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Cloning and Characterization of the Murine β_3 Integrin Gene Promoter: Identification of an Interleukin-4 Responsive Element and Regulation by STAT-6

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Abstract Expression of the $\alpha_v\beta_3$ integrin by murine bone marrow macrophages is regulated by cytokines such as IL-4 and GM-CSF through transcriptional activation of the β_3 subunit gene. To characterize the molecular mechanisms by which such regulation occurs, we isolated the murine β_3 integrin promoter. To this end, we first cloned a full length β_3 cDNA and used the 5′UTR and leader peptide coding sequence to identify genomic clones containing the β_3 promoter region. The transcriptional start site, identified by primer extension and S1 nuclease assay, is 34 nt upstream of the translation initiation codon. A 1.1 kb fragment of the promoter region drives IL-4 responsive transcription in transiently transfected murine bone marrow macrophages. Deletion analysis of the β_3 promoter indicates the IL-4 responsive element lies between −465 to −678 nt relative to the transcriptional start site. This promoter fragment contains two overlapping STAT consensus recognition sites and nuclear extracts from BMMs contain an IL-4-inducible DNA binding factor, identified by super shift analysis, as STAT-6. Furthermore, an oligonucleotide which includes the two STAT recognition sites residing in the IL-4 responsive region of the β_3 promoter, competes for STAT-6 binding. Confirming IL-4 induction of the integrin subunit is specifically mediated by STAT-6, β_3 mRNA is not enhanced in BMMs derived from STAT-6 deleted mice, which however, retain their capacity to respond to GM-CSF. J. Cell. Biochem. 81:320–332, 2001. © 2001 Wiley-Liss, Inc.

Key words: β_3 integrin; promoter analysis; STAT-6; interleukin 4; bone marrow macrophages

Integrins are heterodimeric transmembrane receptors recognizing specific sequences in a range of proteins residing in extracellular matrix and on other cells and viruses. These heterodimers not only mediate cell–cell and cell–matrix attachment but also bidirectionally transmit signals across the plasma membrane [Hynes, 1992]. Consequent to these integrated activities, integrins contribute to a range of activities including migration, spreading, differentiation, apoptosis, and secretion of matrix proteins [Filardo et al., 1996; Gille and Swerlick, 1996; Ruoslahti, 1996].

The integrin $\alpha_v\beta_3$ is expressed in cells including endothelium, migratory keratinocytes, neutrophils, normal and metastatic epithelium and osteoclasts and their precursors, monocytes and macrophages. Given this distribution, it is not surprising that the blockade of $\alpha_v\beta_3$ function impacts angiogenesis, wound healing, metastasis, phagocytosis, and bone resorption [Ross et al., 1993; Felding-Habermann and Cheresh, 1993; Brooks et al., 1994; Ylanne et al., 1995; Gille and Swerlick, 1996].

$\alpha_v\beta_3$ expression, in nontransformed cells, is regulated by steroid hormones [Dedhar et al., 1991; Mimura et al., 1994; Chiba et al., 1996] and a range of cytokines [Defilippi et al., 1991; Kitazawa et al., 1995; Kim and Yamada, 1997]. Treatment of murine osteoclast precursors with IL-4, for example, prompts surface expression of $\alpha_v\beta_3$ via transcriptional activation of the β_3, but not the α_v gene [Kitazawa et al., 1995]. GM-CSF and TNF-α also modulate $\alpha_v\beta_3$ via their effects on β_3 and not α_v mRNA [Inoue et al., 1998, 2000]. Given β_3 is the regulatory subunit of the integrin, we cloned the promoter of the murine gene with the aim of determining the molecular mechanisms governing β_3 expression. Reflecting our observation that IL-4

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accelerates β_3 transcription, the cytokine transactivates a β_3 reporter construct in murine marrow macrophages by a mechanism involving activation of the transcription factor STAT 6.

METHODS

Bone Marrow Macrophage Isolation

Bone marrow cells were collected by flushing the femora and tibiae of 5-week-old male C3H/HeN or C57/Bl6 mice (Harlan, Indianapolis, IN) and STAT-6 deleted mice (generously provided by J. Ihle) [Shimoda et al., 1996] with α-modified Eagle's medium (α-MEM). Cells were cultured for 24 h in α-MEM containing 10% heat-inactivated fetal bovine serum (α-10) and 500 U/ml M-CSF. The non-adherent population was harvested and mononuclear cells were isolated by density gradient centrifugation on Histopaque-1077 (Sigma, St. Louis, MO). Cells isolated in this manner were used directly for osteoclast generation in co-culture with ST2 cells, or grown for up to 7 days, in the presence of 1000 U/ml M-CSF, for RNA isolation, transfection, or nuclear extract preparation.

Construction of a Murine Osteoclast cDNA Library

Poly(A)$^+$ RNA was isolated from in-vitro-generated osteoclast cultures by collagenase-P treatment, guanidiniun isothiocyanate extraction, and chromatography on oligo-dT cellulose. A cDNA library was constructed in the Uni-ZAP XR vector using the ZAP-cDNA Synthesis Kit (Stratagene, La Jolla, CA). The mouse genomic DNA library in the lambda FIX II vector was purchased (Stratagene).

Library Screening

cDNA probes were labeled to high specific activity with α^{32}PdCTP, using the Random Primed DNA labeling Kit (Boehringer Mannheim). More than 2×10^6 plaques were transferred to MAGNA LIFT membranes (MSI, Westboro, MA) and UV-crosslinked. Filters were pre-hybridized 2 h and subsequently hybridized overnight in 5 × SSPE, 5 × Denhardt's solution, 0.1% SDS, 1 × Background Quencher (Tel-Test, Inc., Friendswood, TX), and 50% formamide. Low stringency hybridization was at 35°C and final washing was in 2 × SSPE, 0.1% SDS, at 35°C for 15 min. High stringency hybridization was at 42°C with a final wash in 1 × SSPE, 0.1% SDS, at 42°C for 15 min. Positive clones were rescreened at decreased plating density until single isolates were obtained.

DNA Isolation and Sequencing

cDNA clones in the Uni-ZAP XR vector were rescued by in vivo excision of pBluescript (Stratagene) and plasmid DNA was purified with the Magic Minipreps System (Promega, Madison, WI). DNA from mouse genomic lambda clones was purified from plate lysates using QIAGEN-tip 100 (Qiagen, Chatsworth, CA). DNAs were sequenced from [^{32}P] end-labeled primers with Taq DNA polymerase using the fmol DNA Sequencing System (Promega). Sequencing products were fractionated by electrophoresis on 5% polyacrylamide/8 M urea gels in 0.6 × TBE. Gels were dried and autoradiographed overnight.

Primer Extension Assay

One nanogram of end-labeled exon I antisense primer (5′-CTCCAACAACAACGCCCGCCAG-3′) was added to 50 μg of murine osteoclast total RNA, heat denatured at 95°C for 5 min, and allowed to anneal at 55°C for 1.5 h in 0.15 M KCl, 10 mM Tris (pH 8.3), and 10 mM EDTA. The annealed primer was extended in 30 mM Tris (pH 8.3), 15 mM $MgCl_2$, 8 mM DTT, 0.25 μg/ml actinomycin D, and 0.22 mM 4dNTP mix with 5 U of AMV reverse transcriptase (Promega) for 1 h at 42°C. Autoradiography was performed for 4 weeks with an intensifying screen at −70°C.

S1 Nuclease Assay

A 71nt oligonucleotide was synthesized (OligosEtc, Wilsonville, OR) which is complementary to the first 10 nt 3′ of the translational start site (ATG) plus 53 nt 5′ of the translational start site with an additional 8 nt (underlined) of noncomplementary sequence as a control for S1 activity (5′-GCGCTCGCATCCTGTCCGCCGCCCTTCACGCCGCGCGCGCGCCACGGCGAGCCTGGCACCCGCCTAGTAGG-3′). The oligonucleotide was end-labeled with [γ^{32}P]ATP and T4PNK (Promega) and 10^5 dpm was added to 50 μg of murine osteoclast total RNA in 1 M NaCl, 0.16 M HEPES (pH 7.5), and 0.33 mM EDTA in a total volume of 50 μl. The mixture was heated to 90°C for 4 min and then kept at 60°C overnight. After cooling to 37°C, 800 μl of S1 nuclease mix was added (500 U S1 nuclease (Boehringer Mannheim), 16 μg of ssDNA, 0.3 M NaCl, 50 mM NaOAc, and

4.5 mM $ZnSO_4$). The reaction was incubated at 37°C for 60 min. The reaction was terminated with the addition of EDTA to 2 mM, 1 μg of tRNA was added, and nucleic acids were ethanol-precipitated. The products were fractionated by electrophoresis through a 5% polyacrylamide/8 M urea gel in 0.6 × TBE and autoradiography was performed for 1 week with intensifying screen at −70°C. Fragment lengths were determined by comparison with previously characterized, unrelated, sequencing products.

Restriction Mapping

Mouse genomic lambda clones were digested with the restriction enzyme NotI to release inserts from vector arms. Clones were subsequently digested with a battery of restriction enzymes (Promega), fractionated on 0.7% agarose gels, vacuum-blotted on nylon membranes (Hybond-N Amersham), and probed with end-labeled oligonucleotide probes from exon I (exon I antisense primer above) and the human exon II (nt 105–151) [Zimrin et al., 1990], which shares 42 of 46 bp of the corresponding mouse cDNA sequence. Hybridization and washing were under the low stringency conditions outlined above, except that formamide was excluded from the hybridization solution.

Promoter Constructs

The promoter region between the 5′ end of genomic clone MG011 and translational start site was amplified by PCR using a T3 promoter primer and the exon I antisense primer described above. The 1.1 kb product was cloned in the TA vector pCRII (Invitrogen, San Diego, CA). The fragment was subsequently subcloned in the luciferase reporter vector pGL3basic (Promega) by PCR addition of a *Bgl* II site at +32 (underlined) (5′-AGATCTCCTGTCCGCCGCCCTTCACG-3′), thereby incorporating the entire 5′ UTR and 1084 bp of promoter. Deletions of the −1084 promoter construct to −678, −465, and −227 were generated by restriction digestion with *Sal*I and *Spe*I, *Pst*I, or *Sma*I, respectively. The DNA was blunt-ended with Vent DNA polymerase (New England Biolabs, Beverly, MA) and the vector was religated.

Transient Transfection of Primary BMMs and FD5 Cells

BMMs were grown on bacterial petri dishes for up to 7 days in α-MEM containing 10% FCS, with addition of 1000 U/ml mouse M-CSF on days 1,3, and 5. Cells were washed with PBS, scraped, pooled, and resuspended at 2.5×10^7/ml in optiMEM (GibcoBRL, Gaithersburg, MD). 15×10^6 cells were mixed with 60 μg test DNA (or control) and 6 μg of CMV-β-galactosidase for electroporation. 0.25 ml of the cell/DNA mixture was pulsed using a GENE-Pulser II (BioRad, Hercules, CA) at 950 μF and 250 V. Three such electroporations were pooled, and plated in 10 ml α-10 containing 1000 U/ml M-CSF, with or without IL-4 (Genzyme, Cambridge, MA). Each pooled sample was considered one replicate with three replicates per condition. After 8 h, cells were lysed in 50 μl of Reporter Lysis Buffer (Promega). Luciferase and β-galactosidase activity in the extract were determined using the Luciferase Assay System and the β-galactosidase Enzyme Assay System (Promega), with an OPTOCOMP II luminometer (MGM Instruments, Hamden, CT). The mouse IL-4-dependent cell line FD5 [Welham et al., 1997] was kindly provided by J. W. Schrader. For each replicate of FD5 cells, 2.5×10^6 cells, in 0.25 ml, were mixed with 20 μg of test plasmid and 0.5 μg of CMV-β-galactosidase and electroporated 950 μF and 250 V. Cells were plated in α-MEM containing 10% heat-inactivated fetal bovine serum, with IL-4 or vehicle, and grown 16 h before lysis.

Electrophoretic Mobility Shift Assay

Nuclear extracts were prepared from BMMs, grown for 7 days on bacterial petri dishes, essentially as described [Tetsuka et al., 1996] with the addition of 5 μg/ml Pepstatin A and Leupeptin in the nuclear extraction buffer. For EMSA, nuclear extract, containing 2 μg protein, was mixed with 1 μg poly dI/dC (Pharmacia) in 10 mM Tris–HCl (pH 7.5), 100 mM NaCl, 1 mM EDTA, 10% glycerol, 1 mM DTT, and 5×10^4 CPM of double-stranded oligonucleotide probe from the FcγRI promoter IFN/IL-4 response element 5′-GTATTTCCCAGAAAAGGAAC-3′ [Kotanides and Reich, 1993]. After 15 min incubation at room temperature, 50 × unlabeled competitors or rabbit antibody raised against STAT-6 (kindly provided by S. McKnight) were added and incubation continued for an additional 2.5 h. Complexes were resolved on a pre-cast 4–20% gradient polyacrylamide gel in 1 × TBE (Novex, San Diego, CA). Gels were dried and autoradiographed 48 h.

Ribonuclease Protection Assay

A fragment of the mouse β_3 cDNA was subcloned for use as template for anti-sense RNA transcription. The β_3 cDNA was digested with *Kpn*I and recircularized creating a clone (pMB3Kpn) containing the first 517 bp of β_3 cDNA sequence immediately followed by the bacteriophage T3 promoter. For anti-sense riboprobe generation, plasmid pMB3Kpn, digested to completion with *Bbv*I (New England Biolabs) or control β-actin template (Ambion, Austin, TX) was transcribed with the T3 Maxiscript kit (Ambion) in the presence of [α^{32}P]UTP (DuPont NEN) at 18°C. The probe was purified on Sephadex G-25 Quick Spin columns (Boehringer Mannheim). This 182 nt transcript contains 168 nt of β_3 anti-sense sequence which is homologous to human β_3 cDNA sequence from 339 to 506 bp. This region spans the exon III/exon IV splice junction [Zimrin et al., 1990] and therefore genomic DNA contaminating the RNA preparation will produce a 124 nt protected fragment in contrast to the 168 nt fragment protected in mRNA. RNase protection assays were performed using the RPA II kit (Ambion) with 20 μg of total RNA and 5×10^4 CPM anti-sense probe or mouse β-actin probe per sample. Samples were hybridized for 16 h at 43°C and digested with a T1/RNase mixture for 45 min at 37°C. Samples were precipitated, resuspended in sample-loading buffer, heated to 95°C, and protected fragments were resolved on a 5% polyacrylamide/8 M urea sequencing gel. Autoradiography was performed for 5 h at RT for β-actin samples or 3 days at −70°C with an intensifying screen for β_3 probes.

RESULTS

Cloning the Mouse Integrin β_3 cDNA

Poly-A+ RNA was isolated from purified populations of generated osteoclasts and their committed precursors [Shioi et al., 1994]. An unamplified cDNA library, derived from osteoclast poly-A+ RNA, was screened by low stringency plaque hybridization with a rat β_3 integrin partial cDNA (kindly provided by M. Poncz). From 2×10^6 clones screened, two containing β_3 cDNAs were isolated, one of which (SMOc211) contains sequence-encoding amino acids (AAs) homologous to 242 through 790 of human β_3 integrin [Fitzgerald et al., 1987] as well as the 3′ untranslated region (UTR) and a poly-A tail. The second clone (SMOc631) contains sequences comprising two 5′ UTR nucleotides, the translational start site (AUG), and sequence encoding 204 AAs. A 510 bp restriction fragment from the 5′ end of SMOc631 was used to screen an additional 1×10^6 clones of the mouse osteoclast cDNA library yielding a single cDNA clone (SMOc152) containing 33 nt of 5′ UTR. The cDNA sequences thus isolated represent the 5′ and 3′ ends of the mouse β_3 cDNA molecule with an intervening gap. In order to generate a full length mouse β_3 cDNA, we performed RT-PCR on mouse osteoclast RNA using primers derived from the sequenced partial cDNAs. This effort yielded a fragment spanning the intervening gap, including *Hind*III and *Sac*I sites, at 528 and 1066 bp, relative to the human sequence. The resulting PCR product was digested with *Hind*III and *Sac*I and ligated with *Hind*III digested SMOc152 and *Sac*I digested SMOc211 flanking on the 5′ and 3′ ends, respectively. We co-expressed this full length mouse β_3 cDNA with human α_v in a clone of the human erythroleukemic cell line, K562 which lacks endogenous β_3. These stable transfectants form functional chimeric human α_v/mouse β_3 heterodimers [Blystone et al., 1996]. The sequence of the full length mouse β_3 cDNA has been deposited in GenBank and assigned the accession #AF026509.

The deduced amino acid sequence of the full length mouse and human β_3 integrins are aligned in Figure 1. The DNA coding regions (data not shown) and amino acid sequences of the two species are 85.9 and 90% identical, respectively. The cytoplasmic tails, which are followed by tandem transcriptional termination signals, are identical. The leader peptides, comprising 25 and 26 amino acids in the mouse and human proteins, respectively, exhibit only 68% homology. All 56 cysteine residues are conserved, a hallmark of β-integrin family members with the exception of β_8 [Moyle et al., 1991].

Cloning and Mapping the Murine β_3 Gene Promoter Locus

To identify genomic exon I sequence, we screened an SV129 mouse genomic lambda-FIXII library with a ^{32}P-labeled, PCR-amplified fragment from nt 1 to 165 of clone SMOc152. This 5′ β_3 cDNA was hybridized with approximately three genome equivalents

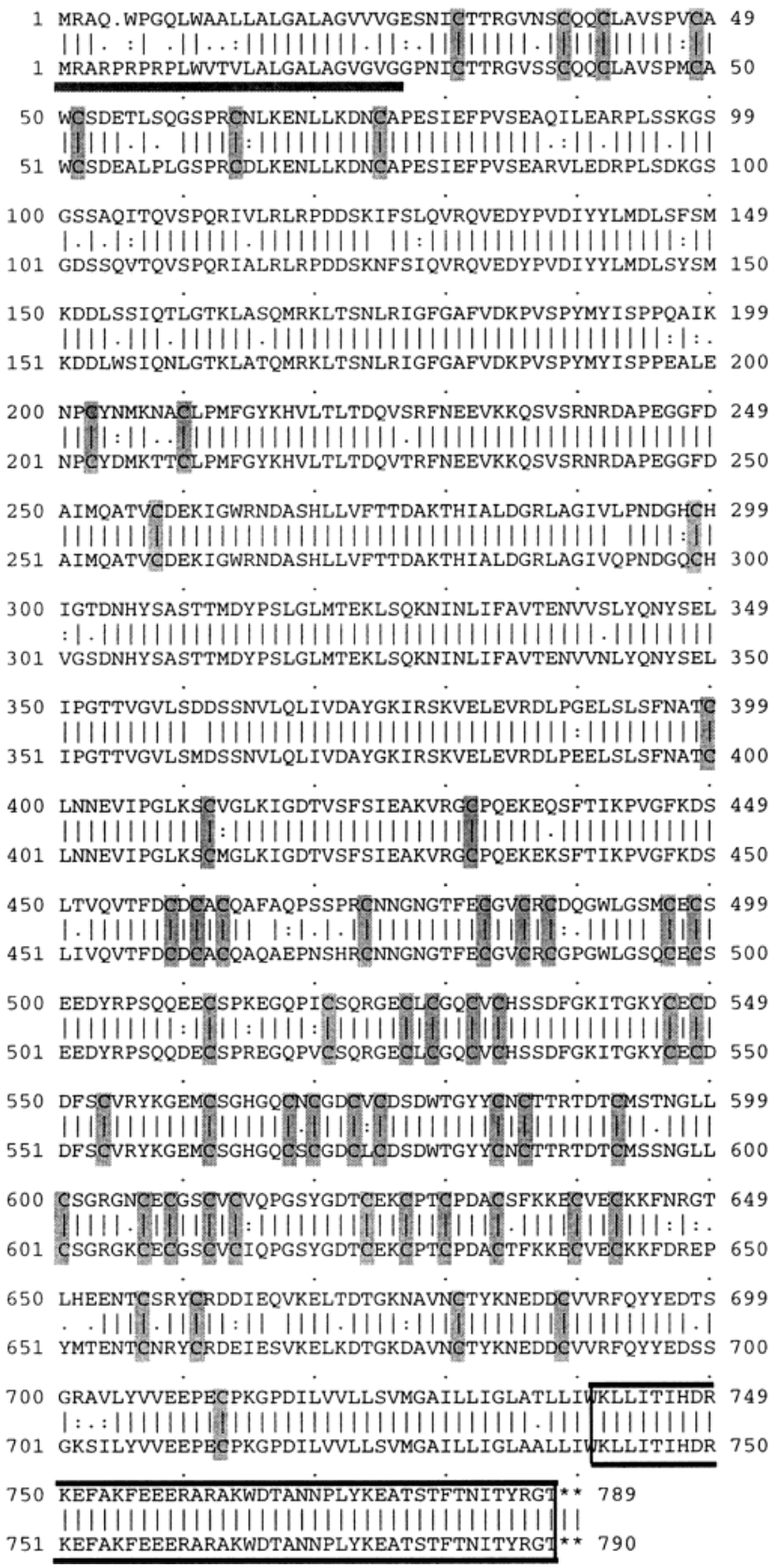

Fig. 1. Complete amino acid sequence for mouse and human integrin β_3 subunits. The deduced amino acid translation (single letter code) of the mouse β_3 integrin subunit (top) is compared with that of the human (bottom). Identity is indicated by ''l'', conservative changes are indicated by '':'', and similar amino acids are denoted ''.''. Transcription termination signals are denoted by asterisks. The human signal peptide sequence is underlined. 56 conserved cysteine residues are shaded and the cytoplasmic tail regions are boxed.

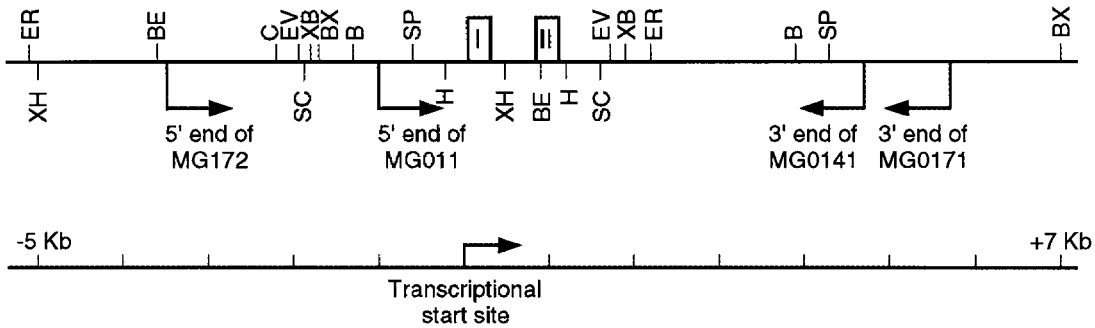

Fig. 2. Restriction map of four mouse genomic DNA clones containing the β_3 promoter region, exons I and II. The restriction sites of several restriction endonucleases were mapped on four mouse genomic DNA clones by Southern hybridization with β_3 exon I oligonucleotide probe (arrow in Fig. 3). The end of each clone nearest to exon I is shown. Restriction enzyme cleavage sites are indicated by B *Bg*lII, BE *Bst*EII, BX *Bst*XI, C *Cla*I, ER *Eco*RI, EV *Eco*RV, H *Hind*III, SC *Sca*I, SP *Spe*I, XB *Xba*I, and XH *Xho*I. The positions of exon I and exon II are indicated by boxes. Clone MG011 is 17 kb, clone MG041 is 26 kb, clone MG171 is 15 kb, and clone MG172 is 18 kb.

of lambda clones. Four overlapping clones containing exon I were isolated. These four genomic clones, MG011, MG041, MG171, and MG172, were digested with both *Not*I (to release inserts) as well as several restriction enzymes. A restriction map of the murine β_3 promotor/exon 1 locus is presented in Figure 2. The distance of exon I from the nearest end of all four clones, and the orientation of these clones in vectors, was confirmed by PCR with exon I sense or anti-sense primers and lambdaFIX T7 or T3 primers (data not shown). An exon II-specific oligonucleotide probe, derived from the human β_3 genomic sequence [Zimrin et al., 1990], was found to hybridize to identical *Hind*III, *Eco*RI, and *Xba*I fragments as the exon I probe yet different size *Xho*I fragments (data not shown). Given that the 1.3 kb *Hind*III fragment is the smallest of the three hybridizing to the probe, we conclude that exon II is within 1.3 kb of exon I. In addition, the DNA sequence was obtained with MG011 template using an exon III anti-sense primer (based on the human genomic organization) [Zimrin et al., 1990], locating exon III within 17 kb of exon I (data not shown). Taken together, these four mouse genomic clones represent sequences which span more than 40 kb of the mouse β_3 locus, including exons I, II, and III.

Sequence of Murine β_3 Integrin Promoter

A T3 promoter primer, complementary to vector sequences in lambdaFIXII, and an exon I anti-sense primer were used to PCR amplify 1.1 kb of sequence between the vector end of MG011 and exon I. This DNA, immediately 5′ of exon I, was subcloned in the pCRII vector. Using additional primers derived from this subclone, we sequenced, in the four genomic clones, 1.1 kb of the promoter region, exon I, and into intron I (GenBank accession #AF026510). Each clone contains identical sequence of this region, including the tetranucleotide repeat, $(TTTA)_{17}$ at −869 to −800 (see Fig. 6). The 5′ UTRs and leader sequences of the human and mouse β_3 cDNAs, and corresponding mouse genomic sequences, are compared in Figure 3. The mouse cDNA and genomic sequences diverge at a consensus 5′ splice site (mammalian consensus is 5′-A**Ggt**(a/g)a**g**t-3′, with invariant bases in bold and intronic sequence in lower case [Nelson and Green, 1989]).

Transcriptional Start Site Mapping

To identify the transcriptional start site of the mouse β_3 integrin promoter we first mapped, by primer extension, the 5′ terminus of the mouse β_3 mRNA. To this end, an anti-sense primer (arrow in Fig. 3) was extended on osteoclast total RNA template. Figure 4 shows the extension products compared with a DNA sequence of genomic clone MG011 using the same primer. The major, if not sole, transcriptional start site is 34 nt 5′ of the translational start codon (ATG). The extension product is 1 nt longer than the 5′ UTR of the longest cDNA clone, SMOc152. The position of the transcriptional start site was confirmed by S1 nuclease mapping. An oligonucleotide was synthesized corresponding to the noncoding strand of mouse β_3 genomic locus, which spans the start

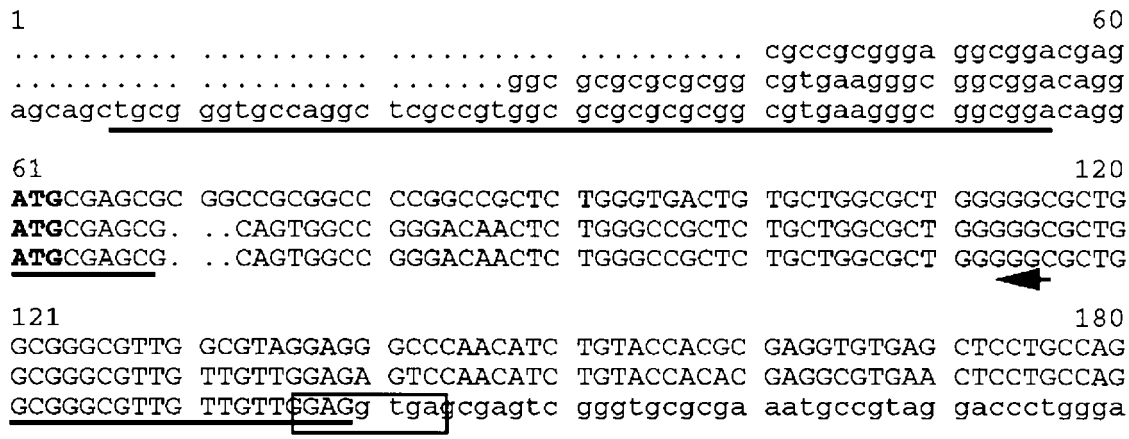

Fig. 3. Alignment of the human β_3 cDNA, mouse β_3 cDNA, and mouse exon I. The human cDNA sequence (top) is the first 140 nt of the published β_3 cDNA. The mouse cDNA (middle) is the first 153 nt of a mouse cDNA clone (SMOC152) which was isolated from an osteoclast cDNA library. The mouse genomic sequence (bottom) represents 180 nt of a mouse genomic clone which encodes exon I. Numbering is for the mouse genomic sequence. The coding regions are upper case letters, the start codon (ATG) is boldface, the putative exon I/intron I junction and its consensus 5′ splice site is boxed (mammalian consensus is 5′-AGgt(a/g)agt-3′). The location of the exon I anti-sense primer is denoted by an arrow and location of the S1 nuclease probe is underlined.

site identified by primer extension (Fig. 3). Eight noncomplementary nucleotides were included as a control for S1 nuclease activity. As seen in Figure 5, the major protected fragments are 44, 43, 42, and 41 nt and a minor one of 63 nt. The 44 nt fragments correspond to 34 nt 5′ of the translational start site. The 63 nt fragment represents the oligonucleotide minus the eight noncomplementary nts and the bands of 43, 42, and 41 nucleotides probably reflect the presence of partially degraded mRNA used in the assay.

Mouse β_3 Gene Promoter Sequence, Activity, and Induction by IL-4

The sequence of the mouse β_3 promoter region, from −1084 to +35, relative to the transcriptional start site, including consensus transcription factor binding sites for SP1, PU.1, NF-κB and STATs, is presented in Figure 6. Of particular interest are the two overlapping potential STAT-6 sites between −646 and −634. The 1.1 kb genomic fragment was subcloned into a luciferase reporter vector (pGL3basic), and M-CSF-dependent mouse BMMs were transfected with the resultant 1.1 kb construct (−1120LUC3) and CMV-β galactosidase. As shown in Figure 7, mouse β_3 promoter-dependent transcription is induced, dose-dependently, by IL-4. The cytokine, in contrast, fails to impact the SV40 promoter-driven construct, pGL3promoter.

Identification of the IL-4 Responsive Region Within the Mouse β_3 Integrin Promoter

To identify the region of the β_3 promoter responsible for IL-4 induction, we prepared a series of promoter 5′ deletions of the intact −1084/Luc3 construct containing −687, −465, or −227 nt. These fragments, as well as the complete −1084 β_3 promoter and SV-40 promoter-driven reporters, were assayed for IL-4 responsiveness by transient transfection of the IL-4-dependent mouse macrophage cell line, FD5 [Welham et al., 1997]. As seen in Figure 8, deletions of up to −678 nt of the β_3 promoter respond to IL-4, whereas the −465 and −227 deletions do not. While IL-4 does not induce −465/Luc, basal transcription in this clone mirrors that of IL-4-induced −678/Luc, suggesting that an inhibitory sequence resides between −678 and −465.

IL-4 Induction of STAT6 in Bone Marrow Macrophages

Loss of IL-4-dependent β_3 gene activation accompanying deletion of overlapping STAT-like sequences at −646 to −634, is consistent with the hypothesis that STAT-6, the only STAT reported to be activated by IL-4, regulates transcription of the β_3 integrin promoter. In order to determine if the cytokine activates and prompts nuclear translocation of STAT-6 in BMMs, we performed EMSA, using

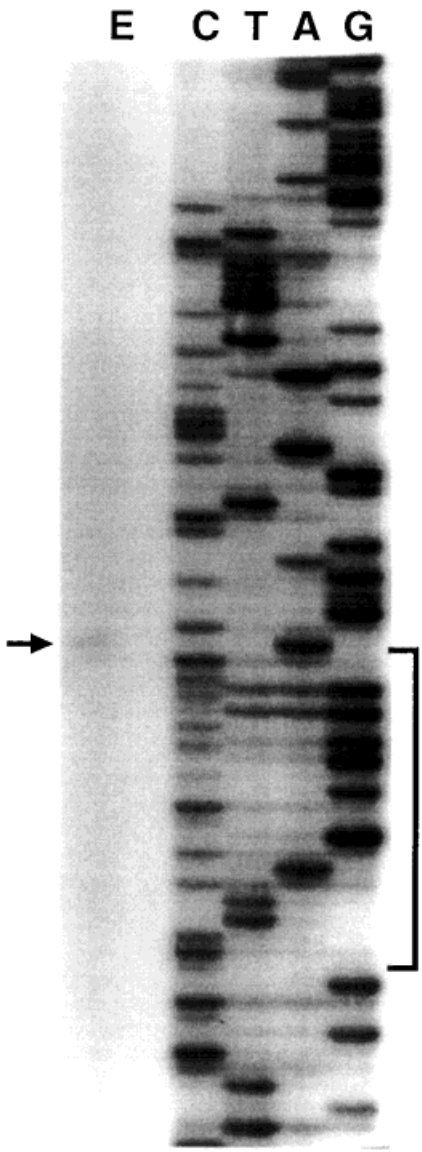

Fig. 4. Primer extension mapping of the 5′ terminus of mouse β_3 mRNA. The anti-sense primer in Figure 3 was extended with reverse transcriptase using osteoclast total RNA as template. Extension products (**Lane E**; arrow) are compared directly to the DNA sequence of genomic DNA clone MG011 using the same anti-sense primer (**Lanes C, T, A, G**). The sequence is the complement of that presented in Fig. 1 and in part reads; 5′-cccttcacgccgcgcgcgcgcca-3′ (bracketed), which is the complement to a portion of the 5′ UTR in Figure 3. The extension product includes 1 nt in addition to the 33 nt of 5′ untranslated sequence of the cDNA clone SMOc152.

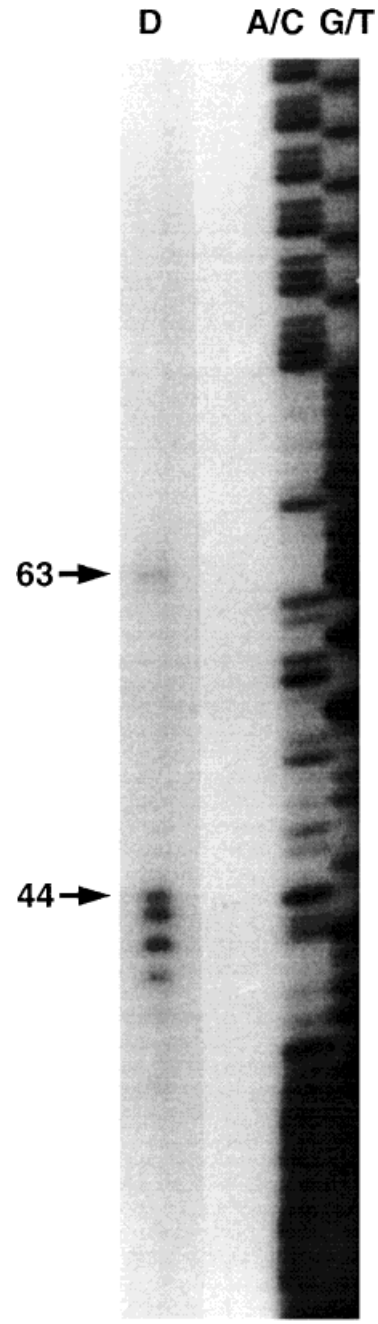

Fig. 5. S1 nuclease mapping of the transcriptional start site. Mouse osteoclast total RNA was annealed with a 71 nt oligonucleotide (Fig. 3, heavy line), which spans the transcriptional start site identified by primer extension, and digested with S1 nuclease. The oligonucleotide contains an additional 8 nt of noncomplementary sequence as a control for S1 activity. **Lane D** shows the digested products. Fragment lengths were determined by comparison with unrelated, previously characterized sequencing products (**Lanes A/C** and **G/T**). Arrows indicate the 63 and 44 nt protected fragments. The 63 nt fragment represents the entire oligonucleotide minus the 8 noncomplementary nts. The 44 nt fragment represents a protected fragment which includes 34 nt 5′ of the translational start site plus 10 nt of the coding region.

as a probe, a consensus STAT-binding sequence (human FcγRI IFNγ/IL-4 response element). As seen in Figure 9, nuclear extracts from IL-4, but not vehicle-treated cells contain a nuclear factor which binds the FcγRI probe. Formation of the DNA–protein complex is diminished by

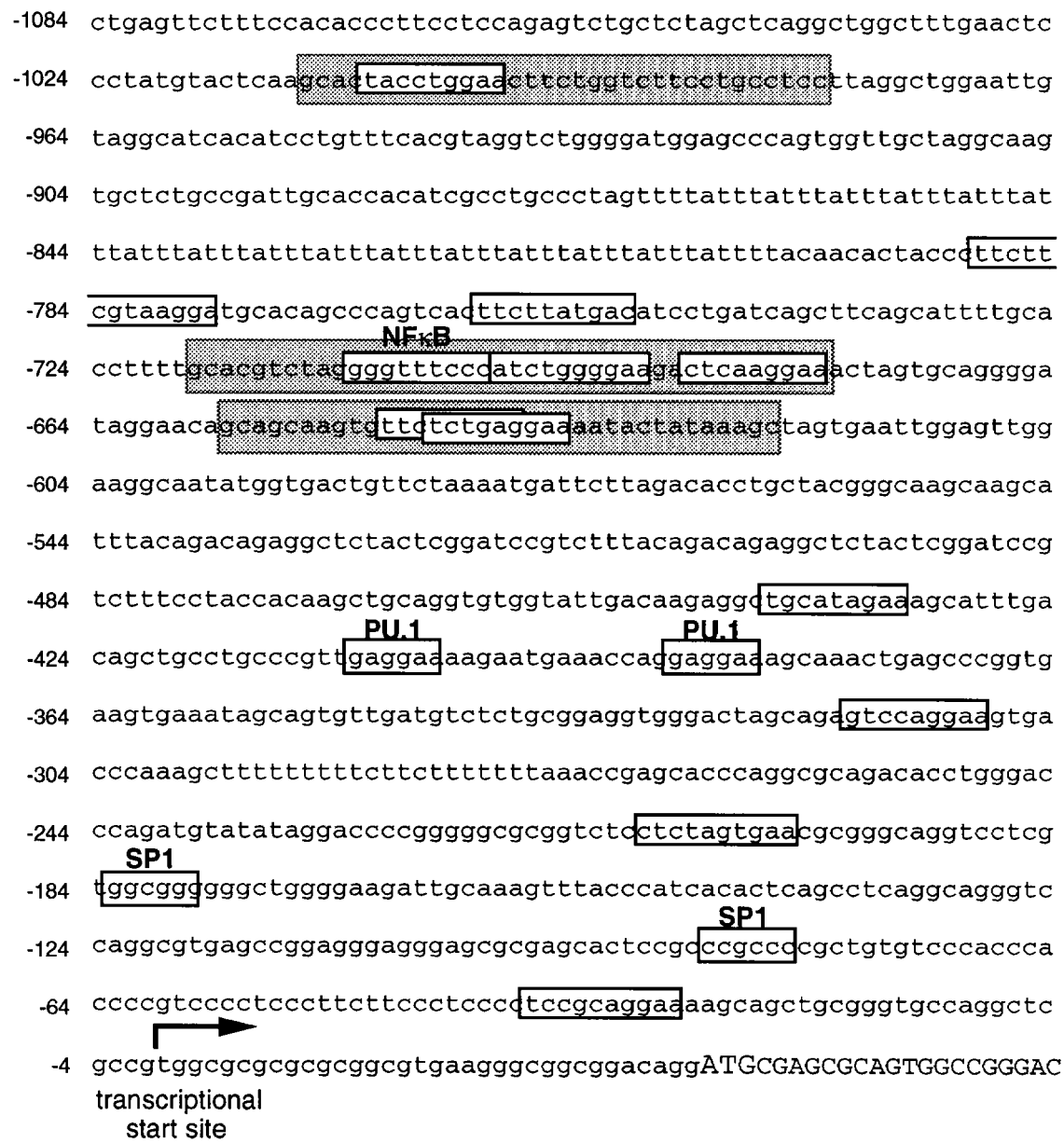

Fig. 6. Sequence of the mouse β_3 promoter region from −1084 to +35. Consensus-binding sites for PU.1, SP1, and NF-κB are boxed and labeled. Potential STAT-binding sites are boxed. Regions used as competitors in gel shift experiments are shaded. The transcriptional start site is marked and the coding region is in uppercase letters. The translational start codon is in larger type.

either unlabeled homologous oligonucleotide or a sequence comprising base pairs −656 to −622 of the murine β_3 promoter containing two overlapping STAT-binding sequences. In contrast, oligonucleotides comprising sequences containing the two more distal putative STAT-binding sites (−678 to −718 and −979 to −1001) are poor inhibitors of binding. The identity of the protein moiety is provided by supershifting of the retarded band with an anti-STAT-6 antibody.

STAT-6 is Required for IL-4 Dependent β_3 Gene Induction

To determine if STAT-6 mediates IL-4 induction of the murine β_3 gene, in vivo, we turned to STAT-6 deleted mice. BMMs of these mutants, and their wild-type counterparts, were treated

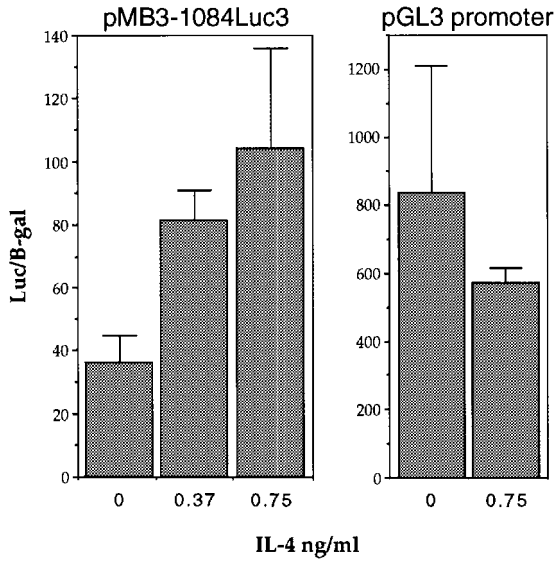

Fig. 7. Transient transfection of primary bone marrow macrophage with −1084/Luciferase construct and response to IL-4. Primary BMMs were transfected by electroporation with −1084Luc3 or pGL3promoter (an SV40-driven construct) and CMV-β galactosidase. Cells were grown 8 h post-transfection with M-CSF (500 U/ml) ± IL-4 at the doses shown. Luciferase activity was measured and normalized with cotransfected β-galactosidase activity. Experiments were performed in triplicate; the average ± SD is shown.

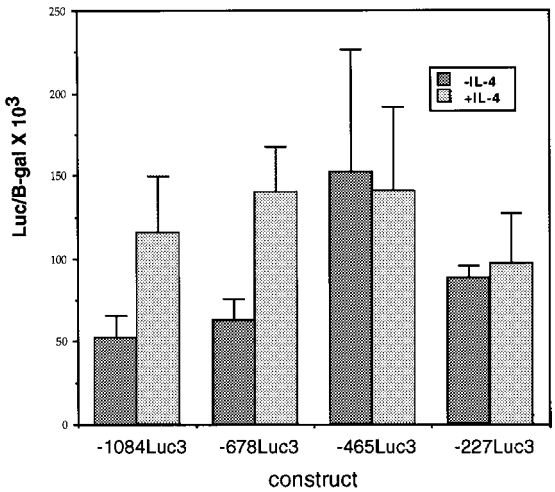

Fig. 8. Transient transfection of FD5 cells with β_3 promoter deletion constructs and response to IL-4. FD5 cells were transfected by electroporation with −1084, −678, −465, and −227 bp promoter constructs. Cells were grown 16 h post-transfection in medium containing WEHI-conditioned medium (5%) ± IL-4 0.75 ng/ml. Cells were harvested and lysed, and luciferase activity was determined and normalized with cotransfected β-galactosidase activity. Filled bars are without IL-4 and open bars are with IL-4. Experiments were performed in triplicate; the average ± SD is shown.

with IL-4, GM-CSF, or vehicle. Figure 10 shows GM-CSF induces β_3 message in BMMs of wild type and STAT-6-deficient mice (lanes 5 and 8). Whereas IL-4 also increases β_3 mRNA in wild-type cells (lane 4), this cytokine does not alter the integrin's message in STAT-6-deleted BMMs (lane 7) in contrast to GM-CSF (lane 8).

DISCUSSION

In the context of bone biology, the integrin $\alpha_v\beta_5$ is expressed abundantly in immature osteoclast precursors while $\alpha_v\beta_3$ is undetectable. With differentiation and formation of multinucleated osteoclasts, surface expression of $\alpha_v\beta_5$ falls, as $\alpha_v\beta_3$ increases [Inoue et al., 1998, 2000]. Thus, while $\alpha_v\beta_5$ functions as a matrix receptor for precursors, blockade of $\alpha_v\beta_3$ blunts bone resorption by mature osteoclasts, in vivo and in vitro [Horton et al., 1991; Ross et al., 1993; Engleman et al., 1997]. We find that the osteoclastogenic cytokine GM-CSF, promotes $\alpha_v\beta_3$ expression by enhancing β_3 but not α_v mRNA [Inoue et al., 1998]. This observation is consistent with the supposition that synthesis of the "monogamous," as opposed to "promiscuous," integrin subunit is the rate-limiting event in the appearance of a given heterodimer. IL-4 is an immunoregulatory cytokine which prompts development of macrophage precursors along a host-defense pathway while at the same time dampening osteoclastogenesis. IL-4 also induces $\alpha_v\beta_3$ expression by these same differentiating macrophages [Kitazawa et al., 1995]. This observation indicates the integrin is not specific to the osteoclast phenotype but is also present in members of the monocyte/macrophage family whose primary function is immunoregulatory. Similar to GM-CSF induction of $\alpha_v\beta_3$, IL-4 mediates its effect by transactivating the β_3 gene. Because of its central role in $\alpha_v\beta_3$ expression, and its regulation by diverse cytokines, the β_3 subunit promoter presented itself as an attractive cloning target.

The human β_3 integrin gene promoter contains a large first intron which resisted cloning by chromosome walking [Zimrin et al., 1990; Cieutat et al., 1993]. This fact, taken with the frequent conservation of intron/exon structure, prompted us to approach the murine β_3 promoter by first obtaining cDNA sequences representing the 5′ UTR of the mouse β_3 mRNA. Having isolated murine β_3 sequences (from

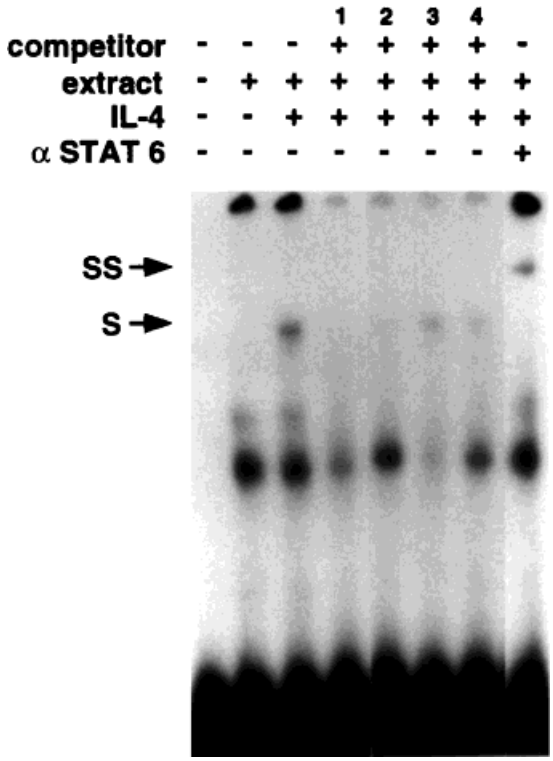

Fig. 9. Nuclear extracts from BMMs treated with IL-4 contain STAT6. Electrophoretic mobility shift assay with a consensus STAT-binding site probe (FcγRI) and nuclear extracts from vehicle-treated or IL-4-treated BMMs. The IL-4-induced complex is denoted by "S" and an arrow. Unlabeled competitors are; (competitor 1) homologous FcγRI probe, (competitor 2) β_3 promoter region −622 to −656. (competitor 3) β_3 promoter region −678 to −718, (competitor 4) β_3 promoter region −979 to −1011. The complex supershifted with rabbit anti-STAT-6 is denoted by "SS" and an arrow.

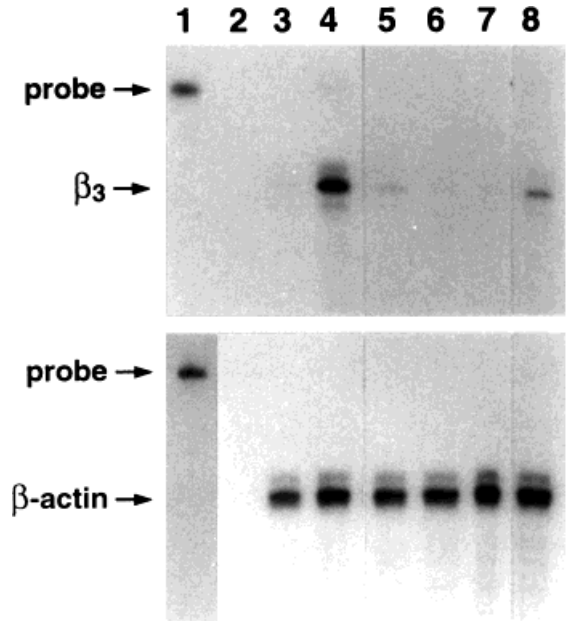

Fig. 10. BMMs from STAT-6-deficient mice cannot upregulate β_3 mRNA with IL-4 treatment but retain their response to GM-CSF. Ribonuclease protection assay on RNA from wild-type BMMs, **Lanes 3–5**, and STAT-6 −/− BMMs, **Lanes 6–8**, with a β_3 anti-sense probe (top) or mouse β-actin anti-sense probe (bottom). Yeast tRNA control without RNase treatment, **Lane 1**, and with RNase treatment, **Lane 2**. Control-untreated BMM RNA **Lanes 3** and **6**, with RNA from IL-4-treated BMMs **Lanes 4** and **7**, and RNA from GM-CSF-treated BMMs **Lanes 5** and **8**. Full length probes and protected fragments are indicated with arrows.

cDNA and RT-PCR) we constructed a full length functional β_3 cDNA. Our data, which add 103 N- and 190 C-terminal amino acids to the previously published partial mouse β_3 integrin sequence [Cieutat et al., 1993], are consistent with a recent report identifying the first 66 amino acids of the mature mouse β_3 integrin [Barron-Casella et al., 1999]. We find, in contrast to the reported sequence [Cieutat et al., 1993], that the amino acid homologous to human 105 is valine and not isoleucine, a discrepancy perhaps reflecting the use of degenerate primers for RT-PCR amplification in the earlier study.

Recent studies have led to the characterization of the human β_3 integrin gene, which spans 63 kb and contains a 16.7 kb first intron. Three regulatory elements have been identified within the gene; those at +13 to +22 and −73 to −66, modulate gene expression in both megakaryocyte-like and non-myeloid cell lines, with the region at −73 to −66 shown to bind the ubiquitous transcription factor SP1. In contrast, the sequence −115 to −89 contains a megakaryocyte-specific element, and binds an unidentified nuclear protein found only in megakaryocytic cell lines [Villa-Garcia et al., 1994; Wilhide et al., 1997; Jin et al., 1998].

Using β_3 mRNA sequence as a probe, we isolated four overlapping genomic clones which contain the 5′ UTR and leader peptide. The transcriptional start is 34 nt 5′ of the translation initiation codon. These data are consistent with β_3 mRNAs from other species which have short 5′ UTRs, with 20 nt in the human [Fitzgerald et al., 1987] and 42 nt in the chicken [Cao et al., 1993]. Genomic DNA 5′ of the β_3 transcriptional start site is a functional promoter and responds to IL-4 when transiently transfected into mouse BMMs. Consistent with a direct effect on the β_3 promoter, by IL-4-activated transcription factors, cytokine-induced transactivation occurs within 8 h of treatment.

A major component of intercellular signaling mediated by GM-CSF and IL-4 involves activation of STAT family proteins which, once phosphorylated on tyrosine, dimerize and translocate to the nucleus, where they function as transcription factors. STAT-binding sites consist of the inverted palindrome TTC(N)$_x$GAA where $x = 3$ or 4 [Darnell, Jr. et al., 1994; Schindler and Darnell, Jr., 1995; Schindler et al., 1995]. Furthermore, 10% of STAT-binding sequences contain a single base substitution in one half-site [Schindler et al., 1995]. While the mouse β_3 promoter contains a number of potential STAT-binding sites, our data indicate that a pair of overlapping sequences, located at −646 to −634, are responsible for regulating IL-4 induction of the gene. Evidence supporting this contention includes the observation that nuclear extracts from IL-4-treated BMMs contain a macromolecule which binds to a consensus STAT site oligonucleotide. Furthermore, such binding is supershifted by an anti-STAT-6 antibody, and binding is abrogated by the overlapping STAT sequences, located at −646 to −634 of the murine β_3 promoter. However, our most compelling evidence that STAT-6 regulates IL-4 induced β_3 expression is the fact that mice lacking this transcription factor fail to express the integrin subunit in response to the cytokine.

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REFERENCES

Barron-Casella EA, Nebbia G, Rogers OC, King KE, Kickler, TS, Casella JF. 1999. Construction of a human platelet alloantigen-1a epitope(s) within murine glycoprotein IIIa: identification of residues critical to the conformation of the antibody binding site(s). Blood 93:2959–2967.

Blystone SD, Lindberg FP, Williams MP, McHugh KP, Brown EJ. 1996. Inducible tyrosine phosphorylation of the β_3 integrin requires the α_v integrin cytoplasmic tail. J Biol Chem 271:31458–31462.

Brooks PC, Montgomery AM, Rosenfeld M, Reisfeld RA, Hu T, Klier G, Cheresh DA. 1994. Integrin alpha v beta 3 antagonists promote tumor regression by inducing apoptosis of angiogenic blood vessels. Cell 79:1157–1164.

Cao X, Ross FP, Zhang L, MacDonald PN, Chappel J, Teitelbaum SL. 1993. Cloning of the promoter for the avian integrin β_3 subunit gene and its regulation by 1,25-dihydroxyvitamin D_3. J Biol Chem 268:27371–27380.

Chiba M, Teitelbaum SL, Cao X, Ross FP. 1996. Retinoic acid stimulates expression of the functional osteoclast integrin $\alpha_v\beta_3$. Transcriptional activation of the β_3 but not α_v gene. J Cell Biochem 62:467–475.

Cieutat AM, Rosa JP, Letourneur F, Poncz M, Rifat S. 1993. A comparative analysis of cDNA-derived sequences for rat and mouse beta 3 integrins (GPIIIA) with their human counterpart. Biochem Biophys Res Commun 193: 771–778.

Darnell JE, Jr., Kerr IM, Stark GR. 1994. Jak-STAT pathways and transcriptional activation in response to IFNs and other extracellular signaling proteins. Science 264:1415–1421.

Dedhar S, Robertson K, Gray V. 1991. Induction of expression of the alpha v beta 1 and alpha v beta 3 integrin heterodimers during retinoic acid-induced neuronal differentiation of murine embryonal carcinoma cells. J Biol Chem 266:21846–21852.

Defilippi P, Truffa G, Stefanuto G, Altruda F, Silengo L, Tarone G. 1991. Tumor necrosis factor alpha and interferon gamma modulate the expression of the vitronectin receptor (integrin beta 3) in human endothelial cells. J Biol Chem 266:7638–7645.

Engleman VW, Nickols GA, Ross FP, Horton MA, Settle SL, Ruminski PG, Teitelbaum SL. 1997. A peptidomimetic antagonist of the $\alpha_v\beta_3$ integrin inhibits bone resorption in vitro and prevents osteoporosis in vivo. J Clin Invest 99:2284–2292.

Felding-Habermann B, Cheresh DA. 1993. Vitronectin and its receptors. Curr Opin Cell Biol 5:864–868.

Filardo EJ, Deming SL, Cheresh DA. 1996. Regulation of cell migration by the integrin beta subunit ectodomain. J Cell Sci 109:1615–1622.

Fitzgerald LA, Steiner B, Rall SC, Jr., Lo SS, Phillips DR. 1987. Protein sequence of endothelial glycoprotein IIIa derived from a cDNA clone. Identity with platelet glycoprotein IIIa and similarity to "integrin." J Biol Chem 262:3936–3939.

Gille J, Swerlick RA. 1996. Integrins: role in cell adhesion and communication. Ann N Y Acad Sci 797:93–106.

Horton MA, Taylor ML, Arnett TR, Helfrich MH. 1991. Arg-gly-asp (RGD) peptides and the anti-vitronectin receptor antibody 23C6 inhibit dentine resorption and cell spreading by osteoclasts. Exp Cell Res 195:368–375.

Hynes RO. 1992. Integrins: versatility, modulation, and signaling in cell adhesion. Cell 69:11–25.

Inoue M, Namba N, Chappel J, Teitelbaum SL, Ross FP. 1998. Granulocyte-macrophage colony stimulating factor reciprocally regulates α_v-associated integrins on murine osteoclast precursors. Mol Endocrinol 12:1955–1962.

Inoue M, Ross FP, Erdmann J, Abu-Amer Y, Teitelbaum SL. 2000. Tumor necrosis factor α regulates $\alpha_v\beta_5$ integrin expression by osteoclast precursors in vitro and in vivo. Endocrinol 141:284–290.

Jin Y, Wilhide CC, Dang C, Li L, Li SX, Villa-Garcia M, Bray PF. 1998. Human integrin β_3 gene expression: evidence for a megakaryocytic cell-specific *cis*-acting element. Blood 92:2777–2790.

Kim LT, Yamada KM. 1997. The regulation of expression of integrin receptors. Proc Soc Exp Biol Med 214:123–131.

Kitazawa S, Ross FP, McHugh K, Teitelbaum SL. 1995. Interleukin-4 induces expression of the integrin $\alpha_v\beta_3$ via transactivation of the β_3 gene. J Biol Chem 270:4115–4120.

Kotanides H, Reich NC. 1993. Requirement of tyrosine phosphorylation for rapid activation of a DNA binding factor by IL-4. Science 262:1265–1267.

Mimura H, Cao X, Ross FP, Chiba M, Teitelbaum SL. 1994. $1,25(OH)_2D_3$ vitamin D_3 transcriptionally activates the β_3-integrin subunit gene in avian osteoclast precursors. Endocrinol 134:1061–1066.

Moyle M, Napier MA, McLean JW. 1991. Cloning and expression of a divergent integrin subunit beta 8. J Biol Chem 266:19650–19658.

Nelson KK, Green MR. 1989. Mammalian U2 snRNP has a sequence-specific RNA-binding activity. Genes Dev 3:1562–1571.

Ross FP, Alvarez JI, Chappel J, Sander D, Butler WT, Farach-Carson MC, Mintz KA, Robey PG, Teitelbaum SL, Cheresh DA. 1993. Interactions between the bone matrix proteins osteopontin and bone sialoprotein and the osetoclast integrin $\alpha_v\beta_3$ potentiate bone resorption. J Biol Chem 268:9901–9907.

Ruoslahti E. 1996. Integrin signaling and matrix assembly. Tumour Biol 17:117–124.

Schindler C, Darnell JE Jr. 1995. Transcriptional responses to polypeptide ligands: the JAK-STAT pathway. Annu Rev Biochem 64:621–651.

Schindler U, Wu P, Rothe M, Brasseur M, McKnight SL. 1995. Components of a Stat recognition code: evidence for two layers of molecular selectivity. Immunity 2:689–697.

Shimoda K, van Deursen J, Sangster MY, Sarawar SR, Carson RT, Tripp RA, Chu C, Quelle FW, Nosaka T, Vignali DAA, Doherty PC, Grosveld G, Paul WE, Ihle JN. 1996. Lack of IL-4-induced Th2 response and IgE class switching in mice with disrupted Stat6 gene. Nature 380:630–633.

Shioi A, Ross FP, Teitelbaum SL. 1994. Enrichment of generated murine osteoclasts. Calcif Tissue Int 55:387–394.

Tetsuka T, Srivastava SK, Morrison AR. 1996. Tyrosine kinase inhibitors, genistein and herbimycin A, do not block interleukin-1 beta-induced activation of NF-kappa B in rat mesangial cells. Biochem Biophys Res Commun 218:808–812.

Villa-Garcia M, Li L, Riely G, Bray PF. 1994. Isolation and characterization of a TATA-less promoter for the human beta 3 integrin gene. Blood 83:668–676.

Welham MJ, Bone H, Levings M, Learmonth L, Wang L-M, Leslie KB, Pierce JH, Schrader JW. 1997. Insulin receptor substrate-2 is the major 170-kDa protein phosphorylated on tyrosine in response to cytokines in murine lymphohemopoietic cells. J Biol Chem 272:1377–1381.

Wilhide CC, Jin Y, Guo QB, Li L, Li SX, Rubin E, Bray, PF. 1997. The human integrin β_3 gene is 63 Kb and contains a 5′-UTR sequence regulating expression. Blood 90:3951–3961.

Ylanne J, Huuskonen J, O'Toole TE, Ginsberg MH, Virtanen I, Gahmberg CG. 1995. Mutation of the cytoplasmic domain of the integrin beta 3 subunit. Differential effects on cell spreading, recruitment to adhesion plaques, endocytosis, and phagocytosis. J Biol Chem 270:9550–9557.

Zimrin AB, Gidwitz S, Lord S, Schwartz E, Bennett JS, White GC, Poncz M. 1990. The genomic organization of platelet glycoprotein IIIa. J Biol Chem 265:8590–8595.