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A Customized Retroviral Vector Confers Marker Gene Expression in Osteoclast Lineage Cells

Daniel J. Selski and Denis R. Clohisy*

Department of Orthopaedic Surgery, The University of Minnesota, Minneapolis, Minnesota

Abstract Osteoclasts play a seminal role in many skeletal diseases and therefore are candidates for cell-based gene delivery systems to treat disorders of bone. As an initial step toward developing osteoclast-mediated gene delivery systems, we have made and analyzed a customized Molony–Murine leukemia virus (MMLV)-based retroviral vector containing elements of the osteoclast-specific tartrate-resistant acid phosphatase (*TRAP*) gene. RAW 264.7 cells were transduced with the customized vector (E3) and differentiated along macrophage or osteoclast lineages. E3 contained a truncated form of the human nerve growth factor receptor (*NGFR*) as a reporter gene. *NGFR* expression increased with RANK-ligand (RANK-L) treatment but not with macrophage (γ-IFN/LPS treatment) differentiation. Enhanced *NGFR* expression peaked 48 h after RANK-L treatment. Electrophoretic mobility shift assays (EMSA) analysis of the *TRAP* gene regulatory elements in E3 identified a single 27 bp DNA probe, which specifically bound protein from RANK-L-treated cells. DNA sequence revealed AP-1 binding sites, and analysis with mutant probes implied that the sites were functional. EMSA supershift analysis identified Fos protein interacting with the 27 bp probe. In summary, insertion of sequence −962 to −868 from the *TRAP* gene into the U3 region of the MMLV LTR confers RANK-L induced retroviral gene expression via Fos family protein interaction at AP-1 sites. J. Cell. Biochem. 97: 641–650, 2006.

Key words: Acp5; retrovirus; RAW 264.7; gene therapy

The pathophysiologic basis for many diseases of the skeleton can be linked, at least in part, to osteoclasts. Osteoclasts are multinucleated cells of monocytic origin whose specialized function is to resorb mineralized bone matrix. Skeletal diseases involving rapid bone loss and intense osteoclastogenesis include cancer-induced osteolysis, inflammatory arthritis, and orthopedic implant-associated osteolysis [Kong et al., 1999; Mundy, 2002]. Novel approaches to treating these disorders are needed.

In recent years, a new paradigm has developed using cell-based gene delivery systems. In these systems, host cells are utilized to deliver therapeutic genes to sites of disease. An example is utilization of transduced endothelial cells or transduced glial cells to deliver therapies for angiogenic cancers and gliomas, respectively [Barresi et al., 2003; Wei et al., 2004]. As osteoclasts are of hematopoietic lineage, it has been determined that bone marrow transplantation in experimental animal models and in humans produces osteoclasts and osteoclast precursor cells of donor origin. Furthermore, it has been shown that transplanted bone marrow cells will form osteoclasts at sites of tumor osteolysis [Clohisy et al., 2000; Honore et al., 2000]. Based on these findings, a new treatment paradigm for osteoclast-based diseases could involve osteoclast-mediated gene delivery systems. In this paradigm, osteoclast precursor cells would be engineered with a therapeutic gene, and after transplantation, expression of the osteoclast-delivered gene at sites of disease would have a therapeutic benefit, either alone or in combination with other treatments.

As the initial step toward developing an osteoclast-mediated gene delivery system, we have created a customized retroviral vector and have analyzed its expression in osteoclast

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*Correspondence to: Denis R. Clohisy, MD, Department of Orthopaedic Surgery, 420 Delaware Street SE, MMC 806, Minneapolis, MN 55455. E-mail: clohi001@umn.edu

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lineage cells. Vector design considerations were based on principles learned from the development of cell type-specific vectors targeted at melanin-producing and chorioembryonic antigen-producing cells [Marks and Walker, 1981; Clohisy and Ramnaraine, 1998; Adachi et al., 2000; Erbs et al., 2000; Luchin et al., 2000; Chung-Faye et al., 2001; Reddy et al., 2001; Chiu et al., 2004]. Specifically, the viral enhancer sequence in the U3 region of the Maloney-murine retroviral LTR is replaced by potential enhancer regions from a tissue-specific gene. Customized retroviral constructs analyzed in this report contained elements from the tartrate-resistant acid phosphatase (*TRAP*) gene and the marker gene human nerve growth factor receptor. Findings indicate that (1) the customized vector was preferentially expressed during osteoclast differentiation, but not during macrophage differentiation; (2) the DNA binding site within the customized vector, which conferred specificity was an AP-1 binding site; and (3) the AP-1 binding protein was a member of the Fos family.

MATERIALS AND METHODS

Retrovirus Construct Design

Subcloning was carried out via standard recombinant DNA techniques [Sambrook and Maniatis, 1989]. Using a Stratagene Robocycler Gradient 96 thermal cycler, a 113 bp fragment was amplified by PCR from mouse genomic DNA. The sense and antisense primers were 5′-CGCTCTAGACCTGTAATTCCAACAC-3′, and 5′-CGCTCTAGATTGTTTTGAGACAGTG-3′, respectively. This fragment contains 95 bp of the TRAP E region [Reddy et al., 1995] plus the underlined nucleotides at the 5′ end of each primer to introduce Xba I restriction endonuclease sites. The PCR conditions were 1 cycle of 94°C for 4 min, then 30 cycles of 94°C for 30 s, 65°C for 30 s, 72°C for 1 min, and 1 cycle of 72°C for 7 min. The amplified TRAP sequence corresponds to nucleotides 885–979 of Acc# M85212, 1193–1287 of Acc# M99054, and 894–988 of Acc# AY187311. The PCR products were then digested with Xba I, and the purified fragments were ligated to a 2,833 bp fragment of Nhe I/Xba I-digested pSK LTR del shuttle vector, which contains a single copy of the wild-type MoMLV 3′ LTR (a gift from Richard Vile, Mayo Clinic, Rochester, MN [Diaz et al., 1998]). PCR-based Sanger dideoxy sequencing on an ABI377 (University of Minnesota's Microchemical Facility) confirmed three repetitions of the TRAP E region in the pSK LTR. An 865 bp truncated form of human nerve growth factor receptor (NGFR) was obtained by digesting MFG-TKiNG vector [Orchard et al., 2002, HGT] at the BamH I sites. The purified piece was inserted at the BamH I site of the parental retroviral plasmid vector, pBabe Puro (also a gift from Richard Vile, Mayo Clinic [Morgenstern and Land, 1990]). The modified pSK LTR was cloned into the pBabe Puro tNGFR construct at Cla I/Sca I sites. The resulting retrovirus vector was simply named E3.

DNA Transfection and Retroviral Transduction of Cells

PA317 packaging cells (ATCC) (3×10^5) were plated in a 35 mm dish the day before transfection. Two micrograms of phenol and chloroform purified retroviral plasmid DNA was transfected using FuGENE 6 Transfection Reagent (Roche) according to the manufacturer's instructions. Two days later, cells were split 1:20 into medium containing 3.0 μg/ml puromycin (Sigma-Aldrich). Ten days after selection cells were frozen in liquid nitrogen. After being thawed, producer cells were further selected in 7.5 μg/ml puromycin and grown from individual clones so that the clone with highest titer could be used for infection of various cell lines. After screening 37 PA317 clones the highest titers attainable were 5×10^3 and 1×10^4 CFU/ml.

The relatively low titers of viral supernatant required infection at an MOI of approximately 0.01. Successful transduction of all cell lines was achieved using puromycin selection at 2 μg/ml for HeLa cells and 10 μg/ml for RAW264. For each parental cell line, pools of transduced cells from multiple infections were maintained and characterized. Raw cells with the E3 virus construct were named R*E3, and HeLa with the E3 were named H*E3.

Cell Culture and Histochemical Techniques

Cells were plated at a density of 4×10^4 per well of a 24-well plate in α-MEM/9% FBS. They were treated with media alone; with 20 ng/ml recombinant, soluble RANK-ligand (RANK-L)(Amgen); with 10 ng/ml IL-4 (R&D Systems); with 30 ng/ml m-CSF (R&D Systems); with 5 ng/ml γ-IFN (R&D Systems), or with 500 ng/ml LPS (Sigma-Aldrich). Treatment times were as indicated in the figures, generally 1–6 days.

Cultures were rinsed briefly with PBS, fixed for 20 min on ice in citrate buffered 3% formaldehyde in 66% acetone (Sigma's CAF fixative). For TRAP staining, the instructions to Sigma Kit 387 were followed with a 20-min incubation at 37°C. For immunocytochemistry, cultures were blocked in 10% normal horse serum (HS) for 1 h at room temperature, incubated in primary antibody ME20.4 (anti-NGFR, a gift from Paul Orchard, University of Minnesota) diluted to 5.4 μg/ml in 1% HS in PBS overnight at 4°C, incubated in biotinylated secondary antibody (Jackson Immunoresearch) diluted to 1.1 μg/ml in 1% HS in PBS at room temperature for 2 h, incubated in avidin–biotin complex per Vector's recommendations, incubated in DAB per Zymed's recommendations for 30 min at 30°C. Several rinses of PBS were done in between each step except the 10% HS and primary incubations. Stained cultures were visualized and photographed on a Nikon TE200 inverted microscope with a CCD digital camera.

Electrophoretic Mobility Shift Assay (EMSA)

Design and labeling of DNA probes: the 113 bp amplicon from the *TRAP* gene was used as a template to design 11 overlapping 27 bp probes to be used in EMSA. Each probe was constructed by purchasing single-stranded 23 base oligodeoxyribonucleotides (IDT). The two oligos that annealed for each probe were designed to generate a 4-base overhang on each 5-prime end with two Ts in each overhang. Double stranded, annealed probes were labeled by a fill-in reaction with the Klenow fragment of DNA Polymerase I (Roche) with α-^{32}P-dATP (Perkin-Elmer), cold dCTP, dGTP, and dTTP. Labeled probes were purified with Sephadex G-25 spin columns (Amersham-Pharmacia). Probe sequences: NFκB control probe (5′-GGTTTA-CAGGGGACTTCCCTCGGGTT-3′ and 5′-TTG-CAACCCGAGGGAAGTCCCCTGTA-3′), based on [Ohmori et al., 1994]. TRAP T7 probe: (5′-TTGCTACCCAGGGAGGAAGTTAG-3′ and 5′-TTCCCTAACTTCCTCCCTGGGTA-3′). TRAP T4 probe with putative AP-1 sites underlined: (5′-CTTCTTGAGACACTGTCTCAAAA-3′ and 5′-ATTGTTTTGAGACAGTGTCTCAA-3′). 5′mut probe (of T4 sequence) with mutation indicated in lowercase: (5′-CTTCTTGAGACACgGTCT-CAAAA-3′ and 5′-ATTGTTTTGAGACCcGTG-TCTCAA-3′). 3′mut probe (of T4 sequence) with mutations indicated in lowercase: (5′-CTT-CTgGAGACACTGTCTCcAAA-3′ and 5′-ATT-GTTTgGAGACAGTGTCTCcA-3′). AP-1 probe with AP-1 consensus sequence underlined: (5′-AATTCGCTTGATGAGTCAGCCGGAA-3′ and 5′-AATTTTCCGGCTGACTCATCAAGCG-3′). mAP probe with mutations indicated in lower case: (5′-AATTCGCTTGATGAGTtgGCCGGAA-3′ and 5′-AATTTTCCGGCcaACTCATCAAG-CG-3′).

Cells were plated at a density of 5×10^4 per 25 cm^2 flask in α-MEM/9% FBS. They were treated with media alone or with 20 ng/ml soluble RANK-L for the times indicated in the figures. Whole cell protein was extracted according to the method of Schöler et al. [1989] using Roche's Cømplete protease inhibitors. Protein yield was quantitated with Pierce's Micro-BCA kit. Typically, 10 μg of protein and 1 ng of labeled probe were used per 25 μl EMSA reaction for one lane on a gel. Specifically, protein samples were diluted in water at 4°C. Labeled DNA probe was diluted at 4°C in 40 mM HEPES, pH 7.8, 2 mM $MgCl_2$, 0.2 mM EGTA, 0.8 mM DTT, 80 mM KCl, 8.4% Ficoll (Sigma-Aldrich), 125 μg/ml sheared denatured salmon sperm DNA (Eppendorf). Equal volumes of protein and DNA solutions were mixed together at room temperature for 30 min. For antibody blocking and supershift experiments, 1 μl of antibody (typically 2 μg) was added to the protein diluted in water and incubated at room temperature for 10 min prior to addition of DNA probe solution. Antibodies used were anti-pan-Fos (K-25) Santa Cruz #sc-253X; anti-c-Fos (Ab-5) Oncogene Research #PC38; anti-pan-Jun (D) Santa Cruz #sc-44X; anti-c-Jun (H-79) Santa Cruz #sc-1694X; and anti-c/EBP-β(Δ198) Santa Cruz #sc-746X. Samples were loaded onto a native 4% polyacrylamide gel that had been pre-run for 30 min at 4°C in 0.25× TBE. Gels were run at 270 V for 400 V-h at 4°C (until the free probe was near the bottom of the 20 cm gel), then dried and exposed to Kodak MS-1 film using a transcreen intensifying screen. Autoradiograms were scanned on a flatbed scanner for digitization.

RESULTS

E3 Retrovirus Assembly

The MMLV retroviral vector, pBABE, was used for cloning a portion of the TRAP upstream regulatory region into the enhancer region of the MMLV promoter. As indicated in Figure 1, the enhancer region of the MMLV three-prime

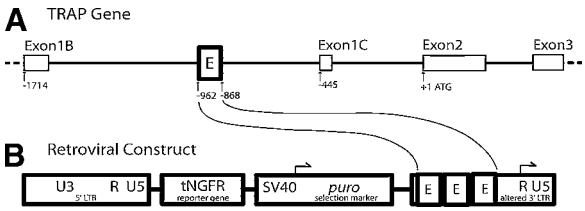

Fig. 1. Element from the tartrate-resistant acid phosphatase (TRAP) promoter replacing the enhancer region of the MoMLV LTR. **Panel A** diagrams a portion of the *TRAP* gene and the relative location of the 95 bp E element used for the retrovirus construct. **Panel B** shows the E3 retrovirus that was constructed using the pBABE-puro vector. Truncated human nerve growth factor receptor (*tNGFR*) was inserted as a reporter gene, and the U3 region of the 3′LTR was replaced with a triple repeat of the TRAP E element. (After retroviral DNA integration, the 3′LTR is positioned 5′ to the reporter gene in the host genome.)

long terminal repeat (3′LTR) was excised, leaving only the basal promoter, and an element from the TRAP regulatory region, called the E region (bases −962 to −868, [Reddy et al., 1995]), was PCR amplified and cloned into the MMLV LTR as a concatamer of three repeats; hence the name E3, for the final retrovirus construct. As a marker gene, a truncated form of human *NGFR* [Orchard et al., 2002] was cloned into pBABE under the transcriptional control of the altered retroviral LTR.

E3 Expression in RAW Cells

The osteoclastogenic precursor cell line, RAW 264.7, was used to test the responsiveness of the E3 construct to induction by RANK-L. RAW cells stably transduced with the E3 retrovirus (R*E3 cells), were treated for 2 days with RANK-L. As shown in Figure 2A, R*E3 cells expressed a basal level of the marker gene, when grown in culture media alone. Treatment with RANK-L dramatically induced the expression of NGFR (Fig. 2B). Intensity of staining of all cells increased, and the number of positive cells increased with RANK-L treatment.

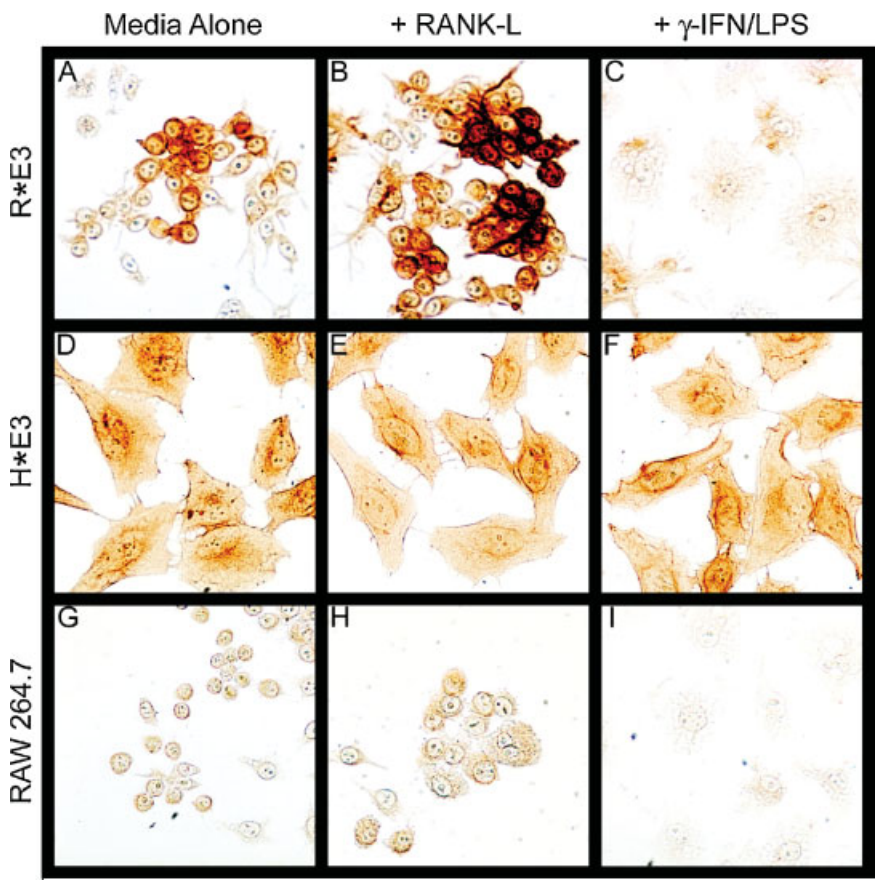

Fig. 2. E3 retrovirus is expressed during osteoclast differentiation. RAW cells transduced with the E3 retrovirus (R*E3) (**A**–**C**), HeLa cells with E3 (H*E3) (**D**–**F**), or untrasduced RAW cells (**G**–**I**) were treated with media alone (A, D, G), 20 ng/ml RANK-ligand (RANK-L) (B, E, H), or 5 ng/ml γ-interferon and 0.5 μg/ml lipopolysaccharide (C, F, I). RANK-L-induced reporter gene (*NGFR*) expression in R*E3 cells, but not in H*E3 cells (IHC for NGFR). No staining was detected in untransduced cells (G–I).

Toward characterizing the specificity of RANK-L induced expression, the influence of other cytokines on R*E3 cells was determined. Neither m-CSF, Interleukin-4, γ-interferon (γ-IFN) nor lipopolysaccharide (LPS), alone or in combinations induced reporter gene expression, and none synergized with RANK-L to increase the induction of tNGFR (data not shown).

Interestingly, both γ-IFN and LPS caused a decrease in NGFR expression (Fig. 2C). As expected, γ-IFN and LPS treatment was associated with macrophage differentiation. The specificity of the induction was further characterized by observation of the HeLa fibroblastic cell line transduced with the E3 retrovirus (H*E3). H*E3 cells expressed basal NGFR, but expression was neither induced with RANK-L nor reduced with γ-IFN and LPS (Fig. 2D–F). Taken together these results indicate that the customized E3 construct promotes retroviral gene expression under osteoclastogenic but not macrophage-differentiating conditions.

E3 Expression During Osteoclast Differentiation

To test whether the E3 construct mimicked expression of the *TRAP* gene, endogenous TRAP expression was compared to NGFR expression following RANK-L treatment. Endogenous TRAP was induced in R*E3 cells (Fig. 3D–F) with a similar time course to the parental RAW cells (Fig. 3G–I). After 1 day of treatment, TRAP expression (Fig. 3D) and NGFR expression (Fig. 3A) was low. After 2 days of treatment, endogenous TRAP and NGFR expression were increased (Fig. 3B,E). Interestingly, as cells fused and differentiated into osteoclast-like cells, the expression of NGFR decreased (Fig. 3C) despite high levels of endogenous

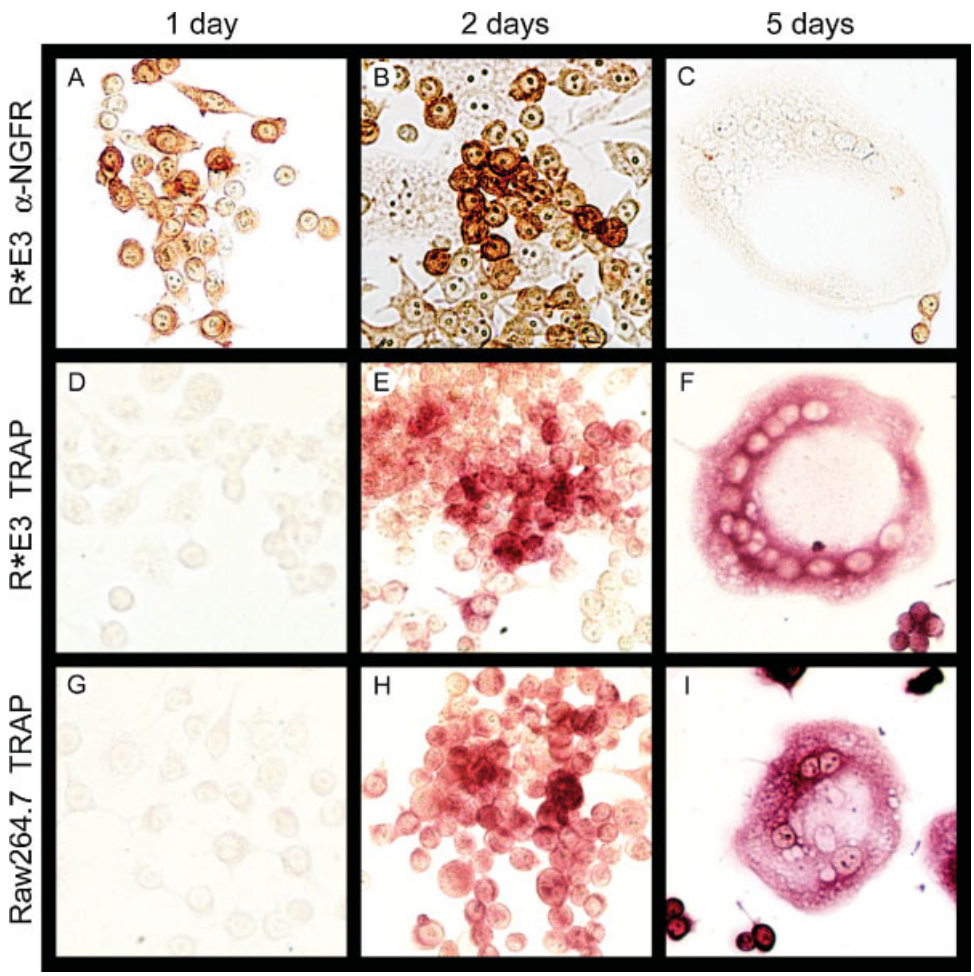

Fig. 3. E3 construct expression is transient during osteoclast differentiation. R*E3 cells were treated with 20 ng/ml RANK-L for 1, 2, or 5 days. Cells were stained for endogenous TRAP (**D**–**F**) or for NGFR (**A**–**C**).

TRAP expression (Fig. 3F,I). Thus the E3 construct was only transiently responsive to RANK-L treatment.

DNA Binding Proteins and Analysis of the E3 Region

EMSA were employed to define that portion(s) of the E region that was interacting with DNA binding proteins in transduced cells. The E region was used as template to design overlapping 27 bp DNA probes for EMSA analysis (Fig. 4A). Although several probes showed electrophoretic mobility shift when incubated with protein extracted from RAW cells (not shown), only one probe (designated T4) showed an increase in protein binding after RAW cells had been incubated with RANK-L. Figure 4B shows three bands seen with the T4 probe under control conditions, and an increase in band intensity after RANK-L treatment. The induction of these DNA binding proteins was maximal 8 h after addition of RANK-L, and thereafter decreased. A positive control NFκB probe showed the predicted increase in protein binding following treatment with RANK-L (Fig. 4B). The pattern and intensity of bands seen with probe T7 was unchanged after treatment with RANK-L, confirming specificity of the T4 probe response.

Evaluation of Mutated AP-1 Binding Sites in T4 Probe

Sequence analysis of the T4 probe revealed an inverted repeat of two AP-1 binding sites. To determine if the T4 probe interacted with AP-1 like proteins, customized T4 probes with point mutations in the 5′ end of one, or the 3′ ends of both AP-1 binding sites were examined (Materials and Methods and Fig. 5A). Both mutations eliminated protein binding, suggesting that the DNA binding protein(s) in the RAW cell lysates following RANK-L treatment was an AP-1 like protein (Fig. 5B).

Further evidence of AP-1 interaction was shown by using an unlabeled (cold) AP-1 probe as competitor against radiolabel led T4 probe. An AP-1 probe was designed with a single 7 bp AP-1 consensus sequence in it, and a mutated version was also designed with 2 bp changes in the 3′ end of the consensus sequence (see Materials and Methods for details). A 200-fold excess of cold AP-1 probe blocked the T4 shift as efficiently as cold T4 probe. Additionally, the mutated AP-1 did not block the shift of the three

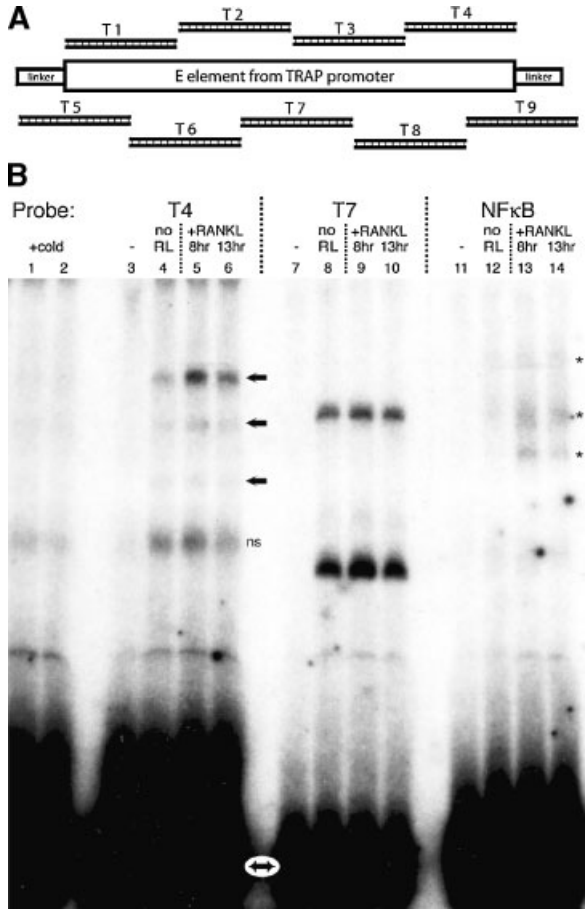

Fig. 4. Electrophoretic mobility shift assays (EMSA) reveal RANK-L-induced protein binds to the E element. **A**: EMSA probe design: oligonucleotide probes, designated T1–T9, were designed that overlap the sequence of the TRAP E element, including linkers used in the retroviral construct. **B**: EMSA with T4 (**lanes 1–6**), T7 (7–10), and NFκB probes. Proteins binding the T4 probe (indicated by arrows) were induced by RANK-L at 8 h and then declined (lanes 4–6). Protein binding to the T7 probe was not affected by RANK-L treatment (**lanes 8–10**). Proteins binding the NFκB probe were induced with RANK-L (**lanes 12–14**). Specificity of binding was shown by blocking the shifted bands with 50× and 100× excess cold probe (lanes 1–2, respectively). Arrows indicate shifted bands with T4. Stars indicate shifted bands with NFκB probe. (–) no protein, probe only (**lanes 3**, 7, and 11). Lanes 1, 2, 4, 8, and 12: Protein from untreated RAW cells. Lanes 5, 9, and **13** protein from RAW cells treated for 8 h with RANK-L. Lanes 6, 10, and **14**; 13 h with RANK-L.

T4 bands (Fig. 5C). As negative control for the sequence specificity, an NFκB probe did not block the shifted T4 bands.

EMSA Antibody Blocked Shift of Candidate AP-1 Binding Proteins

To further characterize the proteins binding to the T4 sequence, antibodies to AP-1 proteins

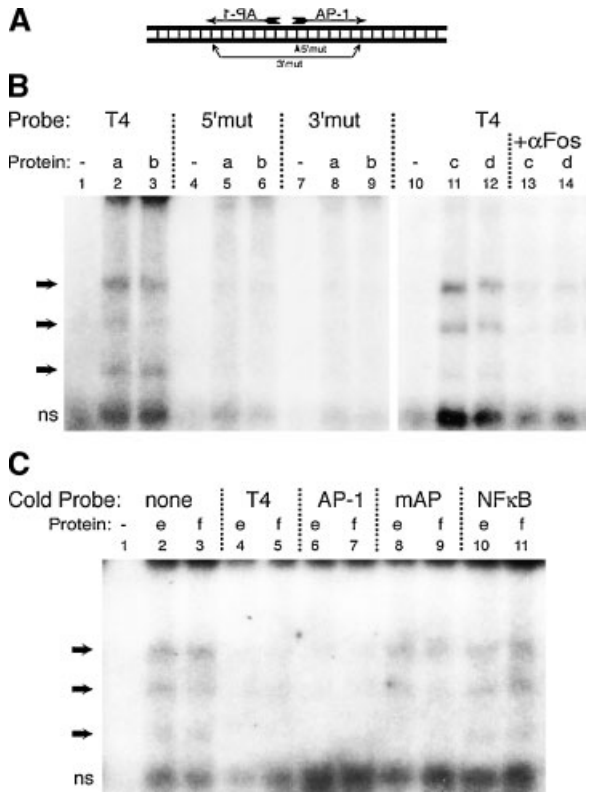

Fig. 5. The factor binding the T4 probe is Fos. **A**: Altered T4 probes were made with point mutations in either the 5′ end (5′mut) or 3′ end (3′mut) of two candidatel AP-1 binding sites identified in the T4 sequence of the E element. **B**: When incubated with duplicate protein lysates (a and b), these mutated T4 probes showed strongly diminshed shifts (**lanes 5**, **6**, **8**, and **9**) relative to the wild-type T4 probe (2 and 3). Antibody against Fos proteins (**lanes 13**, **14**) inhibited binding of protein from duplicate lysates (c and d) relative to control with no antibody (11, 12). **C**: All lanes are T4 probe with duplicate lysates (e and f). Cold AP-1 probe blocked formation of the shifts (lanes 6 and **7**) as well as cold T4 probe (4 and 5). A cold mutant form, mAP, of the AP-1 probe did not block the shifts (8 and 9). A negative control, unrelated NFκB cold probe also did not block the shifts (10 and 11). (–) is no protein lysate.

were used to attempt to block the protein–DNA-probe interactions. Anti-Fos antibody effectively blocked the appearance of the T4 bands on EMSA. Incubation of RAW cell protein with the T4 probe and Jun or c/EBPβ directed antibodies did not block the T4 bands or cause a supershift (not shown). However, incubation of RAW cell protein with the T4 probe and Fos directed antibody eliminated the T4 probe bands (Fig. 5B). This finding indicates that a Fos family protein, and not a Jun family protein, is interacting with the T4 probe. As expected, incubation of RAW cell protein with either a control c/EBP probe and c/EBPβ antibody, or with a control AP-1 probe, and either Jun-directed antibody or Fos-directed antibody caused supershifts (not shown). These data confirm that these transcription factors known to interact with Fos were expressed and detectable in RAW extracts.

DISCUSSION

In this report, we hypothesized that exchange of the E region from the *TRAP* gene (−962 to −868) for the enhancer region of the MMLV would create a retroviral construct with specific expression during osteoclast differentiation. Our findings supported the hypothesis by revealing increased retroviral marker gene (*NGFR*) expression following osteoclastic (RANK-L), but not macrophage (γIFN/LPS) differentiation of E3 transduced RAW cells. Selection of the LTR U3 enhancer region exchange was based on previous descriptions of tissue-specific vector development targeting melanin-producing and CEA-producing cells [Marks and Walker, 1981; Clohisy and Ramnaraine, 1998; Adachi et al., 2000; Erbs et al., 2000; Luchin et al., 2000; Chung-Faye et al., 2001; Reddy et al., 2001; Chiu et al., 2004]. In those reports, the most effective vectors for conferring tissue-specific gene expression replaced the viral enhancer sequence in the U3 region with elements of the tissue specific gene repeated as a concatamer of three repeats [Clohisy and Ramnaraine, 1998; Erbs et al., 2000; Luchin et al., 2000].

While RANK-L treatment induced expression from the retroviral construct, this enhanced expression was transient, peaking 2 days after treatment and disappearing by 5 days after treatment. Because expression from a retrovirally integrated gene has the potential to be stably expressed over time following RANK-L treatment, the observations that reporter gene expression actually decreased after 2 days and was not detectable in multinucleated osteoclasts suggest that the regulatory elements in the E3 retroviral construct are not as complete as the regulatory elements in the full endogenous TRAP promoter. Presumably some of the transcription factors controlling TRAP induction bind to sequence in the E region, but for sustained expression, other transcription factors must binding to other regions of the TRAP promoter to recapitulate endogenous gene expression. Potential regulatory regions outside the E3 region include previously characterized

binding sites for PU.1 [Oddie et al., 2000], MITF [Luchin et al., 2000], and Pip/IRF4 [Matsumoto et al., 2001], located downstream of the E region, and sites for YY-1, USF1 & 2 [Liu et al., 2003], and Myc/Max [Daumer et al., 2002] upstream from the E region. Using the sequence around the *TRAP* gene from NCBI database, we have been able to identify additional putative regulatory elements upstream at exon 1B. These potential DNA binding proteins include Pip/IRF4, NFκB, USF1 & 2, YY-1, AP-1, and PU.1 (data not shown). Because these transcription factors outside the E region may interact with binding sites withing the E region, we sought to first characterize potential DNA binding sites in the E region. Future work will focus on identifying regions outside this E region, which regulate sustained expression of reporter gene.

A more detailed analysis of the E region identified a 27 bp sequence (termed T4), which revealed increased protein binding in lysates from RANK-L treated cells. The T4 probe identified three shifted bands with EMSA analysis. These bands could represent three different DNA-binding proteins with different gel mobility characteristics. Alternatively, the lower band could represent a DNA-binding protein, and the upper bands could represent proteins such as transcriptional co-activators, which interact with the DNA-binding protein. The fact that all three bands increased equally in intensity following RANK-L treatment favors the interpretation that one protein (represented by the lower band) is induced by RANK-L.

Inspection of the T4 sequence revealed an inverted repeat of two AP-1 binding sites that appeared functional since point mutations of AP-1 sites eliminated protein binding in lysates from RANK-L-treated cells. Both mutations diagrammed in Figure 5A eliminated protein binding to the probe, suggesting that both AP-1 sites are functionally important in the E3 retrovirus, as well as in the endogenous TRAP regulatory region. Additional evidence of AP-1 involvement was shown by blocking the interaction between labeled T4 probe and protein with unlabelled (cold) AP-1 probe; but not with cold AP-1 probe containing a mutation in the binding site. Specific to the retrovirus construct, however, is the triple repeat of the E region, which generates six functional AP-1 sites within a relatively small regulatory region. Identification of the AP-1 binding sequence as an active site within the T4 probe following RANK-L treatment suggests that AP-1 proteins are likely involved in regulating expression from the E3 retrovirus. AP-1 is a complex of two proteins dimerized by a basic leucine zipper (bZIP) [Landschulz et al., 1988]. The proteins that comprise AP-1 vary depending on cell phenotype and activation or proliferation state. Often, the proto-oncogene product, c-Jun, or its homologue JunB or JunD bind as homodimers to AP-1 DNA sites. Alternatively, Jun proteins can heterodimerize with c-Fos, one of the Fos homologues (FosB, Fra1, or Fra2), c/EBPβ, or members of the ATF/CREB family [Nakabeppu et al., 1988; Eferl and Wagner, 2003].

Experiments to block the shifted bands showed alterations with antibodies to Fos but not Jun family proteins. Specifically, an antibody to c-Fos did not alter the T4 protein shift pattern, but the pan-Fos antibody, which recognizes all four Fos family members did block the interaction of T4 probe and protein. The importance of Fos family transcription factors in osteoclast differentiation has been demonstrated by the phenotype of *c-fos* knockout mice being osteopetrotic [Grigoriadis et al., 1994]. Additionally, Fra-1 can rescue this phenotype [Fleischmann et al., 2000], and Fra-1 overexpressing mice have increased bone mass [Jochum et al., 2000]. Thus, the function of Fos proteins in osteoclasts is complex and may be redundant.

The link between RANK on the cell surface and Fos action in the nucleus has been characterized in osteoclasts via the canonical Fos activation by the mitogen activated protein kinase (MAPK) second messenger pathway involving Ras/Raf, MEK 1/2, and ERK 1/2 [Miyazaki et al., 2000]. The involvement of other AP-1 factors such as c-Jun and JunB have been well documented in osteoclasts [Ikeda et al., 2004; Kenner et al., 2004] acting through the JNK1 second messenger pathway downstream of RANK [David et al., 2002]. The current findings of Fos binding to the T4 region within the E element of the TRAP regulatory region suggest a site where the biologically relevant actions of Fos activation in osteoclasts could be taking place, and further, where they might interact with Jun or other AP-1 proteins, providing a link between the two second-messenger systems activated by RANK. This demonstration of Fos binding to the T4 enhancer region explains only part of the activation of the endogenous *TRAP* gene, since the induction

of reporter gene in the retrovirus construct, and the binding of protein(s) to the T4 probe, which were all blocked by Fos antibody, were transient following RANK-L stimulation. Clearly, to maintain high levels of induction, other regulatory regions of the *TRAP* gene are required to bind different transcription factors.

We have begun to explore the possibility of osteoclast-based gene delivery systems. This approach may be feasible based on the ability to provide osteoclasts in human and experimental animals via bone marrow transplantation [Walker, 1975a,b; Coccia et al., 1980; Orchard et al., 1987; Tondravi et al., 1997; Eapen et al., 1998]. However, such an approach will require gene transduction constructs expressed preferentially by osteoclast lineage cells. We developed a customized, TRAP-based retroviral vector and determined that its expression is induced early during osteoclast differentiation, but not in mature osteoclasts. This finding may serve as an initial step toward developing gene delivery vectors that ultimately permit development of osteoclast-based gene delivery therapies.

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REFERENCES

Adachi Y, Tamiya T, Ichikawa T, Terada K, Ono Y, Matsumoto K, Furuta T, Hamada H, Ohmoto T. 2000. Experimental gene therapy for brain tumors using adenovirus-mediated transfer of cytosine deaminase gene and uracil phosphoribosyltransferase gene with 5-fluorocytosine. Hum Gene Ther 11:77–89.

Barresi V, Belluardo N, Sipione S, Mudo G, Cattaneo E, Condorelli DF. 2003. Transplantation of prodrug-converting neural progenitor cells for brain tumor therapy. Cancer Gene Ther 10:396–402.

Chiu WS, McManus JF, Notini AJ, Cassady AI, Zajac JD, Davey RA. 2004. Transgenic mice that express Cre recombinase in osteoclasts. Genesis 39:178–185.

Chung-Faye GA, Chen MJ, Green NK, Burton A, Anderson D, Mautner V, Searle PF, Kerr DJ. 2001. In vivo gene therapy for colon cancer using adenovirus-mediated, transfer of the fusion gene cytosine deaminase and uracil phosphoribosyltransferase. Gene Ther 8:1547–1554.

Clohisy DR, Ramnaraine ML. 1998. Osteoclasts are required for bone tumors to grow and destroy bone. J Orthop Res 16:660–666.

Clohisy DR, Ramnaraine ML, Scully S, Qi M, Van G, Tan HL, Lacey DL. 2000. Osteoprotegerin inhibits tumor-induced osteoclastogenesis and bone tumor growth in osteopetrotic mice. J Orthop Res 18:967–976.

Coccia PF, Krivit W, Cervenka J, Clawson C, Kersey JH, Kim TH, Nesbit ME, Ramsay NK, Warkentin PI, Teitelbaum SL, Kahn AJ, Brown DM. 1980. Successful bone-marrow transplantation for infantile malignant osteopetrosis. N Engl J Med 302:701–708.

Daumer KM, Taparowsky EJ, Hall DJ, Steinbeck MJ. 2002. Transcription from the tartrate-resistant acid phosphatase promoter is negatively regulated by the Myc oncoprotein. J Bone Miner Res 17:1701–1709.

David JP, Sabapathy K, Hoffmann O, Idarraga MH, Wagner EF. 2002. JNK1 modulates osteoclastogenesis through both c-Jun phosphorylation-dependent and-independent mechanisms. J Cell Sci 115:4317–4325.

Diaz RM, Eisen T, Hart IR, Vile RG. 1998. Exchange of viral promoter/enhancer elements with heterologous regulatory sequences generates targeted hybrid long terminal repeat vectors for gene therapy of melanoma. J Virol 72:789–795.

Eapen M, Davies SM, Ramsay NK, Orchard PJ. 1998. Hematopoietic stem cell transplantation for infantile osteopetrosis. Bone Marrow Transplant 22:941–946.

Eferl R, Wagner EF. 2003. AP-1: A double-edged sword in tumorigenesis. Nat Rev Cancer 3:859–868.

Erbs P, Regulier E, Kintz J, Leroy P, Poitevin Y, Exinger F, Jund R, Mehtali M. 2000. In vivo cancer gene therapy by adenovirus-mediated transfer of a bifunctional yeast cytosine deaminase/uracil phosphoribosyltransferase fusion gene. Cancer Res 60:3813–3822.

Fleischmann A, Hafezi F, Elliott C, Reme CE, Ruther U, Wagner EF. 2000. Fra-1 replaces c-Fos-dependent functions in mice. Genes Dev 14:2695–2700.

Grigoriadis AE, Wang ZQ, Cecchini MG, Hofstetter W, Felix R, Fleisch HA, Wagner EF. 1994. c-Fos: A key regulator of osteoclast-macrophage lineage determination and bone remodeling. Science 266:443–448.

Honore P, Luger NM, Sabino MA, Schwei MJ, Rogers SD, Mach DB, O'Keefe PF, Ramnaraine ML, Clohisy DR, Mantyh PW. 2000. Osteoprotegerin blocks bone cancer-induced skeletal destruction, skeletal pain and pain-related neurochemical reorganization of the spinal cord. Nat Med 6:521–528.

Ikeda F, Nishimura R, Matsubara T, Tanaka S, Inoue J, Reddy SV, Hata K, Yamashita K, Hiraga T, Watanabe T, Kukita T, Yoshioka K, Rao A, Yoneda T. 2004. Critical roles of c-Jun signaling in regulation of NFAT family and RANKL-regulated osteoclast differentiation. J Clin Invest 114:475–484.

Jochum W, David JP, Elliott C, Wutz A, Plenk H, Jr., Matsuo K, Wagner EF. 2000. Increased bone formation and osteosclerosis in mice overexpressing the transcription factor Fra-1. Nat Med 6:980–984.

Kenner L, Hoebertz A, Beil T, Keon N, Karreth F, Eferl R, Scheuch H, Szremska A, Amling M, Schorpp-Kistner M, Angel P, Wagner EF. 2004. Mice lacking JunB are

osteopenic due to cell-autonomous osteoblast and osteoclast defects. J Cell Biol 164:613–623.

Kong YY, Feige U, Sarosi I, Bolon B, Tafuri A, Morony S, Capparelli C, Li J, Elliott R, McCabe S, Wong T, Campagnuolo G, Moran E, Bogoch ER, Van G, Nguyen LT, Ohashi PS, Lacey DL, Fish E, Boyle WJ, Penninger JM. 1999. Activated T cells regulate bone loss and joint destruction in adjuvant arthritis through osteoprotegerin ligand. Nature 402:304–309.

Landschulz WH, Johnson PF, McKnight SL. 1988. The leucine zipper: A hypothetical structure common to a new class of DNA binding proteins. Science 240:1759–1764.

Liu Y, Shi Z, Silveira A, Liu J, Sawadogo M, Yang H, Feng X. 2003. Involvement of upstream stimulatory factors 1 and 2 in RANKL-induced transcription of tartrate-resistant acid phosphatase gene during osteoclast differentiation. J Biol Chem 278:20603–20611.

Luchin A, Purdom G, Murphy K, Clark MY, Angel N, Cassady AI, Hume DA, Ostrowski MC. 2000. The microphthalmia transcription factor regulates expression of the tartrate-resistant acid phosphatase gene during terminal differentiation of osteoclasts. J Bone Miner Res 15:451–460.

Marks SC, Jr., Walker DG. 1981. The hematogenous origin of osteoclasts: Experimental evidence from osteopetrotic (microphthalmic) mice treated with spleen cells from beige mouse donors. Am J Anat 161:1–110.

Matsumoto M, Hisatake K, Nogi Y, Tsujimoto M. 2001. Regulation of receptor activator of NF-kappaB ligand-induced tartrate-resistant acid phosphatase gene expression by PU.1-interacting protein/interferon regulatory factor-4. Synergism with microphthalmia transcription factor. J Biol Chem 276:33086–33092.

Miyazaki T, Katagiri H, Kanegae Y, Takayanagi H, Sawada Y, Yamamoto A, Pando MP, Asano T, Verma IM, Oda H, Nakamura K, Tanaka S. 2000. Reciprocal role of ERK and NF-kappaB pathways in survival and activation of osteoclasts. J Cell Biol 148:333–342.

Morgenstern JP, Land H. 1990. Advanced mammalian gene transfer: High titre retroviral vectors with multiple drug selection markers and a complementary helper-free packaging cell line. Nucleic Acids Res 18:3587–596.

Mundy GR. 2002. Metastasis to bone: Causes, consequences and therapeutic opportunities. Nat Rev Cancer 2:584–593.

Nakabeppu Y, Ryder K, Nathans D. 1988. DNA binding activities of three murine Jun proteins: Stimulation by Fos. Cell 55:907–915.

Oddie GW, Schenk G, Angel NZ, Walsh N, Guddat LW, de Jersey J, Cassady AI, Hamilton SE, Hume DA. 2000. Structure, function, and regulation of tartrate-resistant acid phosphatase. Bone 27:575–584.

Ohmori Y, Tebo J, Nedospasov S, Hamilton TA. 1994. Kappa B binding activity in a murine macrophage-like cell line. Sequence-specific differences in kappa B binding and transcriptional activation functions. J Biol Chem 269:17684–17690.

Orchard PJ, Dickerman JD, Mathews CH, Frierdich S, Hong R, Trigg ME, Shahidi NT, Finlay JL, Sondel PM. 1987. Haploidentical bone marrow transplantation for osteopetrosis. Am J Pediatr Hematol Oncol 9:335–340.

Orchard PJ, Blazar BR, Burger S, Levine B, Basso L, Nelson DM, Gordon K, McIvor RS, Wagner JE, Miller JS. 2002. Clinical-scale selection of anti-CD3/CD28-activated T cells after transduction with a retroviral vector expressing herpes simplex virus thymidine kinase and truncated nerve growth factor receptor. Hum Gene Ther 13:979–988.

Reddy SV, Hundley JE, Windle JJ, Alcantara O, Linn R, Leach RJ, Boldt DH, Roodman GD. 1995. Characterization of the mouse tartrate-resistant acid phosphatase (*TRAP*) gene promoter. J Bone Miner Res 10: 601–606.

Reddy SV, Kurihara N, Menaa C, Landucci G, Forthal D, Koop BA, Windle JJ, Roodman GD. 2001. Osteoclasts formed by measles virus-infected osteoclast precursors from hCD46 transgenic mice express characteristics of pagetic osteoclasts. Endocrinology 142:2898–2905.

Sambrook JFE, Maniatis T. 1989. Molecular cloning: A laboratory manual. Cold Spring Harbor: Cold Spring Harbor Press.

Scholer HR, Hatzopoulos AK, Balling R, Suzuki N, Gruss P. 1989. A family of octamer-specific proteins present during mouse embryogenesis: Evidence for germline-specific expression of an Oct factor. EMBO J 8:2543–2550.

Tondravi MM, McKercher SR, Anderson K, Erdmann JM, Quiroz M, Maki R, Teitelbaum SL. 1997. Osteopetrosis in mice lacking haematopoietic transcription factor PU.1. Nature 386:81–84.

Walker DG. 1975a. Bone resorption restored in osteopetrotic mice by transplants of normal bone marrow and spleen cells. Science 190:784–785.

Walker DG. 1975b. Control of bone resorption by hematopoietic tissue. The induction and reversal of congenital osteopetrosis in mice through use of bone marrow and splenic transplants. J Exp Med 142:651–663.

Wei J, Blum S, Unger M, Jarmy G, Lamparter M, Geishauser A, Vlastos GA, Chan G, Fischer KD, Rattat D, Debatin KM, Hatzopoulos AK, Beltinger C. 2004. Embryonic endothelial progenitor cells armed with a suicide gene target hypoxic lung metastases after intravenous delivery. Cancer Cell 5:477–488.