Transgenic mice expressing human T cell leukemia virus type I (HTLV-I)-tax under the control of HTLV-I-long terminal repeat (LTR) promoter develop skeletal abnormalities with high bone turnover and myelofibrosis. In these animals, Tax is highly expressed in bone with a pattern of expression restricted to osteoclasts and spindle-shaped cells within the endosteal myelofibrosis. To test the hypothesis that lineage-specific transcription factors promote transgene expression from the HTLV-I-LTR in osteoclasts, we first examined tax expression in transgenic bone marrow cultures. Expression was dependent on 1α,25-dihydroxycholecalciferol and coincided with tartrate-resistant acid phosphatase (TRAP) expression, a marker of osteoclast differentiation. Furthermore, Tax was expressed in vitronectin receptor-positive mononuclear precursors as well as in mature osteoclast-like cells (OCLs). Consistent with our hypothesis, electrophoretic mobility shift assays revealed the presence of an OCL nuclear factor (NFOC-1) that binds to the LTR 21-base pair direct repeat, a region critical for the promoter activity. This binding is further enhanced by Tax. Since NFOC-1 is absent in macrophages and conserved in osteoclasts among species including human, such a factor may play a role in lineage determination and/or in expression of the differentiated osteoclast phenotype.

Osteoclasts are multinucleated cells that resorb bone. Although evidence indicates that they are derived from monocye-macrophage lineage cells (1–3), the exact identity of their precursors and the mechanism by which commitment to the osteoclast lineage is occurring are currently unclear. The data most relevant to this issue comes from the effects of the targeted deletion of the c-fos gene in mice (4, 5), which causes a lineage shift from osteoclasts to macrophages, resulting in an accumulation of bone marrow macrophages and severe osteopenosis due to the absence of mature osteoclasts (6). Although the exact mechanism by which c-Fos is acting is mostly unknown, these observations clearly indicate that c-Fos expression is required for commitment to the osteoclast lineage. Considering the inducibility of c-Fos expression in various cell types, however, it seems likely that the determination of osteoclast differentiation involves other lineage-specific factors or signals that may function together with or independently of c-Fos.

Human T-cell leukemia virus type I (HTLV-I)\(^1\) is a retrovirus etiologically linked to adult T cell leukemia and a neurodegenerative disease called tropical spastic paraparesis (7, 8). One of the viral products that is crucial to its pathogenicity is Tax, a 40-kDa protein that transactivates its own viral expression from the long terminal repeat (LTR) promoter (9–11). This transactivation involves the interaction of Tax with the CREB/ATF family of transcription factors and the binding of the resultant complex to three 21-bp direct repeats in the LTR (12–15). Tax also transactivates various host cellular genes including many growth-related factors considered to be essential to the transformational activity of the virus (16–20).

More directly relevant to the issue of osteoclast differentiation is the fact that transgenic mice expressing tax under the control of its natural promoter, LTR, not only develop neurofibromatosis and Sjögren-like exocrinopathies (21–23) but also exhibit profound skeletal abnormalities, characterized by high bone turnover and myelofibrosis with a markedly increased number of osteoclasts (24). High level of expression of the transgene was observed only in the affected tissues: tumors, bone, and salivary glands. Interestingly, in bone, tax expression is restricted to only two cell types, osteoclasts and spindle-shaped cells within the endosteal myelofibrosis (24). Since, in the same animals, bone marrow and tissue macrophages fail to express tax whether in vivo or in vitro, the relatively specific activation of the HTLV-I-LTR promoter in osteoclasts may be related to a transcription factor that would be expressed in this cell type and promote expression of the transgene.

To test this hypothesis, we have examined differentiation-dependent expression of tax in the osteoclast lineage in cultures of total bone marrow cells from the tax-transgenic mice. Expression of tax was induced by 1α,25-dihydroxycholecalciferol (calcitriol) and occurred mostly in vitronectin receptor (VNR)-positive mononuclear precursors of the osteoclast. Consistent with our hypothesis, a unique nuclear factor was observed in osteoclasts which binds to the 21-bp direct repeat (DR) in the LTR even in the absence of Tax. The factor, designated as NFOC-1 (nuclear factor osteoclast-1), was different from other factors described in the literature and showed specificity for the osteoclast lineage.

\(1\) The abbreviations used are: HTLV-I, human T cell leukemia virus type I; LTR, long terminal repeat; DR, direct repeat; OCL, osteoclast-like cell; TRAP, tartrate-resistant acid phosphatase; VNR, vitronectin receptor; EMSA, electrophoretic mobility shift assay; CRE, cyclic AMP-responsive element; TBP, TPA-responsive element; CREB, CRE-binding protein; ATF, activating transcription factor; AP-1, activator protein-1; bp, base pair(s); GST, glutathione S-transferase; BrdUrd, bromodeoxyuridine; bZIP, basic leucine-zipper.

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Identification of an Osteoclast Transcription Factor That Binds to the Human T Cell Leukemia Virus Type I-Long Terminal Repeat Enhancer Element*

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from DR-binding factors expressed in the spindle-shaped myelofibrotic cells and was not used in macrophages. Furthermore, NFCO-1 was also present in human and rabbit osteoclasts, suggesting that this nuclear DNA-binding factor may normally be associated with lineage determination in several species.

**MATERIALS AND METHODS**

**Chemicals and Antibodies**—All the chemicals, enzymes, and proteinase inhibitors were obtained from Sigma unless specified. Dispase was from Boehringer Mannheim. Anti-Tax polyclonal antibody was described previously (24). Anti-integrin-αv-5 receptor and anti-β3 subunit polyclonal antibodies were kindly provided by Dr. James Gailit (State University of New York, Stony Brook). Anti-actin antibody was purchased from Cortex Biochem (San Leandro, CA). All the antibodies against transcription factors for supershift assays were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

**Animals**—Newborn and 8-week-old male CD-1 mice were purchased from Charles River Laboratories (Wilmington, MA). tax-transgenic mice with a genetic background of C57BL/6L were previously described (24). Transgene-positive mice were identified by slot blot analysis of the tail genomic DNA according to the standard method (25).

**Cells and Cell Cultures**—Murine macrophage cell lines, P388D1 and IC5, were obtained from American Type Culture Collection (Rockville, MD) and maintained in RPMI 1640 supplemented with 10% fetal bovine serum. Primary osteoclastic cells were isolated from newborn CD1 mouse calvariae by digestion with 0.1% collagenase type IA and 0.2% dispase as described previously (26). To obtain myelofibrotic spindle-shaped cell lines, fibroblasts were isolated from tax-transgenic mice, and the marrow cavity was flushed extensively to remove marrow cells. The cleaned bone was treated with collagenase, and cells thus obtained were cultured in α-minimum Eagle's medium containing 10% fetal bovine serum. After more than 10 passages, cells were plated at a density of 100 cells/ml, and clones were isolated with cloning rings (Bel-Art Products, Pequannock, NJ). A representative cell line, characterized by its morphology and high levels of tax expression, was designated as TXB-1, which has been maintained for more than 2 years. Marrow cells were obtained from tibiae and femurs of 8–12 week-old transgenic C57BL/6L or wild type CD1 mice. For total bone marrow cultures (27), cells were plated at a density of 8 × 10^5/ml and cultured for 7 days in the presence or absence of 10 μM calcitriol. Half of the medium was changed every other day during the culture period. In the time course experiments, marrow cells were cultured in a 24-well dish for 3–7 days under the conditions described above. To minimize above-well variance on one occasion, for each day, three wells in a dish were stained for tartrate-resistant acid phosphatase (TRAP), and cells from three different wells in the same dish were lysed and analyzed for Tax by Western blot.

**Osteoclast Preparation and Analysis**—To generate osteoclast-like cells (OCLs) in vitro, 2 × 10^7 marrow cells were co-cultured with 2 × 10^5 primary osteoclasts in a 10-cm culture dish in the presence of 10 nM calcitriol (28). After 6 days, cells were treated sequentially with collagenase-dispase and 5 μM EDTA to remove osteoclastic cells, and OCLs remained strongly attached to the culture dish. In every preparation, more than 80% of the OCL-enriched population was positive for TRAP staining. Human osteoclast-like cells purified from osteoscleroma (29) and authentic rabbit osteoclasts isolated from bone (29) were prepared according to the described methods. For TRAP staining, cells were fixed with 3.7% formaldehyde in phosphate-buffered saline for 10 min, treated with acetone/ethanol (1:1) for 1 min, and stained with red violet LB salt in acetate buffer (40 mM sodium acetate and 10 mM sodium tartrate, pH 5.0) containing naphthol AS-MX phosphate as a substrate (27).

**Cell Sorting**—To enrich putative osteoclast precursors, total bone marrow was cultured for 6 days in the presence of 10 ng/ml 25(OH)D_3, and cells were lifted with collagenase-dispase and 5 μM EDTA/phosphate-buffered saline. At this point, very few multinucleated OCLs had remained strongly attached to the culture dish. In every preparation, 90% of the cells were used as VNR-negative cells.

**RNA Analysis**—Total RNA was prepared as described (30), and the concentration was determined by spectrophotometry. For Northern blot analysis, 10 μg of RNA was separated on a 1% denaturing agarose gel, transferred to a Hybond N membrane (Amersham Life Sciences Inc.), hybridized with ^32P-labeled tax or glyceraldehyde-3-phosphate dehydrogenase cDNA probe (10^6 cpm/ml) prepared by the random primer labeling method (Boehringer Mannheim), and autoradiographed. The intensity of the bands was quantitated by densitometry.

**Protein Analysis**—For Western blots, total cell lysates were obtained by lysis cells in lysing buffer (1% SDS, 66 mM Tris-Cl, pH 7.5, 0.1 mM phenylmethylsulfonyl fluoride, and 2 μg/ml aprotinin, leupeptin, and pepstatin) and resolved on a 10% SDS-polyacrylamide gel, and transferred onto a nitrocellulose membrane. After sequential incubation with a primary and secondary goat anti-rabbit IgG antibody (Promega Co., Madison, WI), the protein bands were visualized by the enhanced chemiluminescence method (Amersham Corp.). As primary antibodies, anti-Tax, anti-integrin β3, and anti-actin antibodies were used at a dilution of 1:2000, 1:1000, and 1:2000, respectively. For quantitation, the intensity of the bands was determined by densitometry.

**Fusion Protein**—To make GST-Tax fusion protein, a tax cDNA fragment corresponding to amino acids 2–353 with BamHI and EcoRI sites introduced at each end was obtained by polymerase chain reaction and subcloned into the PGEX-2T vector (31) in frame. The resultant plasmid was directly sequenced to exclude any misincorporation and cloning errors. Bacterially expressed fusion protein was purified with GSH beads (Pharmacia Biotech Inc.) according to the method recommended by the supplier, released from the beads by incubating with 10 μM free GSH, dialyzed against the protein storage buffer (10 mM Tris-Cl, pH 8.0, 50 mM NaCl, 10% glycerol, 0.5 μM diithiothreitol, and 0.1 mM phenylmethylsulfonyl fluoride) and stored at ~70 °C until use. Purity of the fusion proteins was ensured by Coomassie staining on a 10% SDS-polyacrylamide gel (data not shown).

**Electrophoretic Mobility Shift Assay (EMSA)**—Small scale nuclear extracts were obtained according to the previously described method (32). Briefly, cells were washed, collected by scraping in ice-cold phosphate-buffered saline, incubated in 5 × packed cell volume of hypotonic solution (10 mM HEPES, pH 7.6, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 0.75 mM spermine, 0.15 mM spermidine, 1 mM dithiothreitol, 10 mM Na_2MoO₄, and 0.5 mM phenylmethylsulfonyl fluoride) for 15 min on ice and lysed by adding 0.6% Nonidet P-40 and vortexing 10 x. Nuclei were pelleted and extracted in 50 μl of nuclear lysis buffer (NBL) containing 20 mM HEPES, pH 7.6, 0.4 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 10 mM Na_2MoO₄, and 0.5 mM phenylmethylsulfonyl fluoride and 2 μg/ml aprotinin, leupeptin, and pepstatin. Osteoclast-like cells (OCLs) generated by the co-culture system yielded 10–20 μg of nuclear extracts per 10-cm culture dish. Each reaction for EMSA (20 μl) consisted of 0.5 × NBL, 4 μg of poly(dI-dC) (Pharmacia), 4–20 μg of nuclear extracts, and 30,000 cpm of a double-stranded oligonucleotide probe end-labeled with ^32P by Klenow enzyme (New England Biolabs, Inc., Beverly, MA). The synthetic oligonucleotide probe corresponding to the second DR 5'-GCTAGT-3' was a mutant DR 5'-ACTGACGTAGGCG- CAGGTGCCTCCCTGAAAG-3' (the mutated nucleotides are underlined) and was previously described (12). For competition studies of NFCO-1, three mutants which contain substitutions in each subdomain of the DR (33) (MBC, 5'-CTCATTAAGGGCCTGGACTGCCCTT- GAAG-3'; ANC, 5'-CTCAGGCTAGGGCCTGGACTGCCTTGAAGG-3'; TF, 5'-CTCAGGCTAGGGCCTGGACTGCCTGAAGG-3'), somatostatin CRE (34), 5'-CAGAGATTGCCCTGACCTGAAGAGA GAAGCT-3', and the human collagenase TPA-responsive element (TRE) containing AP-1 binding site (35) 5'-GTCAGGCTAGGT- CACGGCCC-3' were used as competitors. The reaction mixture was incubated for 15 min at room temperature, resolved on a 4.5% non-denaturing polyacrylamide gel, transferred to Whatman 3MM paper, and autoradiographed. For supershift assays, 1 μg of antibody was added to the final mixture and further incubated for 30 min at room temperature. In some experiments, an anti-IG antibody secondary antibody was further added and incubated for another 30 min at room temperature.

**UV Cross-linking Analysis**—10 μg of nuclear extracts of osteoclast-like cells purified from osteoscleroma was incubated with a BrdUrd-substituted DR probe as follows: 5'-CTCAGGCTAGGGCCTGGACTGCCTGAAAG-3' (underlined T residues were substituted by BrdUrd) in EMSA reaction for 20 min. The whole reaction was exposed to ultraviolet light at 4 °C for 30 min by placing the reaction tubes 6 cm from a UV transilluminator (312 nm, 8000 microwatts/cm²), separated on a 10% SDS-polyacrylamide gel, and autoradiographed. To ensure the specificity, a 100 x molar excess of unlabeled probe was included in a parallel reaction.

**RESULTS**

To verify in vitro that the activation of the HTLV-I-LTR is indeed an event associated with the differentiation of cells within the osteoclast lineage, as observed in vitro (24), bone...
The absence or presence of calcitriol for 7 days as described under cultured alone (BM or co-cultured (Co-C) with primary osteoblastic cells in the absence or presence of calcitriol for 7 days as described under "Materials and Methods." A, 30 μg of total cell lysates were run on a 10% SDS-polyacrylamide gel and analyzed for Tax by Western blots. 40-kDa protein, Tax, is indicated by an arrow. B, 10 μg of total RNA was analyzed by Northern blot using tax (upper panel) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH, lower panel) cDNA probes. The intensity of the bands was quantitated by densitometry, and the fold induction by calcitriol was calculated based on tax/glyceraldehyde-3-phosphate dehydrogenase values. EDTA-resistant populations (shown in Fig. 3), the major complex in TXB-1 being recognized by an antibody that is reactive to both CREB and ATF-1 (Fig. 4). A similar DNA-protein complex supershifted by the anti-CREB/ATF-1 antibody was also present in nuclear extracts of tumor cells from the transgenic mice (data not shown). These findings are consistent with previous reports on DR-binding factors in other cells including T cells (12, 13). In contrast, none of the antibodies against the members of the CREB/ATF family (38), including CREB, CREB-2, CRE modulator-1, ATF-1, ATF-2, ATF-3, and ATF-4, recognized NFOC-1 (data not shown). These results indicate that the transgene expression from the LTR promoter in a few restricted cell types in the tax transgenic mice correlates with the presence of two different sets of transcription factors as follows: CREB and/or ATF-1, which has been shown to be capable of mediating transactivation by Tax, is found in tumors and the spindle-shaped cells in bone myelofibrosis; and NFOC-1 is restricted to cells of the osteoclast lineage at least among bone cells and may consist of an as yet unidentified member of the CREB/ATF family and/or a novel factor.

To characterize further the DNA-binding properties of NFOC-1, we examined the effects of various competitors including DR mutants, CRE (34), and TRE (35) in EMSA. DR sequences can be divided into three distinct domains named A, B, and C (33). As shown in Fig. 5, substitutions in the domain A (MBC) or C (ABM) did not affect the competition, whereas mutations in the B domain (AMC) almost completely abolished the ability to compete NFOC-1 binding, indicating that the B domain in the DR which contains CRE-like sequences is critical for NFOC-1 binding. This is consistent with the fact that an authentic CRE was also a potent competitor (Fig. 5). Thus, although antibodies against known members of the CREB/ATF family did not induce a supershift, NFOC-1 has binding properties that resemble members of the CREB/ATF family.

Interestingly, a somewhat weaker competition was also observed with TRE that contains an authentic AP-1 binding site from the human collagenase gene (Fig. 5). Since c-Fos has been

**Fig. 1.** Induction of the *tax* transgene expression by calcitriol in *vitro*. Bone marrow from the *tax*-transgenic mice was either cultured alone (BM) or co-cultured (Co-C) with primary osteoblastic cells in the absence or presence of calcitriol for 7 days as described under "Materials and Methods." A, 30 μg of total cell lysates were run on a 10% SDS-polyacrylamide gel and analyzed for Tax by Western blots. 40-kDa protein, Tax, is indicated by an arrow. B, 10 μg of total RNA was analyzed by Northern blot using tax (upper panel) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH, lower panel) cDNA probes. The intensity of the bands was quantitated by densitometry, and the fold induction by calcitriol was calculated based on tax/glyceraldehyde-3-phosphate dehydrogenase values. EDTA-resistant populations (shown in Fig. 3), the major complex in TXB-1 being recognized by an antibody that is reactive to both CREB and ATF-1 (Fig. 4). A similar DNA-protein complex supershifted by the anti-CREB/ATF-1 antibody was also present in nuclear extracts of tumor cells from the transgenic mice (data not shown). These findings are consistent with previous reports on DR-binding factors in other cells including T cells (12, 13). In contrast, none of the antibodies against the members of the CREB/ATF family (38), including CREB, CREB-2, CRE modulator-1, ATF-1, ATF-2, ATF-3, and ATF-4, recognized NFOC-1 (data not shown). These results indicate that the transgene expression from the LTR promoter in a few restricted cell types in the *tax* transgenic mice correlates with the presence of two different sets of transcription factors as follows: CREB and/or ATF-1, which has been shown to be capable of mediating transactivation by Tax, is found in tumors and the spindle-shaped cells in bone myelofibrosis; and NFOC-1 is restricted to cells of the osteoclast lineage at least among bone cells and may consist of an as yet unidentified member of the CREB/ATF family and/or a novel factor.

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Interestingly, a somewhat weaker competition was also observed with TRE that contains an authentic AP-1 binding site from the human collagenase gene (Fig. 5). Since c-Fos has been
shown to be important for osteoclast differentiation (6), we then examined the possibility that NFOC-1 contains members of the Fos and Jun families, i.e. proteins which together form the AP-1 transcription factor complex (39). As shown in Fig. 6A, a polyclonal antibody that recognizes all the members of the Jun family consistently, albeit moderately, diminished the intensity of the NFOC-1 band, and a clear supershifted complex was observed after longer exposure (Fig. 6B). When each Jun family member was tested, antibodies against JunD were the only ones that produced an appreciable supershift. In contrast, antibodies against each member of the Fos family had no effects, although an antibody recognizing all the members of the Fos family showed some cross-reactivity. Further analysis showed that this particular antibody cross-reacts with other members of the basic leucine-zipper (bZIP) transcription factor family (data not shown). It is therefore possible that the Fos antibody recognizes a component of NFOC-1 which, although different from the Fos family members, retained the bZIP motif. The ability of the antibodies against c-Fos and JunD to bind to their respective antigens under our experimental conditions was verified in EMSA using the AP-1 probe and Cos-7 cells transfected with mouse c-Fos or JunD expression vectors (data not shown). We also confirmed that the antibodies against all Jun and JunD are not cross-reactive to the DR binding factor found in the spindle-shaped cell line, TXB-1, i.e. CREB and/or ATF-1 (data not shown). These results suggest that c-Fos itself is not present in NFOC-1 but that NFOC-1 may contain JunD or a closely related factor.

Given the potential importance of this lineage-specific transcription factor, we then determined whether it was present in other species and in authentic osteoclasts. As shown in Fig. 7, protein-DNA complexes with the same mobility as mouse NFOC-1 were also detected in assays using nuclear extracts from both human OCLs purified from osteoclastoma and purified authentic rabbit osteoclasts. These results suggest that NFOC-1 is conserved among species and is expressed not only in osteoclast-like cells generated in vitro but also in authentic osteoclasts freshly isolated from bone. Since NFOC-1 appeared to be expressed at the highest levels in human osteoclastoma, we took advantage of this fact and used this abundant source of NFOC-1 for further characterization by cross-linking. For this purpose, DR binding factors present in human osteoclastoma nuclear extracts were UV cross-linked to the BrdUrd-substituted probe and separated on a 10% SDS-polyacrylamide gel. As shown in Fig. 8, two major bands were detected; only the lower band was competed by a cold probe and thereby considered specific. These results suggest that human NFOC-1 contains a specific DR binding factor with an apparent molecular mass of approximately 70 kDa. Since the whole extract was cross-linked in this experiment, however, we cannot completely rule out the possibility that the 70-kDa protein is derived from another specific complex.

Although our results clearly show that NFOC-1 can bind to the LTR in the absence of Tax expression, Tax has been shown to enhance DNA binding activities of various transcription factors, especially members of the bZIP protein family (15). Therefore, NFOC-1 may also be involved in subsequent LTR transactivation by Tax. We consequently examined the effects
of Tax on the NFOC-1 binding activity using bacterially expressed fusion proteins. As shown in Fig. 7, GST-Tax fusion proteins, but not GST alone, enhanced the NFOC-1 binding in human OCLs (Fig. 7A) and in authentic rabbit osteoclasts (Fig. 7B). Densitometric analysis revealed that GST-Tax increased the binding of human NFOC-1 by 6-fold compared with the GST control. These results indicate that the binding of NFOC-1 is enhanced by Tax and further suggest that NFOC-1 plays a role not only in initial activation but also in subsequent Tax-dependent transactivation of the LTR promoter in osteoclasts.

DISCUSSION

We have previously reported that the viral oncogene, tax, when under the control of its natural promoter, HTLV-I-LTR, shows a restricted pattern of cellular expression in transgenic animals which includes cells of the osteoclast lineage (24). Based upon these findings, we hypothesized that, although Tax itself is capable of transactivating the HTLV-I-LTR, its initial expression required activation of the LTR by cellular transcription factors, an event that was occurring in only a restricted number of cell types. The fact that cells of the osteoclast lineage were expressing Tax in the transgenic animals thus implied that some factor(s) expressed in this cell type was capable of activating the HTLV-I-LTR.

In the present study, we provide further evidence for the differentiation-dependent expression of the HTLV-I LTR-tax transgene in cells of the osteoclast lineage. First, the expression of tax in marrow cultures was dependent on the presence of calcitriol, which is a strong inducer of osteoclast differentiation (40). Second, time course studies showed that Tax expression coincides with the expression of TRAP, one of the specific markers for the osteoclast lineage (41). Third, in early marrow cultures, Tax was expressed almost exclusively in mononuclear cells expressing the vitronectin receptor at high levels, another phenotypic marker of osteoclasts and their precursors (36). Together, these findings indicate that tax induction occurs in mononuclear osteoclast precursors at or soon after the point of commitment to their lineage.

Given the potential importance of nuclear factors in determining the differentiation of precursor cells toward the osteoclast lineage as opposed to that of macrophages, and because monocytes and macrophages did not express detectable levels of Tax protein in the transgenic mice, we searched for the presence of a lineage-specific nuclear factor in mouse OCLs. Using a 21-bp direct repeat (DR) that is known to be involved in the activation of transcription from the LTR, we have found evidence of a DR-binding nuclear factor (NFOC-1) that is expressed in osteoclasts and OCLs but not in macrophages or osteoblasts. Interestingly, this factor was not immunologically related to any members of the CREB/ATF family that are known to be involved in the LTR activation by Tax in other cell types (12–14, 33, 37). However, in contrast with these results in OCLs, CREB and/or ATF-1 appeared to be responsible for HTLV-I-LTR activation in the other cell type found to express Tax at high levels in bone in transgenic animals, i.e. the spindle-shaped cell present in the myelofibrotic tissue (24).
Thus, the HTLV-I-LTR is activated in cells induced to differentiate into the osteoclast lineage, and the nuclear factor, NFOC-1, which is distinct from the CREB/ATF family members tested in this study, including CREB and ATF-1, appears to be a likely candidate for the factor responsible for this activation. Since NFOC-1 is not detectable in macrophages, our observations suggest that NFOC-1 is induced in early osteoclast precursors at or very soon after the branching point from the macrophage lineage. NFOC-1 may therefore contribute to the determination of the pattern of gene expression that characterizes cells of the osteoclast lineage. Because expression of c-Fos has also been shown to be necessary for commitment to this lineage, but not required for progression to the macrophage phenotype (6), we have tested whether NFOC-1 contained c-Fos itself or other related factors participating in the formation of the AP-1 transcription factor complex. Our results show that NFOC-1 does not contain c-Fos itself but may indeed contain Jun-related protein(s). Interestingly, activation of the HTLV-I-LTR promoter by members of the Jun family has also been reported (42). The reactivity of NFOC-1 to the anti-JunD antibody is of particular interest in light of recent reports which have shown that 1) expression of JunD is up-regulated by calcitriol in a human myeloid cell line and may be one of the primary targets of vitamin D for its anti-proliferative effects (43), and 2) JunD is highly expressed in both mouse and rabbit OCLs (Ref. 44 and data not shown), suggesting that JunD may be involved in the process of osteoclast differentiation. The transcription factors CREB/ATF (38) and Fos/Jun (AP-1) (39) each represent a complex family consisting of several members that can selectively homo- or heterodimerize within the same family and binds preferentially to CRE and TRE, respectively. These two families share the basic region/leucine zipper motif, and in fact, it has been demonstrated that both cross-family heterodimerization and functional cross-talk between the two families can occur (45–47), adding a second level of complexity to the regulation of gene transcription through CRE and TRE. The fact that most cross-family heterodimers show preference to CRE (45, 46) is of particular interest in light of the results of our competition studies showing that NFOC-1 has a higher affinity to CRE than to TRE. Since Jun proteins alone are not expected to bind to CRE efficiently, NFOC-1, most likely, is a heterodimer of an unidentified CREB/ATF-like factor of 70-kDa and Jun proteins part of which, at least, appear to be JunD.

Regarding c-Fos, it is noteworthy that although its temporal profile of expression in cells of the osteoclast lineage has not...
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key factors associated with the differentiated osteoclast phenotype. Alternatively, NFOC-1 may participate in the regulation of osteoclast differentiation like c-Fos but at later stages.

Finally, the potential importance of NFOC-1 in cells of the osteoclast lineage is further supported by the presence of nuclear factors showing exactly the same mobility in EMSA in nuclear extracts of osteoclastic cells from three different species, i.e., mouse, rabbit, and human. Although we have not been able to fully characterize the factor in each species to ensure their identity, two reasons other than their similar mobility lead us to believe that they are the same. First, there are only one or two major protein-DNA complexes detected in EMSA in each of the three species, and second, their DNA-binding activities were similarly enhanced by GST-Tax fusion proteins. Thus, NFOC-1 may be conserved among species, suggesting that NFOC-1 plays a key role in osteoclasts.

In conclusion, we have shown that the expression of the HTLV-I-tax transgene from the LTR promoter is induced in association with commitment to the osteoclast lineage in vitro. Using the viral promoter as a probe, we have identified NFOC-1, an LTR-binding nuclear factor complex present in osteoclasts but not in macrophages, which may be associated with lineage determinants for osteoclasts. Our results suggest that NFOC-1 consists of a Jun-related protein and a CREB/ATF-like factor. Demonstration of an actual involvement of this factor in the LTR promoter activation and osteoclast differentiation will require identification and molecular cloning of its cDNA. Such an attempt is currently under way.

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FIG. 7. Inter-species conservation of NFOC-1 and its enhanced binding by Tax. Nuclear extracts from human OCLs purified from osteoclastoma (A) and rabbit authentic osteoclasts (B) were analyzed for DR-binding factors by EMSA. The position of NFOC-1 is indicated by an arrow. Lane F indicates free probe without extracts. A, in the left panel, 6 μg of human OCL extracts (lane 1) were shown next to 16 μg of mouse OCL extracts (lane 2) in the same gel for comparison. In the right panel, human OCL extracts were analyzed with a hundred-fold excess of cold competitor DNA (lane 2), 600 ng of GST alone (lane 3), or GST-Tax fusion proteins (lane 4). B, 6 μg of rabbit osteoclast nuclear extracts were analyzed alone (lane 2) or in the presence of 600 ng of GST-Tax fusion proteins (lane 3) or GST alone (lane 4). 12 μg of mouse OCL nuclear extracts are shown in lane 1 for comparison.

FIG. 8. UV cross-linking analysis of human NFOC-1. 10 μg of nuclear extracts of human OCLs from osteoclastoma were cross-linked to a BrdUrd-substituted DR probe and analyzed on a 10% SDS-polyacrylamide gel as described under “Materials and Methods.” No extracts were added in lane 1. Unlabeled DR probe was added in 100-fold excess in lane 3. The arrow indicates the position of a specific DR binding protein with an approximately 70-kDa molecular mass.

been well defined, preliminary results from our laboratory suggest that c-Fos protein is hardly detectable in mature osteoclasts. Therefore, it is unlikely that NFOC-1 would contain and/or associate with c-Fos in fully differentiated cells. It is nevertheless possible that at the precursor stage components of NFOC-1 directly interact with c-Fos or participate in a formation of specific subsets of dimers of bZIP transcription factors which, in a combinatorial way, regulate expression of certain

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