Accelerated Cell Cycle Progression in Osteoblasts Overexpressing the c-fos Proto-oncogene

INDUCTION OF CYCLIN A AND ENHANCED CDK2 ACTIVITY*

Received for publication, September 12, 2003, and in revised form, December 10, 2003
Published, JBC Papers in Press, December 29, 2003, DOI 10.1074/jbc.M310184200

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Transgenic mice overexpressing the c-Fos oncoprotein develop osteosarcomas that are associated with deregulated expression of cell cycle genes. Here we have generated osteoblast cell lines expressing c-fos under the control of a tetracycline-regulatable promoter to investigate the role of c-Fos in osteoblast cell cycle control in vitro. Three stable subclones, AT9.2, AT9.3, and AT9.7, derived from MC3T3-E1 mouse osteoblasts, expressed high levels of exogenous c-fos mRNA and protein in the absence of tetracycline. Functional contribution of ectopic c-Fos to AP-1 complexes was confirmed by electrophoretic mobility shift assays and transactivation of AP-1 reporter constructs. Induction of exogenous c-Fos in quiescent AT9.2 cells caused accelerated S-phase entry following serum stimulation, resulting in enhanced growth rate. Ectopic c-Fos resulted in increased expression of cyclins A and E protein levels, and premature activation of cyclin A-, cyclin E-, and cyclin-dependent kinase (CDK) 2-associated kinase activities, although cyclin D levels and CDK4 activity were not affected significantly in these cell lines. The enhanced CDK2 kinase activity was associated with a rapid, concomitant dissociation of p27 from CDK2-containing complexes. Deregulated cyclin A expression and CDK2 activity was also observed in primary mouse osteoblasts overexpressing c-Fos, but not in fibroblasts, and c-Fos transgenic tumor-derived osteosarcoma cells constitutively expressed high levels of cyclin A protein. These data suggest that overexpression of c-Fos in osteoblasts results in accelerated S phase entry as a result of deregulated cyclin A/E-CDK2 activity. This represents a novel role for c-Fos in osteoblast growth control and may provide c-Fos-overexpressing osteoblasts with a growth advantage during tumorigenesis.

Hyperproliferative and neoplastic disorders of bone are characterized by increased production of bone and are generally caused by changes in the differentiation and/or function of bone-forming osteoblasts. The most common primary malignancy of the skeleton is osteosarcoma, and this provides one of the best examples of uncontrolled proliferation and deregulated growth control in osteoblasts. Although our understanding of the mechanisms of cellular growth control has increased rapidly in recent years, our knowledge of the genes that modulate osteoblast proliferation in the context of bone disease is still lacking. In this regard, we have previously shown that the c-fos proto-oncogene is important in bone biology, because its endogenous function is essential for normal bone development but its deregulated expression leads to bone disease (1, 2).

The c-Fos oncoprotein is a member of the AP-1 transcription factor, which consists of protein dimers of the Fos (c-Fos, FosB, Fra-1, and Fra-2), Jun (c-Jun, JunB, and JunD), and ATF families (3). Fos proteins can only form heterodimers with members of the Jun family, whereas Jun proteins can additionally homo- and heterodimerize with Jun, ATF, and CREB proteins (3). AP-1 lies at the nexus of many signaling pathways and transduces extracellular stimuli such as growth factors, cytokines, and environmental stress, modulating a variety of biological processes, including cell growth, death, differentiation, and oncogenic transformation (2–4). The activity of the AP-1 complex can be regulated at many levels, including changes in the expression levels of constituent proteins, dimerization partners, phosphorylation, and protein-protein interactions with other transcription factors, resulting in regulation of AP-1 target genes (5, 6).

Perturbations in AP-1 function by gain- and loss-of-function approaches can alter tissue homeostasis (2), and in the case of c-Fos, this is particularly evident in bone and cartilage. Mice lacking c-Fos develop osteopetrosis due to a block in the differentiation of bone-resorbing osteoclasts, demonstrating an essential role for c-Fos in the development of the osteoclast lineage and bone remodeling (7). In contrast, ubiquitous overexpression of c-Fos in transgenic mice results in the formation of osteosarcomas and chondrosarcomas due to transformation of osteoblastic and chondroblastic cells, respectively (8, 9). Overexpression of the c-Fos-related proteins, Fra-1 and ΔFosB, in transgenic mice caused enhanced bone formation and osteosclerosis, without any associated malignancy (10, 11). High levels of c-Fos have also been observed in many primary and metastatic human osteosarcomas (12–14), as well as in osteoblasts of patients with Paget’s disease, a bone disorder in which a significant proportion of cases develop secondary osteosarcoma (15). Moreover, osteosarcomas often contain mutations in, or have altered levels of p53, pRb, cyclin D1, CDK4, MDM2, and p16 proteins (12, 16–18). In c-Fos transgenic mice, which develop osteosarcomas, we previously demonstrated that cyclin D1 protein is not generally detectable in osteoblasts but becomes markedly elevated upon initial expression of the c-Fos transgene, prior to tumor formation (19). Because oncogenes...
can directly regulate the expression and activity of key components of the cell cycle machinery, these data strongly suggest that changes in cell cycle gene expression may be involved in the initial stages of c-Fos-induced deregulated osteoblast growth and malignant bone disease.

The control of the eukaryotic cell cycle ensures that all the steps that occur in cell division proceed in the correct temporal order. Cell cycle progression is controlled by cyclins, cyclin-dependent kinases (CDKs) and CDK inhibitors (CKIs) (20, 21). CDKs are serine/threonine kinases, which associate with specific cyclin partners and phosphorylate key substrates to facilitate cell cycle progression. The levels of cyclins oscillate throughout the cell cycle: Cyclin D family members are expressed in G1, cyclin E at G1/S, cyclin A in late G1/S, and G2/M phases, and cyclin B in S and G2 phases. Two CKI families exist that can prevent activation of the cyclins and uncontrolled replication, the Cip/Kip (p21, p27, and p57) and the INK4 (p15, p16, p18, and p19) families (21). Cyclin-CDK complexes phosphorylate a number of specific targets, the best characterized being the retinoblastoma family proteins, pRb, p107, and p130. Phosphorylated pRb proteins dissociate from E2F family members, allowing active E2F to regulate the expression of genes required for cell cycle progression (20, 21).

Cell cycle regulation in bone has not been widely studied. We have observed high endogenous levels of cyclin E in vitro in differentiated osteoid osteoblasts of mouse long bones (19), and others have shown that cyclins E and B are up-regulated during differentiation of primary rat osteoblasts in vitro (22). Recent evidence also suggests that cyclin A-CDK2 complexes may also function in the regulation of clonal expansion during osteoblast differentiation in vitro (23). Primary osteoblasts from p27 null mice form larger colonies and produce larger mineralized bone nodules in vitro, suggesting that this CKI plays an important role in osteoblast differentiation (24). Furthermore, parathyroid hormone, a major regulator of bone metabolism, has been shown to inhibit osteoblast proliferation by increasing p27 levels and inhibiting CDK2 activity (25).

An important role for AP-1 in mediating growth factor-induced cell cycle progression has been demonstrated, because cyclin D1 was shown to be an AP-1-responsive gene (3, 20, 26). Induced cell cycle progression has been demonstrated, because loss-of-function, either by antibody microinjection or in double c-Fos/FosB knockout fibroblasts, causes a failure to induce cyclin D1 and retards cell cycle entry (33, 34). Paradoxically, c-Fos has also been shown to inhibit the expression of cyclin D1, CDC2 and CDC4 (35), whereas c-Fos overexpression in B-lymphocytes correlates with increased p27 stability and a delay in S-phase entry (36). These apparently contradictory reports may reflect cell type-specific effects of c-Fos. Our previous in vitro data would suggest that any effect of c-Fos on cell cycle control may be most physiologically relevant in osteoblastic cells, where deregulated c-Fos expression causes dramatic neoplastic changes (9, 19).

To gain a better understanding of the role of c-Fos in osteoblast growth control, we have focused on cell cycle regulation in osteoblasts in vitro. To this end, we have constructed an inducible c-Fos expression vector and obtained stable transfectants of an immortal osteoblastic cell line. Using this as an in vitro model, we have identified key points of cell cycle control that are regulated by c-Fos in osteoblasts.

### EXPERIMENTAL PROCEDURES

**Antibodies and Plasmids**—Antibodies used were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) as follows: cyclin A (sc-596), cyclin D1 (sc-717), cyclin D3 (sc-182), cyclin E (sc-198), CKD2 (sc-163), CKD4 (sc-260), CKD6 (sc-177), p21 (sc-397), p27 (sc-525), c-Fos (sc-52, sc-52X), Fox-B (sc-48), Fra-1 (sc-183), pan-Fos family (sc-411X), c-Jun (sc-179), activated rabbit immunoglobulins (IX007) and secondary antibody horseradish peroxidase conjugates were obtained from Dako (Denmark) against immunoglobulins from mouse (P0447), rabbit (P0449), or goat (P0448).

The plasmids used were pMFP2-c-fos and pMFP2-luc, where the murine c-fos cDNA and luciferase, respectively, are under the negative control of tetracycline (Te), and p7621, which constitutively expresses murine genomic c-fos (64). pCMV-SPAP (37), which contains the secreted placent al alkaline phosphatase (SPAP) gene under the control of a CMV promoter was used as a transfection control, and AP-1 transactivation studies were performed using pColCAT and pmColCAT, containing +517 to +63 nucleotides of the human collagenase promoter with a wild-type or mutated AP-1 site, respectively (kind gifts of Dr. Peter Angel, Deutsches Krebsforschungszentrum, Heidelberg, Germany).

**Cell Culture and Transfections**—Murine MC3T3-E1 osteoblastic cells and primary murine osteoblasts were cultured in α-minimal essential medium (α-MEM plus ribonucleosides/deoxyribonucleosides, Sigma Chemical Co., Poole, UK) supplemented with 10% fetal calf serum (FCS; Mucedo, Hants, UK), antibiotics (penicillin 50 units/ml, streptomycin 50 µg/ml, and l-glutamine (5 mM) (Invitrogen, Paisley, UK). NIH3T3 fibroblasts as well as the osteosarcoma cell lines P1.7, P1.9, and P1.15, which were isolated from bone tumors formed in c-fos-overexpressing transgenic mice (9), were cultured in Dulbecco’s modified Eagle’s medium (Dulbecco) supplemented with 5% calf serum (NIH3T3 cells) or 10% FCS (osteosarcoma cells), antibiotics and l-glutamine. Primary osteoblasts were isolated according to established protocols (38).

MC3T3-E1 cells were stably transfected with the expression vector pMFP2-c-fos using SuperFect according to the manufacturer’s instructions (Qiagen, Crawley, UK). Stable clones were selected in medium containing 0.5 mg/ml G418 (Invitrogen) and in the presence of 10 µg/ml Te (Sigma) to inhibit transgene expression. Twenty-three G418-resistant clones were isolated, of which 10 were screened by Northern blotting as described below. Additional control clones were isolated from MC3T3-E1 cells transfected with pMFP2-luc, which expresses luciferase under Te control. Twenty-two G418-resistant clones were isolated, and 11 were screened for Te-regulatable luciferase activity by the “Enhanced and Glow” system (Promega, Southampton, UK) according to the manufacturer’s instructions.

Transient transfections were performed using Effectene as specified by the manufacturers (Qiagen). CAT activity was measured using a CAT enzyme-linked immunosorbent assay kit (Roche Applied Science, Lewes, UK), and SPAP activity was measured as described previously (37). Primary mouse osteoblasts and NIH3T3 mouse fibroblasts were transfected with the c-fos expression vector p7621, carrying the murine genomic c-fos gene (39).

For serum deprivation and re-stimulation experiments, cells were plated at 2 × 10^4 cells/cm², in α-MEM containing Te (10 µg/ml) for 48 h and were made quiescent by serum deprivation for 24 h in α-MEM containing 0.5% FCS, antibiotics, l-glutamine, 3 × 10⁻³ M sodium selenite, 10 µg/ml human transferrin (Sigma), and 10 µg/ml Tc. Cells were then incubated in α-MEM containing 0.5% FCS with or without Te as above for 48 h, and then stimulated with α-MEM containing 20% FCS with or without Tc (10 µg/ml). Total cellular extracts were prepared between 0 and 48 h as indicated in each figure, and the 0-h time point refers to cells that are quiescent, i.e., before the addition of media containing 20% serum. For [³H]thymidine uptake measurements, quiescent MC3T3-E1, AT7.8, and AT9.2 cells were cultured in 6-well plates and serum stimulated in media containing 1 µCi/ml [³H]thymidine (specific activity of 50 Ci/mmol) (Amersham Biosciences International, UK) for 12 h. Cells were fixed and washed in ice-cold 10% trichloroacetic acid. Cells were then washed with ice-cold 1 M Meldrum, Hants, UK), antibiotics (penicillin 50 units/ml, streptomycin 50 µg/ml, and l-glutamine (5 mM) (Invitrogen, Paisley, UK). NIH3T3 fibroblasts as well as the osteosarcoma cell lines P1.7, P1.9, and P1.15, which were isolated from bone tumors formed in c-fos-overexpressing transgenic mice (9), were cultured in Dulbecco’s modified Eagle’s medium (Dulbecco) supplemented with 5% calf serum (NIH3T3 cells) or 10% FCS (osteosarcoma cells), antibiotics and l-glutamine. Primary osteoblasts were isolated according to established protocols (38).

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### Flow Cytometry

Cells were processed for flow cytometry as previously described (40). Cells were harvested at the times indicated, fixed
in 70% EtOH, stained with propidium iodide (Sigma), and analyzed using a MoFlo flow cytometer (Cytometry Bioinstruments, Freiburg, Germany) with doublet correction. The data were analyzed using the WinMDI program (University of Wisconsin).

Northern Blotting—Poly(A) mRNA was isolated, Northern-blotted, and hybridized with 32P-labeled probes as described previously (39). Blots were hybridized to a v-fos probe, which recognizes both the exogenous and endogenous forms of c-fos as well as fosB, fosA, and a murine housekeeping gene that cross-hybridizes to v-fos, and which was used as a loading control (39).

Protein Extraction, Western blotting, EMSA, Immunoprecipitation, and Kinase Assays—Cellular protein was extracted for use in Western blotting, EMSA, immunoprecipitation, and kinase assays. Cells were lysed in 1 ml of ice-cold lysis buffer (50 mM HEPES, 2.5 mM EDTA, 10 mM MgCl2, 10 mg/ml poly[dI-dC], pH 7.5), for 1 h. After sonication, protein was diluted to 1 ml in lysis buffer, 1 μg of antibody for 1 h on ice. Complexes were resolved by SDS-PAGE, run on 5%—7.5% gels, and transferred to nitrocellulose membranes (Amersham Biosciences UK). Western blotting experiments were performed using a Mulflo flow cytometer (Cytomation Bioinstruments, Freiburg, Germany) with doublet correction. The data were analyzed using the WinMDI program (University of Wisconsin).

RESULTS

Generation of Stable Osteoblastic Cell Lines with Inducible c-fos Expression.—To establish an in vitro inducible system for studying the effects of c-fos on cell cycle regulation, we transfected the immortalized, non-transformed mouse osteoblast-like cell line, MC3T3-E1, with the Tc-regulatable construct pMFP2-c-fos (39), and selected stable transfectants. From 10 independent stable clones, which were screened by Northern blotting in the presence and absence of Tc, three clones, designated AT9.2, AT9.3, and AT9.7, were isolated, which expressed high levels of the exogenous 1.8-kb c-fos mRNA in the absence of Tc, but not in 10 μg/ml Tc (Fig. 1A). Interestingly, none of the clones shown here expressed the 2.2-kb endogenous c-fos transcript under routine culture conditions. Western blotting experiments demonstrated that clones AT9.2, AT9.3, and AT9.7 all expressed high levels of c-Fos protein, which was repressible by Tc, whereas the parental cell line MC3T3-E1 failed to express exogenous c-fos in the absence of Tc (Fig. 1B). The Western blot results also confirmed that the three Tc-inducible clones expressed no, or undetectable levels of endogenous c-Fos (i.e. –Tc), as did the parental cell line MC3T3-E1 either with or without Tc. Stable clones of MC3T3-E1 were also isolated after transfection with pJMF2-luc. From 11 G418-resistant clones, 5 expressed luciferase, and 1 specific clone, AT7.8 showed high luciferase activity coupled with Tc responsiveness, which was used as a transfection control cell line (data not shown; see also Fig. 1 (C and D)). For the majority of the experiments described here, the AT9.2 clone was used.

Exogenous c-Fos Protein Binds to AP-1 Consensus Oligonucleotides and Transactivates AP-1-Responsive Reporter Constructs.—To confirm that the exogenous c-Fos protein expressed in AT9.2 cells was present in active AP-1 complexes, EMSA and supershift assays were performed. EMSA experiments demonstrated a specific band shift corresponding to the AP-1 complex, which disappeared upon the inclusion of a specific AP-1 competitor oligonucleotide, but not with a nonspecific AP-2 oligonucleotide, demonstrating AP-1 specificity (Fig. 1C). Supershift analysis using a control antibody (rabbit Ig) showed that the parental cell line MC3T3-E1, the control clone AT7.8, and the c-Fos-expressing clone AT9.2 all contain high levels of AP-1 binding activity in exponentially growing cells irrespective of the presence or absence of Tc (Fig. 1C). Supershift analyses demonstrated that the AP-1 binding activity in all cell lines contained Fos family members (Fig. 1C, pan-Fos), however, only AT9.2 cells cultured in the absence of Tc exhibited a defined supershift with a Fos-specific antibody (Fig. 1C, c-Fos). These data demonstrate that the induced exogenous c-Fos protein in AT9.2 cells is able to participate in active AP-1 binding complexes. Furthermore, these data also confirm the absence of detectable endogenous c-Fos and suggest that the other Fos family members must contribute to AP-1 binding activity (see below).

To further confirm the functionality of exogenous c-Fos in the AP-1 complexes, we performed transfection experiments using AP-1-responsive CAT reporter constructs. MC3T3-E1, AT7.8, and AT9.2 cells were transfected with either pColCAT or pmColCAT, which harbors an inactivating mutation in the AP-1 site. The results shown in Fig. 1D clearly demonstrate an increase of 4-fold in collagenase promoter activity in AT9.2 cells following Tc withdrawal (i.e. after exogenous c-Fos induction) but not in MC3T3-E1 or AT7.8 cells. Very low basal CAT expression was observed following transfection with pmColCAT (Fig. 1D). Higher basal levels of CAT activity from the pColCAT vector in all cell lines confirmed the presence of AP-1 binding activity shown earlier. Thus, these experiments clearly demonstrate that the exogenous c-Fos protein induced in AT9.2 cells following Tc withdrawal is capable of regulating the expression of AP-1 target genes.
the expression of Fra-1 and FosB/FosB by Western blotting, because both these Fos-related proteins share some biological functions with c-Fos (10, 11, 34, 42). In quiescent AT9.2 cells grown in the presence of Tc the levels of c-Fos followed classic immediate early gene kinetics: c-Fos expression was negligible in quiescent cells (0 h) but rose dramatically after 4 h following serum stimulation, then was reduced by 8 h, and was barely detectable thereafter (Fig. 2). However, in the absence of Tc, c-Fos expression was readily detectable in unstimulated, quiescent cells (0 h), reflecting expression of exogenous c-Fos. After 4 h following serum stimulation, c-Fos expression was higher than in cells cultured in the presence of Tc, representing both endogenous and exogenous c-Fos proteins. However, this high level was sustained over the 48-h time course in the absence of Tc, representing deregulated expression of exogenous c-Fos protein (Fig. 2, −Tc). Other c-Fos family members, FosB and Fra-1, were also regulated by serum. FosB levels displayed similar induction kinetics to c-Fos in the presence of Tc, with a slower migrating form of FosB detectable after 24 h (Fig. 2). In the presence of ectopic c-Fos, however, the induction of the higher molecular weight band was observed by 8 h, earlier than in control cells, and FosB expression was absent by 24 h (Fig. 2). The induction kinetics of Fra-1 in cells cultured in Tc was delayed when compared with those of c-Fos and FosB, with the peak of expression occurring after 8 h. However, in the presence of exogenous c-Fos there was a clear induction of Fra-1 expression in quiescent cells, which persisted for longer
compared with controls (Fig. 3, E). Periods of time following serum stimulation (Fig. 2, to determine whether ectopic c-Fos expression in osteoblasts affected cell growth, we performed growth curve analyses and determined cell cycle distribution of AT9.2 cells grown in the presence or absence of Tc. In non-synchronized cultures (10% FCS), expression of exogenous c-Fos had no effect on cell cycle protein expression (data not shown) or cell growth (Fig. 3A), which is not surprising in view of the large number of mitogens present in serum-containing media. However, under reduced serum concentrations (1% FCS) we did observe a mitogenic effect following Tc withdrawal (Fig. 3B), suggesting that c-Fos can stimulate cell proliferation. This was further shown by quantifying cell number and thymidine incorporation following serum stimulation of quiescent AT9.2 cells in the presence and absence of Tc, which showed a greater than 2-fold increase in c-Fos-expressing cultures, but not in control AT7.8 cells (Fig. 3, C and D). To investigate the cell cycle kinetics in these cultures, parallel cultures were harvested during the stimulation time course, and the cell cycle phase distribution was analyzed by flow cytometry. AT9.2 cells overexpressing exogenous c-Fos displayed accelerated S-phase entry, which was evident as early as 4 h and being most notable 24 h after stimulation, when the majority of cells had already left G0/G1, compared with controls (Fig. 3, E and F). These data demonstrate a clear cellular effect and strongly indicate that exogenous c-Fos provides a growth advantage to osteoblasts.

Regulation of Cell Cycle Proteins by Exogenous c-Fos—We next used Western blotting to determine whether ectopic c-Fos expression resulted in changes in protein expression of key cell cycle regulators, which may explain the observed accelerated S-phase entry. In non-synchronized cultures, expression of exogenous c-Fos had no effect on cell cycle protein expression (data not shown), consistent with the growth curve observations in 10% serum. We therefore analyzed the effects of exogenous c-Fos induction in quiescent cells and after serum stimulation. In the absence of c-Fos induction, cyclins A, E, and D were expressed at low levels during quiescence, but became elevated following serum stimulation (Fig. 4A, +Tc). The most marked effect of c-Fos overexpression induction was deregulated cyclin A expression: Cyclin A levels were high in quiescent cells following c-Fos induction (−Tc), which were maintained throughout the time course, although the maximal levels of cyclin A were not greater than those in uninduced cells (Fig. 4A). Elevated levels of cyclin E were also observed in quiescent c-Fos-overexpressing cells, which subsequently became reduced to below control levels after 48 h (Fig. 4A). In contrast, no significant correlation was observed between D-type cyclin levels and exogenous c-Fos expression (Fig. 4A).

Examination of CDK expression revealed that induction of ectopic c-Fos did not generally affect CDK2, −4, or −6 levels (Fig. 4B). However, analysis of CKIs of the Cip/Kip family demonstrated altered expression patterns following exogenous c-Fos induction in AT9.2 cells. In the absence of exogenous c-Fos, p21 was expressed at very low levels in quiescent cells, which increased dramatically over 24–48 h, whereas p27 levels were low at quiescence and remained low throughout cell cycle reentry (Fig. 4C, +Tc). Induction of exogenous c-Fos caused an increase in p21 levels from quiescence to 8 h post-stimulation, however, the levels of p21 after 24 and 48 h were lower than in control cultures (Fig. 4C, −Tc). Ectopic expression of c-Fos also caused a slight elevation in p27 expression, most notably in quiescent cells (Fig. 4C, −Tc). Taken together, these data suggest that ectopic c-Fos expression alters the expression levels of specific cell cycle proteins, which may contribute to the observed accelerated S-phase entry.

c-Fos Modulation of CDK2- and CDK4-associated Kinase Activities and Association with p27—To determine whether ectopic c-Fos expression regulated CDK2 activity, immunoprecipitation kinase assays were performed to assess the ability of these complexes to phosphorylate a recombinant GST-Rb fusion protein. When quiescent AT9.2 cells were stimulated with 20% serum to re-enter the cell cycle, CDK2-associated kinase activity was elevated in ectopic c-Fos-expressing cells at all time points compared with control cells, peaking at 16 and 24 h (Fig. 5A). Because both cyclin A and cyclin E contribute to active CDK2-containing kinase complexes in G1 and S-phases, and because both these cyclins were expressed at higher levels following exogenous c-Fos induction, we next sought to determine whether the increased CDK2 kinase activity was associated with either cyclins A or E. The levels of cyclin A-associated kinase activity were increased in ectopic c-Fos-expressing cells, peaking at 16–24 h post stimulation, compared with 24 h in the control cells (Fig. 5B). These kinetics paralleled closely those observed for CDK2-associated kinase activity suggesting that cyclin A-CDK2 complexes may predominate in the presence of exogenous c-Fos. The levels of cyclin E-associated kinase activity were also altered following Tc withdrawal, albeit to a lesser extent; Cyclin E kinase activity peaked earlier in the presence of ectopic c-Fos compared with control cells (Fig. 5C). In combination with the elevated cyclin A-CDK2 activity, these data provide one explanation for the observed accelerated S-phase entry following exogenous c-Fos induction. Consistent with this, we observed that there was a more rapid disappearance of unphosphorylated pRb in the presence of exogenous c-Fos, with almost all pRb being in the hyperphosphorylated state by 12 h following serum stimulation, compared with 16 h in control cells (Fig. 5D).

Because previous results have implicated a role for p27 in modulating osteoblast proliferation (24, 25), we determined the extent of complex formation between p27 and CDK2. Western blotting for CDK2 following immunoprecipitation with an anti-p27 antibody indicated an increased association between CDK2 and p27 in quiescent cells expressing exogenous c-Fos compared with control cells (Fig. 5E). Following serum stimulation, the extent of p27 binding to CDK2 in cells lacking ectopic c-Fos remained relatively unchanged, whereas the p27-CDK complexes formed in the presence of ectopic c-Fos rapidly dissociated over the 24 h following stimulation (Fig. 5E). Interestingly, the kinetics of p27 dissociation from CDK2 closely paralleled the increase in CDK2 kinase activity observed earlier (Fig. 5A, −Tc).

Finally, we performed immunoprecipitation kinase assays to investigate whether CDK4 complexes were altered following induction of exogenous c-Fos. CDK4-associated kinase activity in the absence of exogenous c-Fos became elevated after 8 h, rising to a maximum at 16 h, whereas CDK4 activity in the presence of c-Fos remained low between 0 and 8 h, but remained maximal at 16 h (Fig. 5F). The overall CDK4 activity was not elevated relative to controls at any point by exogenous c-Fos expression. Indeed, at earlier time points (0–12 h) levels were in fact slightly reduced. Western blot analysis confirmed that equivalent levels of CDK4 had been immunoprecipitated. We have also performed similar immunoprecipitation kinase assays to determine the activity of CDK6-containing complexes, which similarly failed to show any c-Fos-dependent increases in activity (data not shown). Taken together, these
data suggest that the regulation of cyclin A/E-CDK2-p27 complexes provides one possible explanation for the observed accelerated cell cycle progression in the presence of ectopic c-Fos.

**Regulation of Cyclin A Expression by Ectopic c-Fos in Primary Mouse Osteoblasts and c-Fos-transformed Osteoblasts**

To confirm that the effect of ectopic c-Fos to regulate cyclin A-CDK2 activity occurs in other independent c-Fos-expressing clones, and to rule out potential clonal variability, we tested two additional stable clones that express inducible c-Fos, AT9.3 and AT9.7. Following Tc withdrawal, there was a clear increase in cyclin A expression in quiescent AT9.2 cells cultured in the presence (+) or absence (-) of Tc were harvested at the indicated times, fixed in 70% ethanol, and analyzed by flow cytometry for quantification of cell cycle phase distribution (G0/G1, S, and G2/M).

**Fig. 3.** AT9.2 cells overexpressing exogenous c-Fos display increased cell proliferation and accelerated S-phase entry. Growth curve analysis of AT9.2 cells ± Tc grown in (A) 10% FCS or in (B) 1% FCS. C, AT9.2 cells were made quiescent in low serum ± Tc and were harvested at the indicated times following serum stimulation and cell numbers were quantified. D, AT7.8 and AT9.2 cells ± Tc were labeled with [3H]thymidine, and DNA synthesis was measured in acid-precipitable material as described under “Experimental Procedures.” Quiescent AT9.2 cells cultured in the presence (E) or absence (F) of Tc were harvested at the indicated times, fixed in 70% ethanol, and analyzed by flow cytometry for quantification of cell cycle phase distribution (G0/G1, S, and G2/M).
Role of c-Fos in Osteoblast Cell Cycle Control

FIG. 4. Modulation of cell cycle protein expression by exogenous c-Fos. AT9.2 cells were made quiescent in low serum (−Tc), and total cell extracts were prepared between 0 and 48 h following serum stimulation. Western blotting was performed using antibodies to cyclin A, cyclin E, cyclin D1, cyclin D2, and cyclin D3 (A); CDK2, CDK4, CDK6 (B); and p21 and p27 (C). The Western blots shown are representative of three independent experiments, which gave similar results. Even loading of the gels was confirmed by transillumination of the PVDF filter and by Western blotting with an actin antibody.

cent cells (0 h) in all of the c-Fos-overexpressing clones, and this was paralleled by an increase in CDK2-associated kinase activity 16 h after serum stimulation (Fig. 6A). To investigate whether the increased expression of cyclin A in response to exogenous c-Fos also occurs in primary murine osteoblasts, and to further define cell type specificity, we transiently transfected primary mouse osteoblasts and NIH3T3 mouse fibroblasts with c-fos. Quiescent osteoblasts transfected with control DNA expressed low levels of cyclin A, whereas osteoblasts transfected with c-fos showed elevated cyclin A protein expression (Fig. 6B). In contrast, transfection of c-fos into NIH3T3 fibroblasts did not result in up-regulated cyclin A expression in quiescent cells (Fig. 6B). Following serum stimulation, cyclin A expression was readily detectable in all cells consistent with their cell cycle re-entry (Fig. 6B). Finally, we investigated the implications of altered cyclin A in the context of c-Fos-induced osteoblast transformation, by analyzing cyclin A protein levels in c-Fos-overexpressing osteoblastic cells derived from c-Fos transgenic osteosarcomas: These clonal cell lines, designated P1.7, P1.9, and P1.15, have been shown previously to express exogenous c-fos and to induce osteogenic tumors when injected into nude mice (9). Western blot analysis demonstrated that, in contrast to primary wild-type osteoblasts, the three independent c-Fos-overexpressing clones showed high basal levels of cyclin A expression at quiescence, which were further enhanced upon serum stimulation (Fig. 6C). These data suggest that overexpression of c-Fos may result in deregulation of cyclin A in a cell-specific manner, in this case, osteoblasts but not fibroblasts, and that this may be a critical step for c-Fos-induced transformation of osteoblasts.

DISCUSSION

In this study, we have used a tetracycline-regulatable system and have demonstrated that c-Fos plays a key role in osteoblast cell cycle control. Following Tc withdrawal, the osteoblastic clones AT9.2, AT9.3, and AT9.7 all expressed high levels of functional exogenous c-Fos protein that could transactivate AP-1 reporter genes. Endogenous c-Fos expression was not detectable in cycling cells, and although it could be induced with classic immediate early gene kinetics, the observed effects of c-Fos induction on cell cycle protein expression were manifested predominantly at quiescence and during its high constitutive expression when the endogenous gene was not expressed. Thus, the observed cellular and molecular changes are likely to be a direct consequence of deregulated expression of the c-fos transgene.

c-Fos-induced Accelerated Cell Cycle Progression Is Dependent upon Cyclin A/CDK2—The most dramatic effect of c-Fos overexpression was the constitutive up-regulation of cyclin A protein levels, with a corresponding increase in cyclin A- and CDK2-associated kinase activity. This was confirmed in clones AT9.3, AT9.7, and, more importantly, in primary osteoblasts, thus providing the first demonstration that c-Fos can regulate cyclin A levels in osteoblasts. The maximal levels of cyclin A protein in the presence of c-Fos were not higher than those in non-induced cells, suggesting that it is the deregulated expression of cyclin A, rather than its overexpression that was responsible for the accelerated entry into S-phase. Deregulated cyclin A expression has been shown previously to function as a G1 cyclin, because overexpression of cyclin A can cause premature S-phase entry (43, 44). Several reports have demonstrated that cyclin A is a target for c-Fos and AP-1 in other cells, including chondrocytes, although the mechanisms may be indirect, involving the CRE and E2F sites within the cyclin A promoter (45–49). Our preliminary results have suggested that induction of exogenous c-Fos in AT9.2 cells causes an increase in cyclin A promoter activity, although the involvement of the CRE site is not yet known.2 Interestingly, recent experiments in NIH3T3 fibroblasts using tethered Jun-Fos proteins have demonstrated that Jun–Fra-2 rather than Jun–c-Fos dimers predominantly regulate cyclin A expression (50). Thus, the preference for different dimer combinations to regulate cyclin A levels might depend on the cellular context. Indeed, we have also shown here that overexpression of c-Fos in NIH3T3 cells failed to induce cyclin A expression, whereas induction was observed in primary osteoblasts. Our data therefore suggest that active cyclin A-CDK2 complexes are predominantly formed in c-Fos-overexpressing osteoblasts, and this is likely to be a major contributing factor to premature S-phase entry.

Induction of exogenous c-Fos in AT9.2 cells also resulted in elevated levels of cyclin E protein, albeit to a lesser extent than cyclin A. This is unlikely to be a direct effect, because there is little evidence for direct transcriptional regulation of cyclin E by AP-1. In contrast to cyclin A levels, however, cyclin E levels were not constitutively high, but became reduced at later times compared with control cells. It is well established that cyclin E transcription is rapidly down-regulated and the protein undergoes proteolysis once cells have entered S-phase (51). Thus, our observed decrease in cyclin E levels at later time points would predict that the majority of AT9.2 cells overexpressing c-Fos

2 A. Sunters and A. E. Grigoriadis, unpublished observations.
Fig. 5. c-Fos modulation of CDK2 and CDK4 activities and complex formation. AT9.2 cells were made quiescent by serum starvation. Western blots were performed using antibodies to CDK2 and cyclin A, and CDK4 (P) and the ability to phosphorylate a recombinant C-terminal GST-Rb fusion protein in vitro was measured as described under "Experimental Procedures." Immunoprecipitations with normal rabbit immunoglobulin (Ab) or CDK2 were used as negative and positive controls, respectively, and the levels of CDK2, CDK4, and p27 were determined by Western blotting. D, Western blot analysis of pRb and phosphorylated pRb (pRb) levels following serum stimulation. Immunoprecipitation kinase assays were performed as described in Fig. 5. A, Western blot analysis of cyclin A and actin, and CDK2 immunoprecipitation kinase assays were performed using antibodies to CDK2 and p27. All autoradiographs shown are representative of three independent experiments, which gave similar results.

Fig. 6. Altered cyclin A expression in c-Fos-overexpressing primary osteoblasts and in c-Fos-transformed osteosarcoma cells. A, AT9.2, AT9.3, and AT9.7 cells were made quiescent in low serum +Tc and total cell extracts were made at 0 and 16 h following serum stimulation. Western blots were performed using antibodies to cyclin A and actin, and CDK2 immunoprecipitation kinase assays were performed as described in Fig. 5. B, Western blot analysis of cyclin A and actin protein levels in primary mouse calvarial osteoblasts (1° OBs) and NIH3T3 fibroblasts transfected with either pcDNA3 (mock), or a c-fos expression vector, p76/21 (c-Fos). Total cell extracts were made at 0 and 16 h following serum stimulation, and the transfection efficiency was confirmed by measuring SPAP activity as described under "Experimental Procedures." C, Western blot analysis of cyclin A and actin protein expression in primary mouse calvarial osteoblasts (1° OBs) and c-fos transformed osteosarcoma cells (P1.7, P1.9, and P1.15) derived from c-fos transgenic osteosarcomas (9). Total cellular extracts were made at 0 and 16 h following serum stimulation.

have passed through S-phase earlier than control cells, and this is indeed what was observed by flow cytometry. The increase in cyclin E-associated kinase activity may also contribute to the c-Fos-induced accelerated S-phase entry, and the kinetics of cyclin E activity correlated well with the flow cytometry analysis. Overexpression of cyclin E and increases in cyclin E-CDK2 activity have been shown previously to accelerate G1 progression, although this may require other active kinase complexes to phosphorylate pRb (52–54). Thus, it remains possible that the effects on S-phase entry caused by cyclin E activity are secondary to those caused by the increases in cyclin A-CDK2 activity.

The lack of correlation between exogenous c-Fos induction and D-type cyclin protein levels was somewhat unexpected, because cyclin D1 has been shown to be an AP-1 target in fibroblasts and chondrocytes in vitro (20, 28, 34, 55, 56). Moreover, we have previously seen a strong correlation in transgenic mice between induction of the c-Fos transgene and induction of cyclin D1 protein expression in osteoblasts (19), although it is yet to be proven that the altered cyclin D1 expression contributes functionally to c-Fos-induced tumor formation. It is possible that, in AT9.2 cells, the serum-induced increases in cyclin D1 protein levels are maximal, such that any further increases by exogenous c-Fos are not observed, or there may be additional translational or post-translational regulation of cyclin D1 in these cells. Alternatively, because c-Fos and FosB can substitute for each other in regulating cyclin D1 expression in fibroblasts (34), it is possible that both these Fos family members may be required for determining the c-Fos-responsiveness of cyclin D1 in AT9.2 cells (see also below). The absence of changes in cyclin D protein levels correlated with the lack of enhancement of CDK4 and CDK6 activity in c-Fos-overexpressing cells, suggesting that cyclin D-CDK4/6 activity is unlikely to play a role in the observed enhanced S-phase entry observed in AT9.2 cells. The lack of enhanced cyclin D1 expression was also observed in the other independent clones, AT9.3 and AT9.7, following induction of exogenous c-Fos (data not shown). Although cyclin D-CDK4/6 are classic regulators of pRb phosphorylation and G1 progression, the G1-S transition can also occur in the absence of cyclin D-CDK4 activity, especially in the presence of deregulated cyclin E activity such as in AT9.2 cells overexpressing c-Fos (52–54, 57). Our data are also consistent with the recent observations that v-Jun-induced cell
cycle entry in chick embryonic fibroblasts is typified by activation of CDK2 but not CDK4/6 (58).

Taken together, our studies suggest that, in AT9.2 osteoblasts, the altered growth control induced by exogenous c-Fos can be explained in part by deregulation of cyclins A and E levels and enhanced cyclin A/E-CDK2 activity and that this can occur in a cyclin D-CDK4-independent fashion.

p27 protein levels were increased in c-Fos-overexpressing AT9.2 cells at quiescence and co-immunoprecipitation experiments demonstrated that c-Fos promoted the association of p27 with CDK2, which rapidly dissociated upon cell cycle re-entry paralleling the increase in cyclin A/E-CDK2 activity. The increased p27 protein levels in the presence of c-Fos may be due to increased p27 protein stability, because a similar effect of c-Fos has been observed in c-Fos-overexpressing B cells (36). Because the protein half-life and timing of p27 degradation is crucial for regulating CDK2 activity (44, 59), our data strongly suggest that, in addition to deregulated cyclin A levels, the amount of p27 present in complexes may be critical in regulating c-Fos-dependent cell cycle progression by contributing to the enhanced CDK2 activity.

The levels of p21 in AT9.2 cells were also higher with exogenous c-Fos at quiescence, and this may have contributed, in part, to the suppressed CDK4 activity. We also observed reduced p21 levels at later time points in the presence of c-Fos, and this is consistent with the general notion that AP-1 inhibits p21 expression: Induction of c-Fos using a c-Fos/estrogen receptor fusion construct inhibited p21 transcription via a putative AP-1 site in the p21 promoter (60), and c-Jun dimers can suppress p21 expression in a p53-dependent manner (28). It is unlikely that the regulation of p21 in AT9.2 cells involves p53, because we have not detected p53 protein expression in these cells (data not shown). However, the regulation of p21 expression is complex, and it remains possible that the observed increases in p21 levels are indirect, involving activation of alternative signaling pathways that regulate p21 transcription and protein stability (61, 62).

**Cell Cycle, Osteoblast Differentiation, and Transformation—** Cyclins A and E and CDK2 activity may well have important roles in osteoblastic cells which go beyond cell cycle regulation. We and others (19, 22) have shown that cyclin E is expressed in roles in osteoblastic cells which go beyond cell cycle regulation. Cyclins A and E and CDK2 activity may well have important correlates with enhanced cyclin A-CDK2 activity.3 Together, c-Fos-induced increase in post-confluence growth of AT9.2 cells A-CDK2-E2F4-p130 complexes and a reduction of cyclin A-

Reference patterns of Fra-1 and FosB were altered in the presence of high exogenous c-Fos. Fra-1 is well established as a c-Fos target gene (42), and recent transgenic rescue experiments as well as gene knock-in studies have demonstrated unequivocally that Fra-1 can substitute for some, but not all the functions of c-Fos (65). The overall time course of FosB expression was also enhanced in the presence of c-Fos, and it is not yet known whether the higher molecular weight species represents cell cycle-dependent phosphorylation of FosB (e.g. Ref. 55). It remains to be seen whether the changes in Fra-1, FosB, or any other Fos/Jun/ATF family member participate in the c-Fos regulation of cyclin A/E expression and CDK2 kinase activity in osteoblasts. Although FosB can substitute for c-Fos in cell cycle regulation in fibroblasts (34), previous gain-of-function analyses suggest that neither up-regulation of Fra-1 nor FosB alone will explain the full effects on osteoblast transformation and osteosarcoma formation that are observed by deregulated c-Fos in vivo. FosB transgenic mice do not develop any tumors (9), and neither do transgenic mice overexpressing Fra-1 or ΔFosB, despite exhibiting marked changes in osteoblast activity leading to osteosclerosis (10, 11). Thus, although Fos family members share some overlapping functions on osteoblast differentiation and/or activity, c-Fos remains unique in its ability to cause deregulated osteoblast proliferation and transformation leading to bone tumor development.

Perturbations in the G1-S transition are thought to contribute to deregulated growth and tumor formation, and this is consistent with our previous observed correlation between cyclin D1 and exogenous c-Fos levels in transgenic mice (19). Together with the studies presented here, and in view of the correlation between cyclin A and tumorigenesis (66–69), we propose that changes in specific cell cycle genes, such as cyclin A and cyclin D1, represent an initial genetic event in the c-Fos-induced transformation of osteoblasts, which may give them a proliferative advantage during early tumor growth. Our recent experiments in c-Fos transgenic mouse tumors support such a hypothesis, because we have observed an increase in bromodeoxyuridine-labeled osteoblasts (normally post-mitotic) in early hyperplastic lesions, which correlates well with their increased number in these lesions (see Ref. 9).3 Although knock-out experiments have indicated that c-Fos is dispensable for cell cycle progression, our data suggest that, in a gain-of-function setting, c-Fos can indeed perturb the osteoblast cell cycle in a specific manner. The regulation of cyclinA/E/CDK2 represents a novel role for ectopic c-Fos expression in osteoblast growth control, and we suggest that this must be considered together with cyclin D1 as a potential downstream target of c-Fos in the cell cycle control of bone cells. It remains to be determined how altered cell cycle expression in osteoblasts might relate to other factors, which have been reported to be involved in c-Fos-induced transformation (Refs. 70–72 and references therein). Other approaches involving modulation of cell cycle proteins in osteoblasts by gain- and loss-of-function mutations in vivo, as well as expression profiling strategies, are currently being employed to identify genes that transform osteoblasts in a c-Fos-dependent manner.

**Acknowledgments**—We thank Drs. Peter Angel (Deutsches Krebsfor- schungszentrum, Heidelberg) and Gordon Peters (Cancer Research UK, London) for CAT reporter and GST-Rb plasmids, respectively, Dr. Adrian Hayday for use of the flow cytometer, and Dr. D. Harney for primary osteoblast cultures. We also thank Drs. E. F. Wagner and J.-P. David for critical review of the manuscript.

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Accelerated Cell Cycle Progression in Osteoblasts Overexpressing the c-fos Proto-oncogene: INDUCTION OF CYCLIN A AND ENHANCED CDK2 ACTIVITY

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J. Biol. Chem. 2004, 279:9882-9891.
doi: 10.1074/jbc.M310184200 originally published online December 29, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M310184200

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