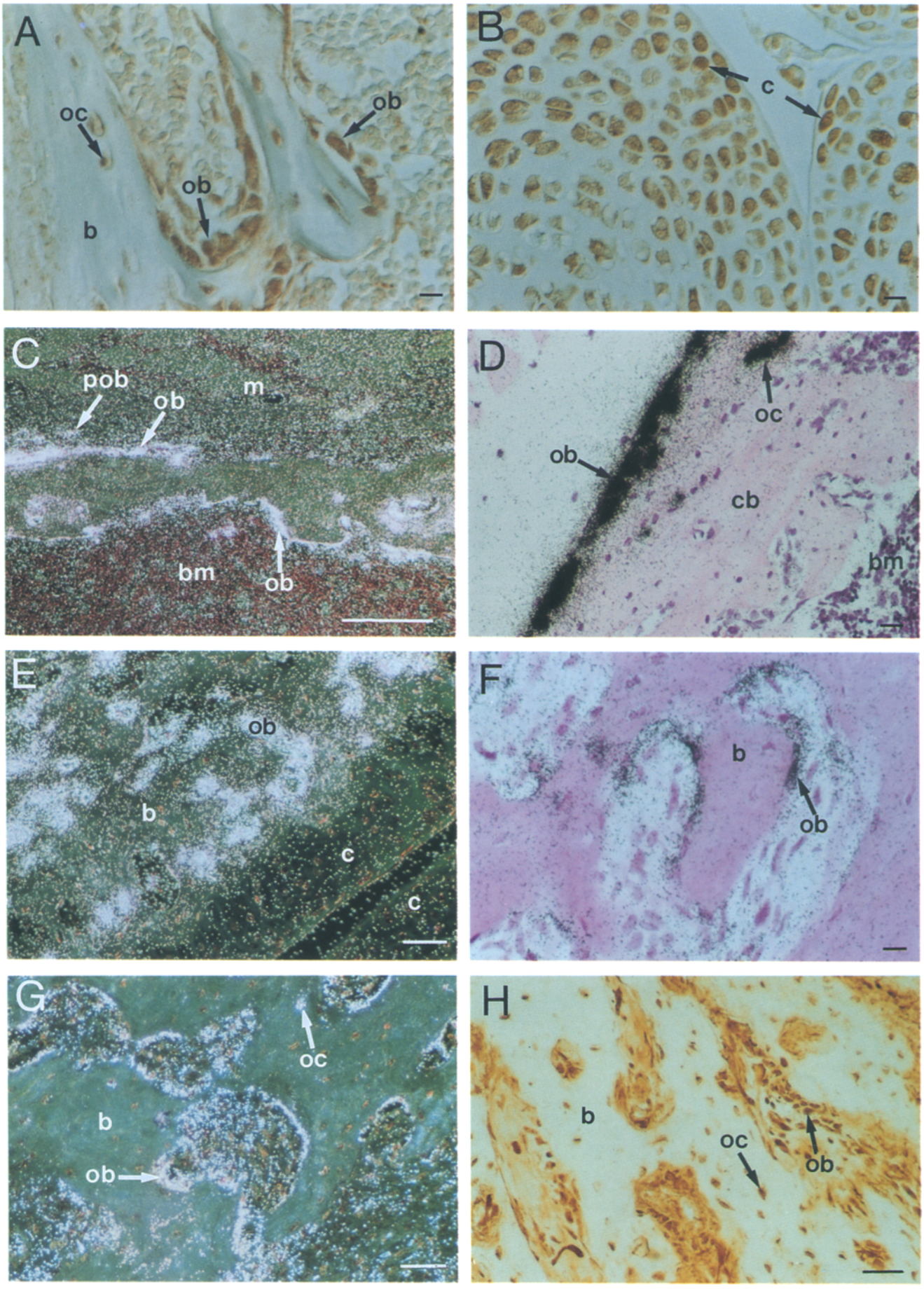
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Table II. Expression of AP-1- and Osteoblast-associated Genes in Primary Osteosarcomas Isolated from c-fos Transgenic Mice

	AP-1 Genes							Osteoblastic Genes				
	c-*fos*											
Tumor	Exo	Endo	*fos*B	*fra*-1	c-*jun*	*jun*B	*jun*D	Type I coll	ALP	OP	OC	BMP-2
1	++++	−	−	++	+	+++	+++	+	+++	+++	++++	±
2	+++++	−	−	+++	++	+++	+++	+	+++	++	++++	−
3	++++	−	−	++++	+	++++	+++	+	++++	+++	+++	−
4	++	−	−	++	+	+++	+++	+	++++	+++	++++	−

Four primary osteosarcomas isolated from different H2-c-*fos*LTR transgenic mice were analyzed for expression of AP-1- and osteoblast-associated marker genes. Data represent the relative abundancies of gene expression as estimated by Northern blot analysis. All blots were hybridized with specific probes against: c-*fos* (*Exo*, exogenous; *Endo*, endogenous), *fos*B, *fra*-1, c-*jun*, *jun*B, *jun*D, *type I collagen* (*type I coll*), alkaline phosphatase (*ALP*), osteopontin/2ar (*OP*), osteocalcin/BGP (*OC*) and bone morphogenetic protein-2 (*BMP-2*). ±, hybridization signal detected after >3-d exposure. Tumors Nos. 2 and 3 also expressed cartilage-specific type II collagen at very low levels (see text for details).

the Fos oncoprotein, and to study the possible molecular alterations which may have occurred in these cells, we isolated cell populations from the c-*fos*–induced tumors. Nine individual primary cell populations were isolated from tumors arising from different origins (i.e., from ribs, pelvis, calvaria, long bones). The tumor-derived cell lines, designated R3, C1, C2, C3, P1, K6, K7, K11, and K14, were characterized with respect to morphology, doubling time, osteoblastic gene expression, differentiation capacity and tumorigenicity. All tumor-derived cell lines could be easily established as continuously growing cell lines, with doubling times ranging from 18–24 h (data not shown). All cells had a typical transformed fibroblastic morphology comprising small, spindle-shaped cells (e.g., P1 cells, Fig. 7 *A*). One newborn calvaria-derived cell line was also isolated which had a morphology typical of normal fibroblasts with a doubling time of ~30 h (data not shown).

With respect to gene expression, the nine tumor-derived cell lines analyzed expressed high levels of exogenous c-*fos* and of these, 5 (C1, C3, K7, K14, and at lower levels P1) also expressed the endogenous c-*fos* gene (Table III). As expected based on the time course of exogenous c-*fos* expression (Fig. 5), newborn calvaria cells did not express the transgene but did express endogenous c-*fos*. All cell lines expressed type I collagen, OP, and ALP mRNAs at varying levels and there did not appear to be any correlation between expression of c-*fos* and these osteoblastic genes (Table III). In contrast, OC expression was either absent or present at very low amounts (e.g., P1 cells) in all cell lines.

To assess the bone forming capacity of the isolated cell lines in vitro, we cultured each population in media containing ascorbic acid, β-glycerophosphate, and the glucocorticoid analogue dexamethasone as previously described (Bellows et al., 1986, 1987). None of the cell lines could differentiate in vitro as judged by the formation of bone nodules, although P1 cells were able to secrete an extracellular matrix which could mineralize under these conditions (data not shown). Since all cells exhibited a transformed morphology, we next investigated their tumorigenic and differentiation potential in vivo by injecting each cell line into nude mice. All tumor-derived cell populations were tumorigenic with a latency period of 2–5 wk (Table III). Interestingly, P1- and K14-induced tumors induced tumors with the phenotypic characteristics of osteosarcoma and chondroblastic osteosarcoma, respectively, while others were diagnosed as anaplastic sarcomas, some containing areas of osteoid deposition and occasional chondrocyte foci (e.g., C3-, K6-, and K11-induced tumors). A representative example of a P1-induced osteosarcoma is shown in Fig. 7 *C*. RNA in situ hybridization for exogenous c-*fos* and immunocytochemistry for Fos protein clearly indicated that the osteogenic cells within these populations express the transgene (Fig. 7, *D* and *E*) although a high proportion of fibroblastic stromal cells in the tumors also expressed the transgene. Finally, newborn

Figure 6. In situ hybridization and immunocytochemistry of unaffected bone and of osteosarcomas which develop in H2-c-*fos*LTR transgenic mice. (*A* and *B*) Immunocytochemistry for Fos protein in 5-d-old mice showing expression of endogenous Fos protein in osteoblasts (*ob*) and osteocytes (*oc*) (*A*) and in articular chondrocytes (*c*) present on joint surfaces (*B*). (*C–G*) In situ hybridization analysis of c-*fos* expression in osteosarcomas and in bone tissues before tumor formation. (*C*) Dark field micrograph of the diaphysis of a 7-mo-old homozygous mouse femur showing intense staining in periosteal and endosteal osteoblasts (*ob*) lining the cortical bone (*cb*), as well as in putative preosteoblasts (*pob*). (*D*) Bright field micrograph showing high c-*fos* expression in osteoblasts (*ob*) and osteocytes (*oc*) present in the tibia of a 6-wk-old homozygous mouse. (*E*) Dark field micrograph showing that c-*fos* is also expressed in osteoblasts (*ob*) present in the vertebral body of a 7-mo-old mouse, but not in the articular chondrocytes (*c*) of the vertebral joint. (*F*) Bright field micrograph of a vertebral body in the same mouse as in (*E*) confirming the expression of c-*fos* in osteoblastic cells (*ob*) lining the bone (*b*). (*G*) Dark field micrograph of an osteosarcoma surrounding the spine of an 8-mo-old mouse showing transgene expression in osteoblasts (*ob*) and osteocytes (*oc*). (*H*) Immunocytochemistry showing exogenous Fos protein in the osteoblasts (*ob*) and osteocytes (*oc*) of an 8-mo-old calvarial osteosarcoma. The probe used for in situ hybridization experiments is an antisense riboprobe specific for exogenous c-*fos* transcripts (see Materials and Methods). Control sense hybridizations revealed no specific signal (data not shown). All sections are 4–6 μm paraffin sections and were processed as described in the Materials and Methods. *b*, bone; *pob*, preosteoblasts; *ob*, osteoblasts; *oc*, osteocytes; *cb*, cortical bone; *bm*, bone marrow; *m*, muscle. Bars, 25 μm.

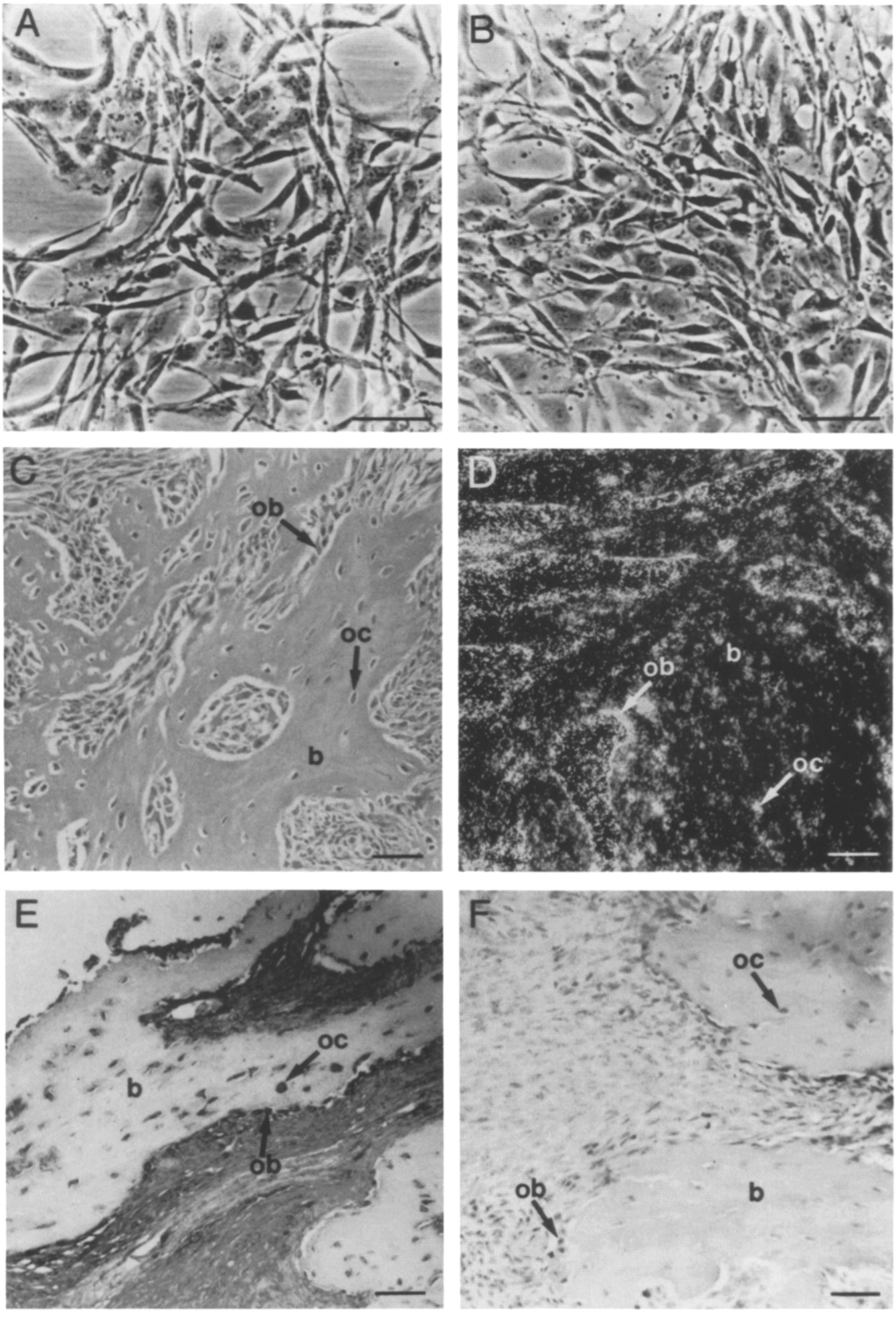
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Table III. Gene Expression and Tumorigenicity of Primary Cell Lines Established from c-fos–Induced Osteosarcomas and Non-Affected Tissues

Cell line	c-fos Exo	c-fos Endo	Type I coll	ALP	OP	OC	Tumor phenotype	Latency period (d)
R3	++++	−	+++	±	++	−	Anaplastic sarcoma	21
C1	+++++	++	+++	+++	+	−	ND	−
C2	+++++	−	++++	++	++	−	Anaplastic sarcoma	21
C3	+++++	++	++	±	++++	−	Anaplastic sarcoma + osteoid	21
P1	++	±	++++	+++	++	±	Osteosarcoma	15
K6	++++	−	++++	++	++	−	Anaplastic sarcoma + osteoid	21
K7	++++	+++	++++	++	+	−	Anaplastic sarcoma	21
K11	+++++	−	+++++	+	+	−	Anaplastic sarcoma + osteoid*	35
K14	++	+	±	+++	+	−	Chondroblastic osteosarcoma	35
NC	−	++	+++	+++	+++	−	None	−

Summary of c-*fos* and osteoblastic gene expression and tumorigenicity of primary cell lines established from c-*fos*–induced osteosarcomas and from non-affected tissues. Data represent the relative abundancies of gene expression as estimated by Northern blot analysis. All blots were hybridized with specific probes against: c-*fos* (*Exo*, exogenous; *Endo*, endogenous), type I collagen (*Type I coll*), alkaline phosphatase (*ALP*), osteopontin/2ar (*OP*) and osteocalcin/BGP (*OC*). ND, not determined; ±, hybridization signal detected after >3-d exposure. Tumorigenicity studies were performed in nude mice and the tumors were diagnosed after histological processing and staining with H+E as described in the Methods.
* Small foci containing chondrocytes observed occasionally.

calvaria cells which did not express exogenous c-*fos* and which did not have a transformed morphology failed to induce tumors in nude mice. Thus, these results suggest that different osteoblastic cell populations can be isolated which are transformed by Fos and which exhibit differences in gene expression and differentiation capacity. Table III summarizes the gene expression and tumorigenic potential of the isolated cell lines.

Characterization of Clonal Bone-forming Cell Lines

The demonstration that the mixed cell populations expressed osteoblastic genes and moreover that some had bone-forming potential prompted us to characterize further the specific cell types which are responsible for c-*fos*–induced osteosarcoma formation. To this end, we cloned osteogenic P1 cells and analyzed the properties of individual clones. 16 clones were isolated by limiting dilution (P1.1 to P1.16) of which six were chosen for further study. All clones generally exhibited a transformed morphology similar to the parental P1 cells, that is, spindle shaped and fibroblastic (e.g., P1.15 cells, Fig. 7 *B*), and had a population doubling time of ~20 h (data not shown). Northern blot analysis indicated that all clones expressed osteoblastic marker genes: expression of ALP and OP was highly variable whereas type I collagen expression was more ubiquitous (Fig. 8 *A*). OC expression was low or absent, although clones P1.15 and P1.16 contained higher OC mRNA levels than the parental P1 cells. With respect to *fos*- and *jun*-related gene expression, all clones expressed exogenous c-*fos* and some also expressed high levels of the endogenous c-*fos* gene (Fig. 8 *B*). The clones also expressed *fra*-1 and c-*jun* mRNAs at high levels, and *jun*B at lower levels. All the clonal cell lines tested were tumorigenic in nude mice with latency periods varying between 15 and 21 d (data not shown). Of greater significance was the observation that a high proportion of them were osteogenic, that is, exhibited bone-forming capacity. Histological analysis revealed that the tumors induced by P1.4, P1.7, P1.15, and P1.16 cell lines were clearly osteosarcomas as judged by the presence of mineralized bone (e.g., Fig. 7 *F*). Similarly to the parental P1 cells, RNA in situ hybridization indicated that exogenous c-*fos* was expressed specifically in the osteoblasts and osteocytes within the bone as well as in many fibroblastic cells present within the tumors (data not shown). Interestingly, tumors induced by P1.4 cells also contained some very small focal areas containing chondrocytes suggesting that this clonal cell population also contained bipotential progenitor cells for both bone and cartilage cell lineages.

Steroid Hormone Regulation of Osteoblast Gene Expression

Since steroid hormones can affect the expression of the osteoblast phenotype and regulate the expression of bone-associated genes we decided to investigate whether the high levels of c-*fos* in the clonal tumor-derived cell lines interfere with the regulation of osteoblast-specific gene expression by vitamin D_3 and glucocorticoid hormones. Treatment of control ROS 17/2.8 osteoblastic cells with 1,25-dihydroxy-

Figure 7. Morphology, in situ hybridization and immunocytochemistry analyses of tumor-derived cell lines and cell-induced osteosarcomas. (*A* and *B*) Phase-contrast micrographs showing a fibroblastic and spindle-shaped cell morphology of the osteosarcoma-derived cell line P1 (A) and one of its clonal derivatives, P1.15 (*B*). (*C*) A typical osteosarcoma induced by P1 cells showing abundant bone formation (*b*) with numerous osteoblasts (*ob*) and osteocytes (*oc*). In situ hybridization analysis of exogenous c-*fos* mRNA (*D*) and immunocytochemistry for Fos protein (*E*) demonstrate high transgene expression in osteoblastic cells (*ob*) and in osteocytes (*oc*) of a P1-induced tumor. (*F*) A representative osteosarcoma induced by the clonal cell line P1.7, showing similar characteristics to the parental P1–induced tumor in (*C*). Sections in (*C–F*) are 4–6 μm paraffin sections and were processed as described in the Materials and Methods. *ob*, osteoblasts; *oc*, osteocytes; *b*, bone. Bars: (*A* and *B*) 120 μm; (*C–F*) 25 μm.

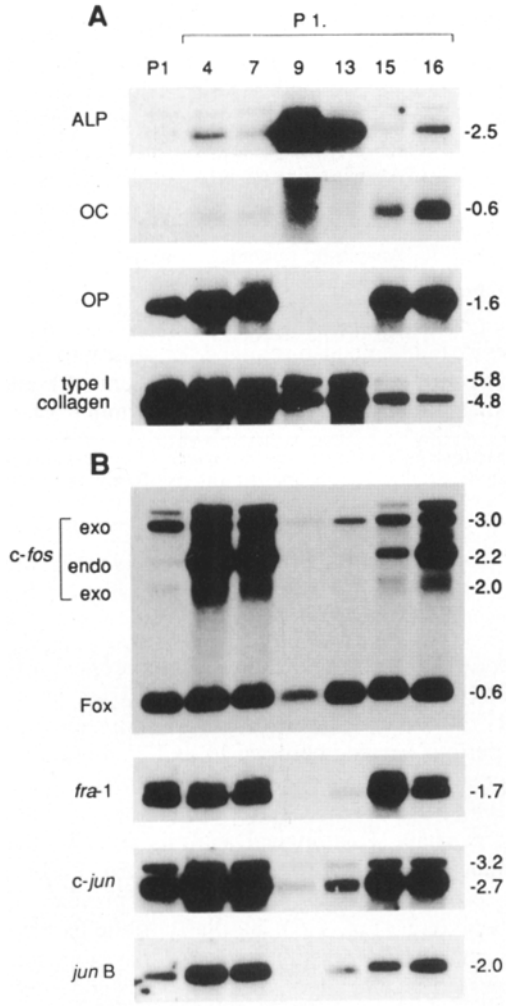

Figure 8. Gene expression in primary and clonal cell lines established from c-*fos* transgenic osteosarcomas. Parental P1 cells and 6 P1-derived clonal cell lines (P1.4, P1.7, P1.9, P1.13, P1.15, and P1.16) were analyzed by Northern blots for expression of (*A*) osteoblastic genes and (*B*) *fos*- and *jun*-related genes. RNA was isolated as described in the Materials and Methods. 5 μg of poly(A)$^+$ RNA were loaded per lane and the blot was hybridized with the following probes as described in the Materials and Methods: and alkaline phosphatase (*ALP*), osteocalcin/BGP (*OC*), osteopontin/2ar (*OP*), type I collagen, v-*fos* (for exogenous [*exo*] and endogenous [*endo*] c-*fos*), *fra*-1, c-*jun*, and *jun*B. The same filter was used for each probe following complete stripping. Transcript sizes for each gene are indicated in kilobases. The endogenous Fox gene serves as a control for RNA loading.

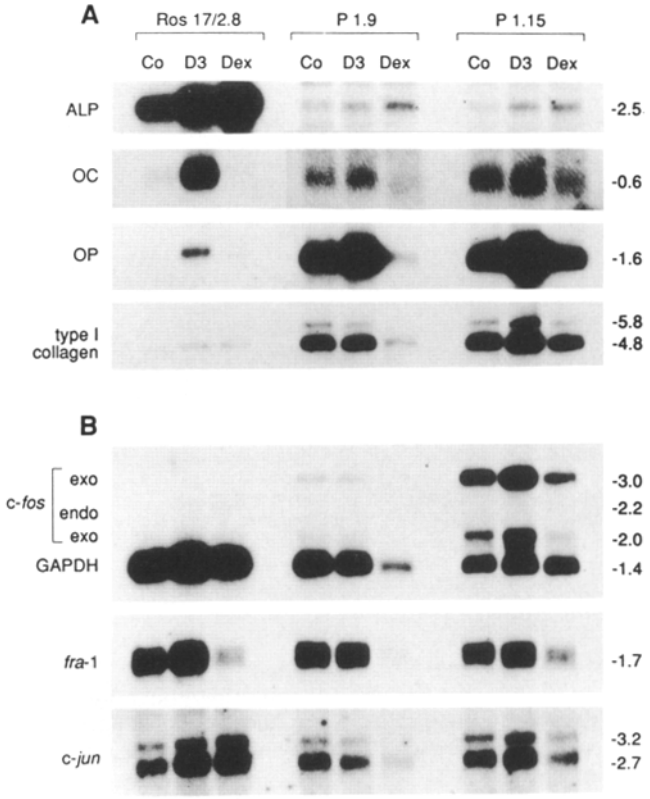

Figure 9. Steroid hormone regulation of gene expression in clonal cell lines derived from c-*fos* transgenic osteosarcomas. P1.9 and P1.15 clonal cell lines, as well as clonal rat osteosarcoma cells which exhibit an osteoblastic phenotype (Ros 17/2.8) were analyzed by Northern blots for expression of (*A*) osteoblastic genes and (*B*) *fos*- and *jun*-related genes. Cells were cultured under standard conditions, and then treated with 10^{-7} M 1,25-dihydroxyvitamin D_3 (D3), 10^{-7} M dexamethasone (*Dex*) or with 0.1% ethanol vehicle (*Co*) for 72 h in media containing 1% FCS. RNA was isolated as described in the Materials and Methods and hybridized with the following probes: alkaline phosphatase (*ALP*), osteocalcin/BGP (*OC*), osteopontin/2ar (*OP*), type I collagen, c-*fos* (*exo*, exogenous; *endo*, endogenous), *fra*-1, and c-*jun*. 5 μg of poly(A)$^+$ RNA were loaded per lane. The same filter was used for each probe following complete stripping. Transcript sizes for each gene are indicated in kilobases. GAPDH hybridization serves as a control for RNA loading.

vitamin D_3 (1,25-$(OH)_2D_3$), the most active metabolite of vitamin D_3 (for review see Minghetti and Norman, 1988), caused an increase in OC, ALP, and OP mRNAs typical of osteoblastic cells (for review see Rodan and Rodan, 1984; Yoon et al., 1987). In contrast, 1,25-$(OH)_2D_3$ had no effect on either OC or ALP expression in the c-*fos*–transformed P1.9 and P1.15 clonal cells. It should be noted that basal ALP expression in P1.9 cells varied between different experiments (see also Fig. 8 *A*). The reasons for this are not entirely clear, but may relate to the different culture conditions (e.g., serum concentration) used in each experiment which could affect basal and hormone-stimulated ALP expression (e.g., see Majeska and Rodan, 1982). 1,25-$(OH)_2D_3$–induced OP expression in all cell lines tested whereas type I collagen expression was not affected (Fig. 9 *A*). Responsiveness to the potent glucocorticoid analogue, dexamethasone (Dex) was not altered in P1.9 and P1.15 cells when compared to ROS 17/2.8 osteoblastic cells: Dex stimulated ALP expression and reduced OP mRNA in both clones and caused a slight reduction in OC mRNA in P1.15 cells. In contrast, type I collagen expression was not affected. With respect to *fos*- and *jun*-related genes, expression of exogenous c-*fos* was inhibited by Dex in P1.15 cells, and interestingly, this was paralleled by decreases in *fra*-1 and c-*jun* expression (Fig. 9 *B*). In ROS 17/2.8 cells, Dex also inhibited *fra*-1 expression, but stimulated c-*jun* expression. 1,25-$(OH)_2D_3$ treatment did not affect c-*fos*, *fra*-1, or c-*jun* expression in any cell line tested. Thus, the differential regulation of osteoblastic genes

and *fos*- and *jun*-related genes by 1,25-$(OH)_2D_3$ and Dex suggests that specific molecular interactions may be taking place in different osteoblastic cells overexpressing c-*fos*.

Discussion

c-fos, But Not fosB or c-jun, Causes Osteosarcomas in Transgenic Mice

In this study we describe a family of H2-c-*fos*LTR transgenic mice which is susceptible to osteosarcoma development with 100% efficiency. Expression of the introduced c-*fos* gene was high in bone-forming osteoblastic cells and in the tumors. The pathology observed was specific for Fos, since transgenic mice expressing the other AP-1 genes *fos*B and c-*jun* in several tissues including bone are phenotypically normal, implying that altered levels of FosB and c-Jun oncoproteins do not affect bone development. Although we cannot completely rule out the possible influence of strain differences on tumor development, for example, as has been shown for v-*fos*–induced osteosarcoma formation (Kelloff et al., 1969), these results further strengthen the notion that c-*fos* is the important component of the AP-1 transcription factor complex which appears to be causal for the development of osteosarcomas in transgenic mice.

Tissue Specificity and Temporal Expression of c-fos

Ectopic c-*fos* expression was observed in H2-c-*fos*LTR transgenic mice in the tumors and bone tissues, as well as in other tissues which do not exhibit any pathology (e.g., heart, lung, brain). The expression levels are higher than those documented in MT-c-*fos*LTR transgenic mice (Rüther et al., 1987), which could be attributed, at least in part, to the change in promoter elements. Although endogenous MHC class I genes are usually transcribed as early as day 10 of embryonic development (Ozato et al., 1985), expression of exogenous c-*fos* in H2-c-*fos*LTR mice was not observed during embryogenesis. The reasons for this are not entirely clear, but may relate to the site of transgene integration. All homozygous mice carrying an H2-c-*fos*LTR construct developed osteosarcomas with a short latency period and transgene expression occurred before any phenotypic changes were observed. Since the 3′ FBJ-LTR ensures high exogenous c-*fos* expression in bone tissues (see also Rüther et al., 1988; Wang et al., 1991), these data strongly suggest that c-*fos* is a causative factor in the genesis of osteosarcoma.

Osteoblastic Cells Are Transformed by Ectopic c-fos Expression

Several lines of evidence suggest that cells of the osteoblast lineage are affected by exogenous Fos. First, all bone tissues were affected regardless of embryonic origin, that is, no selectivity was observed between bones formed via endochondral ossification (e.g., long bones, vertebrae) versus those formed via intramembranous ossification (e.g., calvaria). Second, high levels of exogenous c-*fos* were present in the osteosarcomas, specifically in the osteoblasts and osteocytes. Third, hyperproliferative regions arising apparently from periosteal cells were observed during the early stages of tumor formation which contained active osteoblasts and new bone. Although it is possible that these early lesions may represent induced reactive bone formation, the fact that high transgene expression was demonstrated in periosteal and osteoblastic cells suggests that these lesions are pre-neoplastic and that the transformation of these cells by c-*fos* was, at least in part, responsible for tumor formation. Finally, the isolation of clonal osteosarcoma–derived cell lines has demonstrated the cellular specificity and transforming capacity of Fos. All tumor-derived cell lines expressed high levels of the transgene and were transformed as evidenced by their ability to form tumors when injected in vivo. Not only was each cell line tumorigenic, but some also retained the ability to form bone. This is best represented by the osteogenic P1 and P1–derived clonal cell lines in which bone-forming osteoblasts, osteocytes, and putative osteoprogenitor cells present in the cell-induced tumors expressed the transgene at high levels.

In addition to the ability of some of these cell lines to form bone in vivo, the expression of a number of genes which are associated with the osteoblast phenotype further suggest that they are of osteoblastic origin. All cell lines expressed type I collagen, ALP and OP mRNAs, however, there was no apparent correlation between expression of these marker genes and the levels of exogenous c-*fos*. In contrast, the expression of OC was dramatically reduced or absent in all cell lines analyzed, despite expression in the primary tumors. This is interesting in view of recent observations in vitro that high AP-1 activity is associated with inhibition of OC transcription (Schüle et al., 1990; Owen et al., 1990), and is consistent with the observations in primary cell lines established from MT-c-*fos*LTR transgenic osteosarcomas (Goralczyk et al., 1990; see also discussion below).

That c-*fos* has transformed osteoblastic cells with bone-forming capacity is consistent with previous results indicating that high levels of exogenous Fos can transform different cells of mesenchymal origin, including fibroblasts (Miller et al., 1984), myogenic cells (Lassar et al., 1989), and chondrogenic cells (Wang et al., 1991, 1993). Whether earlier uncommitted cells are also affected in c-*fos* transgenic mice is not clear, but may be possible based on the observations that the clonal cell line P1.4 induced tumors containing both bone and, at a low frequency, cartilage, suggesting that it contains bipotential progenitor cells for both osteogenic and chondrogenic lineages. In addition, the observation that calvarial tumors arise only at parieto-occipital sutures where cartilage is present during development, suggests that cells with both chondrogenic and osteogenic capacities may have been affected by Fos. Interestingly, similar bipotential progenitor cell populations may also be affected in c-*fos* chimeric mice (Wang et al., 1991, 1993).

Cellular Specificity of c-fos–induced Transformation

The notion that osteogenic cells are transformed in c-*fos* transgenic mice is of importance in view of our previous observations that c-*fos* chimeric mice develop chondrosarcomas due to specific transformation of chondrogenic cells (Wang et al., 1991). These differences may be explained, at least in part, by the timing of exogenous c-*fos* expression whereby the postnatal onset of transgene expression in H2-c-*fos*LTR transgenic mice might preclude an effect on earlier progenitor cells present during embryogenesis. An alternative expla-

nation for the cellular specificity of Fos may relate to the presence of tissue-specific or cooperating factors which are necessary for cellular transformation and initiation of multi-step tumorigenesis (Hanahan, 1988; Adams and Cory, 1991). Indeed, the presence of histologically normal areas of bone expressing the transgene together with the observed time lag before tumor formation suggests that cooperating genetic events are occurring subsequent to c-*fos* expression. Since Fos transformation and regulation of target gene expression is dependent upon dimerization with specific *jun*-related genes (Schuermann et al., 1989), it is tempting to speculate that the levels of specific *jun* family members in osteogenic and chondrogenic cells may affect the target cell specificity of c-*fos*. In support of this notion are the observations that levels of c-*jun* in tissues, tumors, and cell lines isolated from H2-c-*fos*LTR transgenic mice are not strictly correlated with the levels of exogenous c-*fos*, which is in contrast to the good correlation observed between the expression of these two genes in c-*fos* chimeric mice (Wang et al., 1991). This is also supported by other observations that c-*jun* is expressed during mouse development in chondroblastic, but apparently not in osteoblastic cells (Wilkinson et al., 1989). Thus, c-*jun* does not seem to be a limiting factor in transgenic mice. With respect to the *fos*-related genes, the correlation between exogenous c-*fos* and *fra*-1 expression in osteogenic cells implies that this gene may be a cooperating factor in the cascade of events initiated by high Fos levels. In this regard, the recent evidence that *fra*-1 is a possible *fos*-regulated gene (Braselmann et al., 1992) may have physiological relevance. Thus, the possibility arises that different Fos–Jun complexes, and specific modifications of these complexes, for example by phosphorylation (Ofir et al., 1990; Pulverer et al., 1991), may have differential activities in osteoblastic and chondroblastic cell populations (see also Baichwal et al., 1991).

The cellular specificity of Fos action may certainly involve other factors which are implicated in tumorigenesis, such as the tumor suppressor gene p53 (for review see Levine et al., 1991; Weinberg, 1991). The association between osteogenesis and p53 function is supported by molecular analyses of p53 gene rearrangements in osteosarcomas (Masuda et al., 1987; Nigro et al., 1989; Strauss et al., 1992), as well as in mice overexpressing mutant alleles of p53 or completely lacking functional p53 protein, both of which are susceptible to different neoplasias including osteosarcomas (Lavigueur et al., 1989; Donehower et al., 1992). Thus, it is possible that potential interactions between Fos–Jun complexes and p53 in tumors and cells isolated from c-*fos* transgenic mice may affect the cellular specificity of Fos action.

A Functional Role for c-fos in the Expression of the Osteoblast Phenotype?

There are numerous studies correlating c-*fos* expression and osteoblast activity (Schön et al., 1986; Schmidt et al., 1986; Wu et al., 1990; Closs et al., 1990; Birek et al., 1991; Ohta et al., 1991). However, Fos activity is clearly not obligatory for osteoblast differentiation and bone formation since osteoblastic cells present in mice lacking functional Fos protein can, to some degree, undergo osteogenesis (Wang et al., 1992; Johnson et al., 1992). Since it is well established that steroid hormones can regulate the expression of osteoblastic genes, and that steroid hormone–receptor complexes can functionally interact with AP-1 complexes in vitro, we decided to investigate whether the role of c-*fos* in the clonal tumor-derived cell lines extended beyond its ability to cause transformation and induce tumorigenesis. The stimulation of OC and ALP gene expression by 1,25-$(OH)_2D_3$ has been shown to be a useful indicator of the osteoblastic phenotype in different model systems (for review see Rodan and Rodan, 1984). In this regard, the most striking observation was that 1,25-$(OH)_2D_3$–stimulated OC and ALP expression was apparently blocked in the Pl-derived osteogenic cell clones. These observations are important in view of the recent reports demonstrating overlapping AP-1 consensus sequences and putative vitamin D_3 responsive elements (VDRE) in the promoter regions of these genes (Schüle et al., 1990; Owen et al., 1990). Moreover, occupancy of the AP-1 sites blocks 1,25-$(OH)_2D_3$-receptor-VDRE interactions and subsequent gene transcription, a phenomenon termed "phenotype suppression" (Owen et al., 1990; Lian et al., 1991). The data presented here represent the first in vivo evidence of a functional interaction between Fos–Jun complexes and steroid hormone response elements in bone cells. It is tempting to speculate that the transformation of osteogenic cells (as represented by Pl-derived clonal cells) by high Fos levels has prevented further differentiation as judged by the lack of 1,25-$(OH)_2D_3$ responsiveness. This block in differentiation, together with the uncontrolled growth due to cellular transformation may explain the induction of osteogenic tumors in c-*fos* transgenic mice. Interestingly, while the responsiveness of P1.9 and P1.15 cells to 1,25-$(OH)_2D_3$ was affected by high Fos levels, Dex responsiveness was not altered. Although interactions between glucocorticoid response elements (GRE) and AP-1 sites have been demonstrated in vitro (for review see Diamond et al., 1990), functional interactions have not been observed in the specific regulation of ALP and OC transcription. In fact, glucocorticoid inhibition of OC gene transcription is apparently due to interactions between the GRE and the TATA box of the OC promoter, that is, separate from the VDRE/AP-1 site (Morrison et al., 1989; Strömstedt et al., 1991).

The apparent block in 1,25-$(OH)_2D_3$ effects was not due to impaired function of 1,25-$(OH)_2D_3$ or its receptor as demonstrated by the fact that 1,25-$(OH)_2D_3$ induced OP expression in all clones, another feature characteristic of osteoblastic cell populations (Yoon et al., 1987; Noda et al., 1990). Interestingly, the murine OP promoter also contains an AP-1 site but it does not overlap with the putative VDRE, suggesting that there may not be a direct interaction between these two elements in regulating OP gene transcription (Noda et al., 1990; Craig and Denhardt, 1991). The inhibition of OP expression by Dex in both clones supports previous observations in transformed osteoblastic cells (Yoon et al., 1987) and further suggests a role for glucocorticoid hormones in the regulation of bone cell activity. Finally, the regulation of OP gene expression by both 1,25-$(OH)_2D_3$ and Dex implies in addition that OP expression in these cells may be biologically relevant to the osteoblast phenotype rather than being a consequence of cellular transformation (Craig et al., 1989; Senger et al., 1989). With respect to the regulation of *fos*- and *jun*-related genes, Dex treatment revealed some interesting patterns of expression. The decrease in exogenous c-*fos* expression in osteogenic P1.15 cells but not in non-osteogenic P1.9 cells was paralleled by a decrease in

both *fra*-1 and c-*jun* expression. Although the biological significance of this result is not entirely clear, the results suggest a role for AP-1 transcription factor interactions in osteoblastic cells. Alternatively, we cannot rule out the possibility that the changes in immediate early gene expression are related to the possible effects of steroid hormones on cell proliferation.

Analysis of the clonal cell lines has suggested that while some phenotypic markers of osteoblastic cells are altered uniformly in c-*fos*–transformed cell lines, others are variable. The altered responsiveness to 1,25-$(OH)_2D_3$ strongly support the possibility that the differences in osteoblastic gene expression in these clones are related to the levels of AP-1 binding activity. In this regard, it will be interesting to determine whether the phenotypic heterogeneity in 1,25-$(OH)_2D_3$ responsiveness in different osteosarcoma cell lines (Spiess et al., 1986; Fournier and Price, 1991; see also Heersche and Aubin, 1990 for review) is related to levels of endogenous AP-1 activity. Indeed, we have also isolated c-*fos*–transformed clonal cells which express osteoblastic marker genes but are non-osteogenic, suggesting that different stages of osteoblast differentiation may be affected differently by high levels of Fos.

We have demonstrated that specific transformation of osteogenic cells is responsible for c-*fos*–induced tumorigenesis in transgenic mice. Together with the osteopetrosis which develops in mice lacking functional Fos protein (Wang et al., 1992; Johnson et al., 1992), these data point to a biologically important role for Fos in the regulation of bone development and bone-specific gene products.

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