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The Murine *DUB-1* Gene Is Specifically Induced by the βc Subunit of the Interleukin-3 Receptor

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Cytokines regulate cell growth and differentiation by inducing the expression of specific target genes. We have recently isolated a cytokine-inducible, immediate-early cDNA, *DUB-1*, that encodes a deubiquitinating enzyme. The *DUB-1* mRNA was specifically induced by the receptors for interleukin-3, granulocyte-macrophage colony-stimulating factor, and interleukin-5, suggesting a role for the β common (βc) subunit known to be shared by these receptors. In order to identify the mechanism of cytokine induction, we isolated a murine genomic clone for *DUB-1* containing a functional promoter region. The *DUB-1* gene contains two exons, and the nucleotide sequence of its coding region is identical to the sequence of *DUB-1* cDNA. Various regions of the 5′ flanking region of the *DUB-1* gene were assayed for cytokine-inducible activity. An enhancer region that retains the βc-specific inducible activity of the *DUB-1* gene was identified. Enhancer activity was localized to a 112-bp fragment located 1.4 kb upstream from the ATG start codon. Gel mobility shift assays revealed two specific protein complexes that bound to this minimal enhancer region. One complex was induced by βc signaling, while the other was noninducible. Finally, the membrane-proximal region of human βc was required for *DUB-1* induction. In conclusion, *DUB-1* is the first example of an immediate-early gene that is induced by a specific subunit of a cytokine receptor. Further analysis of the *DUB-1* enhancer element may reveal specific determinants of a βc-specific signaling pathway.

Interleukin-3 (IL-3) is a glycoprotein hormone that regulates growth of hematopoietic progenitor cells (18). IL-3 activates a specific receptor (IL-3R), which is expressed on the surface of its target cells. The IL-3R complex consists of an IL-3-specific α chain and a β common (βc) chain shared by the granulocyte-macrophage colony-stimulating factor receptor (GM-CSF-R) and the IL-5 receptor (IL-5R) (21, 22, 27, 42). IL-3R activates multiple signal transduction pathways, including the Ras-Raf-MAP kinase pathway and the JAK-STAT pathway, resulting in the induction of immediate-early genes. How these immediate-early genes couple receptor activation to the biochemical machinery of cell growth and cell cycle progression is not known.

Discrete domains of the cytoplasmic region of the βc subunit activate the Ras-Raf-MAP kinase pathway and the JAK-STAT pathway. The distal cytoplasmic domain of βc, for example, activates the $p21^{ras}$-Raf-MAP kinase pathway (35, 36). Activation of this pathway results in suppression of apoptosis and induction of the immediate-early genes c-*fos* and c-*jun*. The membrane-proximal domain of βc, in contrast, is required for activation of the JAK-STAT pathway (50). Activation of this pathway results in cellular proliferation. In addition, the membrane-proximal region is required for induction of the c-*myc* gene (45, 46).

IL-3R, like other cytokine receptors, activates a combination of JAK and STAT proteins (10, 17). JAK kinases are cytoplasmic proteins that constitutively associate with the tails of cytokine receptors and become activated upon receptor-ligand interaction. STAT proteins are latent cytoplasmic transcription factors that are phosphorylated by activated JAK kinases. Phosphorylated STAT proteins form homo- and hetero-oligomeric complexes and translocate to the nucleus, where they activate transcription of a specific set of genes. STAT complexes bind to STAT binding elements that are found in the 5′ regulatory sequences of many inducible genes (10).

Cytokines induce specific proliferative and differentiative responses on their target cells. The biological specificity of each cytokine is determined in part by the activation of unique combinations of JAK kinases and STAT proteins. For instance, IL-3 activates the Janus kinases JAK1 and JAK2 (5, 31) and the STAT proteins STAT5A, STAT5B, and STAT6 (2, 14, 26, 32, 44). IL-4, on the other hand, activates JAK1 and JAK3 (3, 20, 51) and the STAT protein STAT6 (16). Additional cytokine specificity is provided by variations in the sizes and sequences of the palindromic STAT binding elements found 5′ to cytokine-inducible genes (37). Importantly, other components of cytokine-specific signaling that are distinct from the JAK-STAT pathway remain unidentified. For example, IL-3 and erythropoietin (EPO) both activate JAK2 and STAT5 yet each cytokine induces a unique pattern of immediate-early genes.

In order to identify specific *cis*-acting regulatory elements that regulate expression of cytokine-inducible genes, we recently utilized the strategy of differential display (23, 24) to isolate IL-3- and EPO-inducible genes. For this purpose, we used Ba/F3-EPO receptor (EPO-R) cells, which have functional receptors for both IL-3 and EPO. While stimulation of IL-3 results in a proliferative signal, stimulation of EPO-R results in erythroid cell-specific differentiation (6). An IL-3-specific inducible cDNA, *DUB-1*, was isolated (53). *DUB-1* was identified as a hematopoietic cell-specific immediate-early cDNA that encodes a deubiquitinating enzyme.

In this report, we describe the isolation of a full-length genomic clone for *DUB-1* containing its promoter region. Consistent with our cloning strategy, the 5′ region of the *DUB-1* gene was responsive to IL-3 but not to EPO. In addition,

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DUB-1 was induced by GM-CSF and IL-5, suggesting that it is specifically induced by the shared βc subunit. A 112-bp βc-specific enhancer region was identified 5′ to the *DUB-1* gene. This enhancer binds two specific protein complexes as demonstrated by electrophoretic mobility shift assays. Further analysis of the *DUB-1 cis*-acting regulatory sequences and binding proteins should reveal specific determinants of a βc-specific signaling pathway.

MATERIALS AND METHODS

Cells and cell culture. Ba/F3 is an IL-3-dependent murine pro-B cell line (28). Ba/F3–EPO-R cells were generated by stable transfection of Ba/F3 cells with the cDNA encoding the murine EPO-R (8). Ba/F3–EPO-R cells grow in either murine IL-3 or human EPO (6, 25). Ba/F3–GM-CSF-Rα cells were generated by stable transfection of Ba/F3 cells with the cDNA encoding the murine GM-CSF-Rα (30). Ba/F3–GM-CSF-Rα cells grow in either murine IL-3 or murine GM-CSF. Ba/F3–IL-5Rα cells were generated by stable transfection of Ba/F3 cells with the cDNA encoding the murine IL-5Rα chain (11, 41, 42). Ba/F3–IL-5Rα cells grow in either murine IL-3 or murine IL-5 (30a). The Ba/F3–human IL-4R (hIL-4R) cells grow in either murine IL-3 or hIL-4 (38). All Ba/F3 subclones were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum and 10% conditioned medium from WEHI-3B cells as a source of murine IL-3.

For induction assays, the various Ba/F3 subclones were washed three times in phosphate-buffered saline, grown at 37°C without growth factor for 8 h, and then restimulated with murine IL-3 (Kirin) or with one of several other growth factors for 1 h. Ba/F3–EPO-R cells were restimulated with human EPO (1 pM = 10 mU/ml) (Genetics Institute). Ba/F3–GM-CSF-Rα cells were restimulated with murine GM-CSF (Prepro Tech Inc.). Ba/F3–IL-5Rα cells were restimulated with murine IL-5 (R and D Systems). Ba/F3–IL-4R cells were restimulated with IL-4 (Genzyme).

cDNAs and stable transfections. The full-length murine GM-CSF-Rα cDNA was isolated by reverse transcription-PCR with primers derived from its published sequence (30). The IL-5Rα chain (41, 42) was a gift from J. Tavernier. The human GM-CSF-Rα (hGM-CSF-Rα) cDNA (12) and the wild-type and mutant human βc (hβc) cDNAs (15) were generously supplied by A. Miyajima. Stable transfection of Ba/F3 cells with these cDNAs, using G418 selection, was performed as previously described (9).

Generation of Ba/F3 cells expressing a chimeric IL-5Rα subunit. By a PCR strategy (54), a chimeric IL-5R (IL-5Rα–βIL-3) containing the extracytoplasmic region of IL-5Rα (41, 42) and the cytoplasmic tail of βIL-3 (19) was generated. The IL-5Rα–βIL-3 chimeric receptor consists of amino acids 1 to 361 of IL-5Rα fused to amino acids 466 to 882 of βIL-3. The cDNA encoding this chimeric receptor was transfected into Ba/F3 cells, and stable Ba/F3 transfectants were analyzed for factor-dependent growth by the XTT assay (9). Ba/F3(IL-5Rα–βIL-3) cells grow equally well in IL-3 and IL-5 (30a).

Northern (RNA) analysis. RNA samples (10 to 30 μg) were electrophoresed on denaturing formaldehyde gels and blotted onto Duralon UV membranes (Stratagene, La Jolla, Calif.). The indicated cDNA inserts were purified from agarose gels (Qiagen), radiolabeled, and hybridized to the membranes for 1 h at 68°C. Hybridized filters were washed at room temperature in 0.1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and 0.1% sodium dodecyl sulfate (SDS).

Cloning of the *DUB-1* gene and construction of luciferase reporter plasmids. A mouse genomic library prepared in Lambda FIX II (Stratagene) was screened with the open reading frame region of the *DUB-1* cDNA. The probe was labeled with [^{32}P]dCTP by the random primer method (Pharmacia), and hybridization was performed in 0.8 M NaCl–0.02 M PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); pH 6.5]–0.5% SDS–50% deionized formamide–100 μg of denatured, sonicated salmon sperm DNA per ml for 16 h at 42°C. A clone encompassing the *DUB-1* gene and its 5′ and 3′ flanking sequences was recovered as a 13-kb *Not*I fragment. The clone was mapped by restriction enzyme digestion and sequenced by the dideoxy DNA sequencing protocol (United States Biochemicals).

A 1.5-kb *Spe*I-*Xba*I fragment from the 5′ flanking region of *DUB-1* was subcloned into the pGL2Basic plasmid (Pharmacia). The pGL2Basic plasmid contains a luciferase reporter gene without a promoter. Additional DNA fragments from the 5′ flanking region of the *DUB-1* gene were generated by PCR with pairs of 20-mer oligonucleotides designed according to the genomic DNA sequence. These fragments were subcloned into the pGL2Promoter plasmid (Pharmacia), which contains the simian virus 40 basal promoter upstream of the luciferase reporter gene.

Transient transfection and transactivation experiments. All plasmid DNAs were purified with Qiagen (Chatsworth, Calif.) columns. The indicated Ba/F3 subclones (10^7 cells per transfection) were transfected by electroporation with 10 μg of test plasmid. The cells were starved by depletion of cytokine for 5 h before electroporation. After electroporation, cells were divided into pools and restimulated with the cytokines at various concentrations as indicated in the figure legends. Luciferase levels were assayed after 12 h of stimulation according to vendor specifications (with a luciferase assay kit; Analytical Luminescence Laboratory, San Diego, Calif.). Each construct was tested at least three times by independent transfection, with similar results each time.

Electrophoretic mobility shift assay. Nuclear extracts were prepared from unstimulated and IL-3-stimulated Ba/F3 cells, as previously described (1, 48). Probe 1 (nucleotides [nt] −1528 to −1508) was synthesized as 5′-TAACAGGA AATAATGACTAAG-3′. Probe 2 (nt −1512 to −1494) was synthesized as 5′-CTAAGACTGTGGTATGAAG-3′. Probe 3 (nt −1479 to −1461) was synthesized with the sequence 5′-GTAGAAATGGAAAAAAAAG-3′. Probe 4 (nt −1455 to −1432) was synthesized with the sequence 5′-GGTTTCACTGCTTC ATAAGGAGAT-3′. The nonspecific oligonucleotide contained 20 nt which had no homology with the 112-bp enhancer element of *DUB-1*. The probes were end labeled with ^{32}P by using T4 polynucleotide kinase (Boehringer Mannheim) and annealed to the complementary strands. Unincorporated nucleotides were removed with a G-50 column (Pharmacia). Binding reaction mixtures (20 μl) contained nuclear extract, 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid, pH 7.8), 65 mM NaCl, 1 mM dithiothreitol, 0.15 mM EDTA, 8% glycerol, 1 μg of poly(dI-dC), and labeled DNA (1×10^6 to 10×10^6 cpm/pmol). Binding was for 30 min on ice. For competition analysis, unlabeled double-stranded oligonucleotides were added directly to the binding reaction mixture, as indicated in the figure legends. Samples were run for 3 h on a 5% nondenaturing polyacrylamide gel, dried, and exposed on X-ray film.

Isolation of a sequence from the *DUB-3* gene, corresponding to the *DUB-1* enhancer. A 243-bp fragment, corresponding to the enhancer region (nt −1657 to −1416) of the *DUB-1* gene, was amplified by PCR from a genomic clone of the *DUB-3* gene. The *DUB-3* gene encodes a functional deubiquitinating enzyme that is highly related to *DUB-1* but is not induced by IL-3 or EPO (52a). The primers utilized were 5′-GATTTAACAGGAATGGGCAA-3′ and 5′-TCCTTA GTCACTGTTGTATCT-3′. The amplified *DUB-3* genomic product was subcloned in pBluescript and sequenced. The 112-bp fragment derived from *DUB-3* was further subcloned into pGL2Promoter. A 112-bp *DUB-1* mutant fragment [*DUB-1*(Mut)] containing only the G-−1523-to-T mutation was generated by PCR mutagenesis and subcloned in pGL2Promoter. Enhancer activity and gel shift activity were analyzed as described above.

Detection of hβc subunit expression. Ba/F3 cells were transfected with the cDNAs encoding full-length (wild-type) hβc, a truncated βc terminating at position 544 [the βc(544) mutant], or the βc(455) mutant. Stably transfected cells were lysed, and proteins were detected by immunoblot analysis with an anti-hβc antiserum generously provided by A. Lopez.

RESULTS

***DUB-1* is a hematopoietic cell-specific, cytokine-inducible immediate-early gene.** The *DUB-1* mRNA (3.1 kb) was detected in cells growing in IL-3 (Fig. 1A, lane 1). When Ba/F3 cells were depleted of IL-3 for 8 h, the *DUB-1* mRNA disappeared (Fig. 1A, lane 2). When the cells were restimulated with IL-3, the *DUB-1* mRNA, like the c-*myc* mRNA, was induced after 1 h (Fig. 1A, lane 3). The presence of cycloheximide (10 μg/ml) plus IL-3 resulted in a superinduction of the *DUB-1* mRNA (Fig. 1A, lane 4), thereby defining *DUB-1* as an immediate-early gene. Expression reached a maximum at 3 h poststimulation and declined by 6 h.

The full-length *DUB-1* cDNA probe was used to detect mRNA expression in murine cell lines and tissues (Fig. 1B). The mRNA was detected in the IL-3-dependent early hematopoietic progenitor cell lines Ba/F3 and FDCP1 but not in the myeloid cell line 32D. Other cell lines, including MEL, CTLL, and 011 (megakaryocyte), lacked the *DUB-1* mRNA (data not shown). The *DUB-1* mRNA was not detected in normal adult murine tissue samples under the conditions described.

Activation of the βc subunit of IL-3R results in induction of *DUB-1* mRNA. We next tested EPO-, GM-CSF-, IL-5-, and IL-4-responsive cell lines for induction of *DUB-1* mRNA. Ba/F3–EPO-R, Ba/F3–GM-CSF-R, Ba/F3–IL-5R, or Ba/F3–IL-4R cells were starved and restimulated with the appropriate cytokines and assayed by Northern (RNA) blot analysis for the expression of endogenous *DUB-1* mRNA (Fig. 2). Murine IL-3, but not EPO, induced *DUB-1* mRNA in Ba/F3–EPO-R cells. Both murine GM-CSF and murine IL-3 induced *DUB-1* mRNA in Ba/F3–GM-CSF-R cells. Similarly, murine IL-5 and murine IL-3 induced *DUB-1* mRNA in Ba/F3–IL-5R cells. Murine IL-3, but not hIL-4, induced *DUB-1* mRNA in Ba/F3–IL-4R cells. Since the βc subunit of the IL-3R functionally

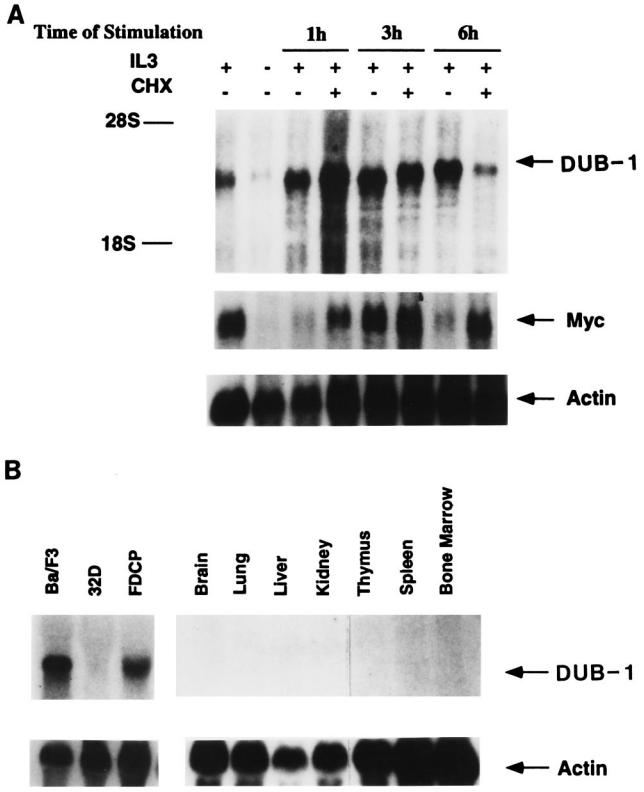

FIG. 1. *DUB-1* is a hematopoietic cell-specific, cytokine-inducible, immediate-early gene. (A) Northern blot analysis of total RNA (10 μg per lane) from Ba/F3 cells under various growth conditions. The ^{32}P-labeled probes included the full-length *DUB-1* cDNA (53), the c-*myc* cDNA, and or the β-actin cDNA. RNA was prepared from cells grown under the various conditions indicated in the figure. Where indicated, cycloheximide (CHX, 10 μg/ml) was added. (B) Total cellular RNA (10 μg) and tissue RNA (20 μg) were subjected to Northern blot analysis and probed with the *DUB-1* cDNA. The blot was stripped and rehybridized with a β-actin cDNA probe as a loading control.

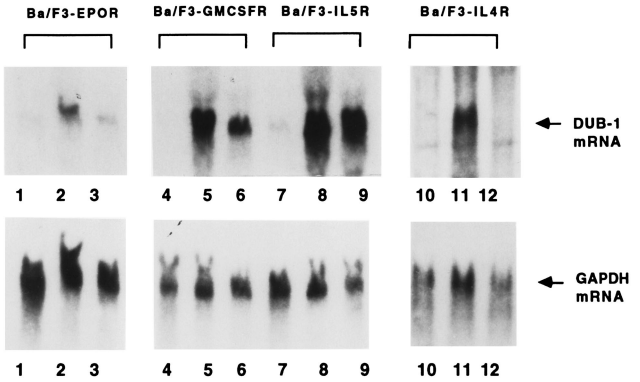

FIG. 2. Activation of the βc subunit results in *DUB-1* induction. The indicated Ba/F3 subclones were starved for 8 h (lanes 1, 4, 7, and 10) and restimulated for 1 h with either IL-3 (10 pM) (lanes 2, 5, 8, and 11), EPO (10 U/ml) (lane 3), GM-CSF (10 ng/ml) (lane 6), IL-5 (10 ng/ml) (lane 9), or IL-4 (50 U/ml) (lane 12). Total RNA (10 μg) was isolated from the cells and analyzed on a Northern blot with the ^{32}P-labeled full-length *DUB-1* cDNA probe (53). As a loading control, the blot was stripped and reprobed with a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA.

interacts with the α chains of the IL-3R, the GM-CSF-R, and the IL-5R (12, 15, 21, 22, 27, 35), our results suggest that *DUB-1* is specifically induced by the βc subunit.

The βc subunit is necessary and sufficient for the induction of *DUB-1*. Recent evidence suggests that the cytoplasmic regions of cytokine receptor α subunits also contribute to receptor signaling (40). For instance, the α subunit of the IL-5R has a 55-amino-acid cytoplasmic region that may be required for signal transduction. In order to test the requirement for the cytoplasmic region of IL-5Rα in *DUB-1* induction, we generated a chimeric receptor (IL-5Rα–βIL-3) that does not contain this region. The chimeric receptor contains the extracytoplasmic region of the IL-5Rα chain and the cytoplasmic tail of murine βIL-3 (Fig. 3A). This receptor forms a high-affinity IL-5 receptor with the endogenous βc subunit expressed in Ba/F3 cells. Stable transfection of this chimeric receptor into Ba/F3 cells conferred high-affinity murine IL-5 binding and IL-5-dependent cell growth (30a). Stimulation of Ba/F3(IL-5Rα–βIL-3) cells with IL-3 or IL-5 resulted in induction of endogenous *DUB-1* mRNA levels (Fig. 3B), demonstrating that the α subunit cytoplasmic region is not required for *DUB-1* induction.

Genomic organization and 5′ regulatory region of the murine *DUB-1* gene. In order to understand the mechanism of cytokine-inducible transcription of *DUB-1*, we isolated the full-length murine *DUB-1* gene. A total of 10^6 recombinant phage were screened with the open reading frame of the *DUB-1* cDNA, and four positive clones were identified. By sequence analysis, one phage clone was found to contain the full-length *DUB-1* coding region (Fig. 4). The sequence was identical to the sequence of the *DUB-1* cDNA clone (53). The other three clones contained genes with open reading frames bearing considerable homology to *DUB-1* (approximately 90% base pair identity) (52a). These genes are presumably *DUB* subfamily members, and we refer to them as *DUB-2* through *DUB-4*. A schematic diagram of the *DUB-1* gene and a restriction map are shown in Fig. 4A. The *DUB-1* gene contains a small exon (exon 1) encoding amino acids 1 to 9 and a larger exon (exon 2) encoding amino acids 10 to 526. The sequence of the intron-exon junction conforms to a consensus sequence for a eukaryotic splice junction. The ATG translation start site is designated +1. The 1.5-kb sequence of the *DUB-1* gene, 5′ to the ATG, was determined and is shown (Fig. 4B). The *DUB-1* cDNA clone (53) contains the sequence (−80 to +1) as a 5′ untranslated sequence. The region between positions −362 and −80 was shown to contain basic promoter activity in reporter assays (data not shown) and contains a putative TATA box at position −318. Therefore, the transcription initiation site was localized to the region between −318 and −80.

The 5′ region of the *DUB-1* gene contains a βc-specific enhancer element. The various Ba/F3 subclones described in

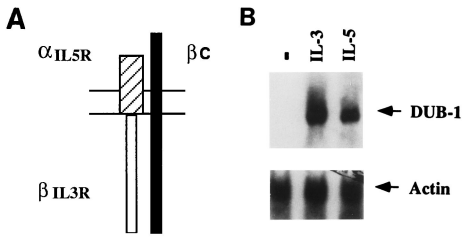

FIG. 3. The βc subunit is necessary and sufficient for the induction of *DUB-1*. (A) A chimeric receptor (IL-5Rα–βIL-3), containing the extracytoplasmic region of the IL-5Rα subunit and the cytoplasmic domain of the βIL-3 chain, was stably expressed in Ba/F3 cells that express the endogenous murine βIL-3 and βc polypeptides. (B) Cells were starved for 5 h (lane −) and restimulated with murine IL-3 (10 pM) or murine IL-5 (10 ng/ml) for 1 h. Total RNA was isolated and probed with the *DUB-1* cDNA or the β-actin cDNA as a loading control.

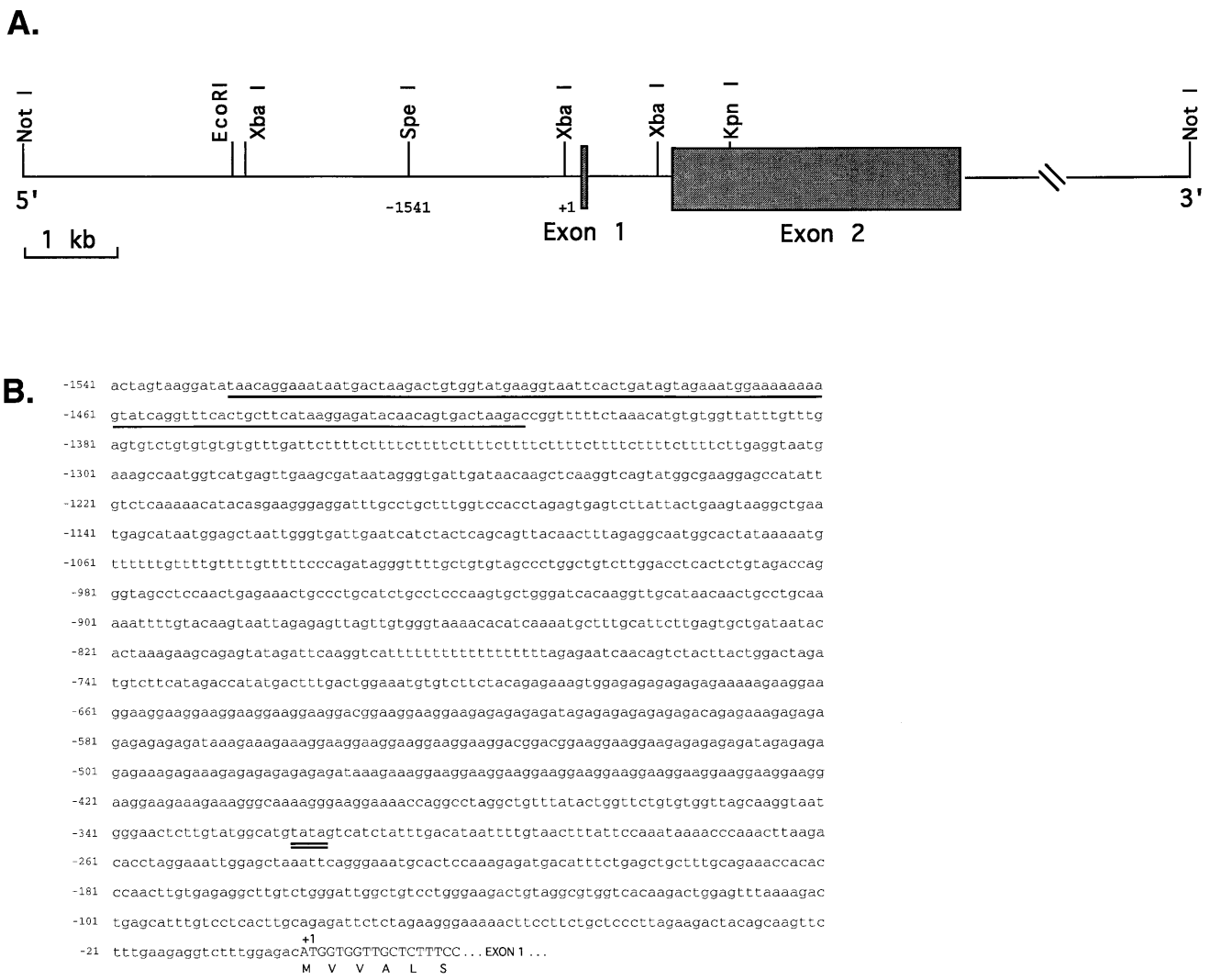

FIG. 4. Genomic organization of the *DUB-1* gene. (A) Schematic diagram of the *DUB-1* gene with partial restriction map. The lengths and positions of the two exons are indicated. +1 refers to the ATG start codon. (B) Nucleotide sequence of the *DUB-1* promoter. A putative TATA box (position −318) is indicated with a double underline. A 112-bp enhancer element is underlined. In addition, a purine-rich microsatellite repeat is found between positions −687 and −388.

Fig. 2 were transiently transfected with a plasmid containing the 1.5-kb 5′ flanking region of the *DUB-1* gene (T14P1.5kb) driving a luciferase reporter (Fig. 5). Luciferase activity was increased in a dose-dependent manner by IL-3, GM-CSF, and IL-5 but not by hIL-4 or EPO. In addition, luciferase activity was not increased by alpha interferon or gamma interferon, despite the expression of functional interferon receptors on Ba/F3 cells (data not shown). For all Ba/F3 subclones, no luciferase activity was induced when the vector alone was transfected (data not shown). Taken together, these data verify that activation of the βc subunit is required for induction of the *DUB-1* gene. In addition, the results demonstrate that the 1.5-kb 5′ flanking region of the *DUB-1* gene contains a functional promoter and regulatory elements that mediate βc-dependent transcriptional activation.

Identification of a minimal βc-responsive enhancer element. To further delineate the specific DNA element required for IL-3 induction, we next divided the 1.5-kb region into several DNA fragments (Fig. 6). Each fragment was subcloned into the vector pGL2Promoter, which contains the luciferase gene driven by a simian virus 40 basic promoter without an enhancer (Fig. 6A). The fragments T14P11/18, T14P17/20, and T14P11/21 had IL-3-dependent enhancer activity (Fig. 6B). Stimulation of cells with 10 pM IL-3 resulted in three- to fourfold-higher levels of luciferase activity than that produced by stimulation with 1 pM IL-3. Stimulation of cells with EPO resulted in no increased luciferase activity (data not shown). The fragments T14P14/17, T14P16/13, and T14P12/18 had no enhancer activity. Since the three fragments overlapped the region from −1541 to −1416, this region (125 bp) was initially identified as a βc-specific, inducible enhancer.

We next performed further truncations of the *DUB-1* enhancer region (Fig. 7). A region of 112 bp (B1NC21), corresponding to bp −1528 to −1416, had activity comparable to that of the T14p11/21 fragment. Further truncations within this region generated DNA fragments with only partial activities. For instance, the B1B2 fragment (5′ end) demonstrated enhancer activity that was 9% of the activity of the T14p11/21 fragment. The B2NC21 fragment (3′ end) had enhancer activity that was 14% of the activity of the T14p11/21 fragment.

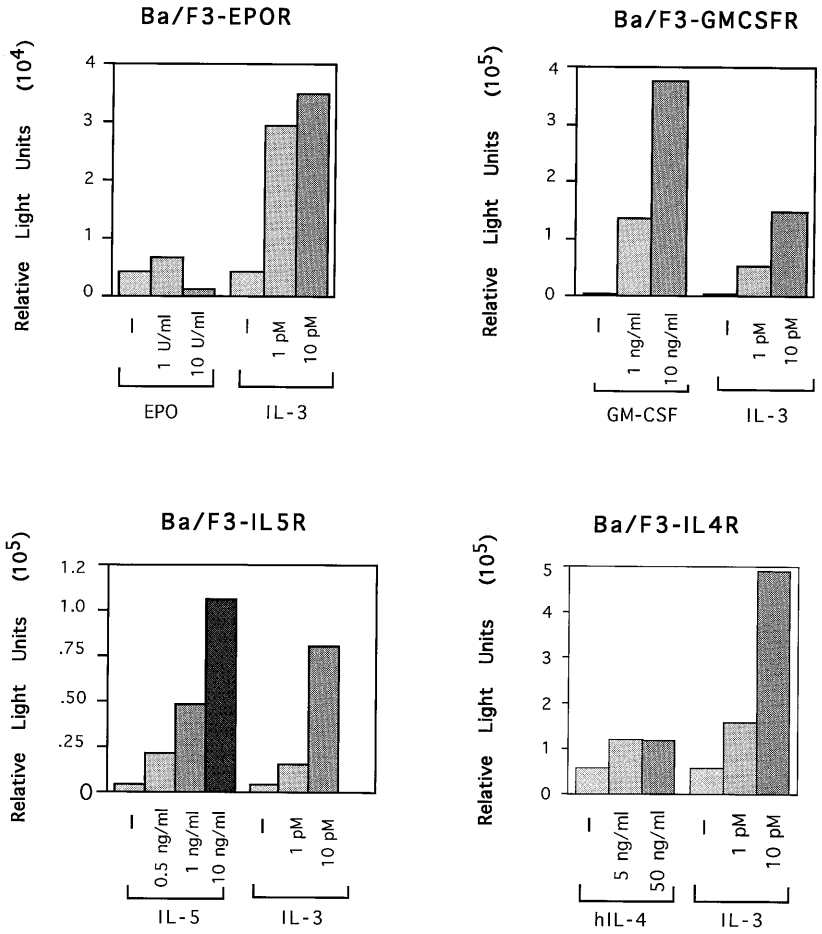

FIG. 5. The 5′ region of the *DUB-1* gene contains a βc-specific enhancer element. Luciferase activity was assayed for the indicated cell lines after transfection with the T14P1.5kb-luc construct. This construct contains the entire 1.5-kb 5′ flanking region of the *DUB-1* gene. Following transfection, the Ba/F3 subclones were stimulated with the indicated concentrations of cytokines. Results are representative of three independent experiments.

These data suggest that the 112-bp fragment is the minimal region required for βc-specific *DUB-1* induction. This fragment was not activated by EPO or IL-4 stimulation (data not shown). The 112-bp sequence (Fig. 4B) may, therefore, contain a novel βc-specific transcriptional activation element.

Specific binding of inducible and noninducible protein complexes to the βc-responsive *DUB-1* enhancer element. In order to determine if regulatory proteins bind to the *DUB-1* enhancer, we constructed four double-stranded oligonucleotides from the minimal enhancer region (Fig. 8A). Initially, probe 1, derived from the 5′ end of the minimal enhancer (−1528 to −1508), was end labeled with ^{32}P and used in a gel mobility shift assay to determine protein binding (Fig. 8B). Interestingly, two major gel shift complexes were identified in nuclear extracts from IL-3-stimulated cells. The complexes were inhibited by unlabeled probe 1 (S) but not by a nonspecific oligonucleotide (NS). These data demonstrate that specific, inducible protein complexes bind to the 5′ region of the 112-bp *DUB-1* enhancer. However, it is not clear whether these two complexes are related.

Probe 3, derived from the 3′ region of the minimal enhancer (−1479 to −1461), was labeled with ^{32}P and used to identify additional protein complexes (Fig. 8C). Another protein complex was identified from both unstimulated and IL-3-stimulated cells. This noninducible protein complex was inhibited by unlabeled probe 3 (S) but not by a nonspecific oligonucleotide (NS). Probe 2 and probe 4 did not reveal significant protein complexes by gel shift analysis (data not shown). Taken together, these data demonstrate binding of two discrete protein complexes, one to the 5′ region and one to the 3′ region of the minimal enhancer. Both complexes may be needed for functional enhancer activity in vivo.

Specific sequences required for *DUB-1* enhancer activity. In order to identify specific base pairs within the *DUB-1* enhancer required for βc responsiveness, we next compared the *DUB-1* enhancer with an analogous region of the *DUB-3* gene (Fig. 9). The *DUB-3* gene was isolated during our genomic screening for the *DUB-1* gene (52b). The *DUB-1* and *DUB-3* genes encode functional deubiquitinating enzymes that are highly homologous throughout their primary amino acid sequences; however, the *DUB-3* mRNA is not induced by IL-3 or EPO. We reasoned that differences in the upstream regulatory sequences of *DUB-1* and *DUB-3* might account for differences in transcriptional activation. The analogous 5′ enhancer region of the *DUB-3* gene differed from the *DUB-1* minimal enhancer region by only 6 bp at five positions (Fig. 9A). A G-to-T mutation fell within probe 1, and a double-A insertion fell within probe 3. These base pair alterations of the *DUB-3* enhancer region ablated IL-3-dependent enhancer activity (Fig. 9B).

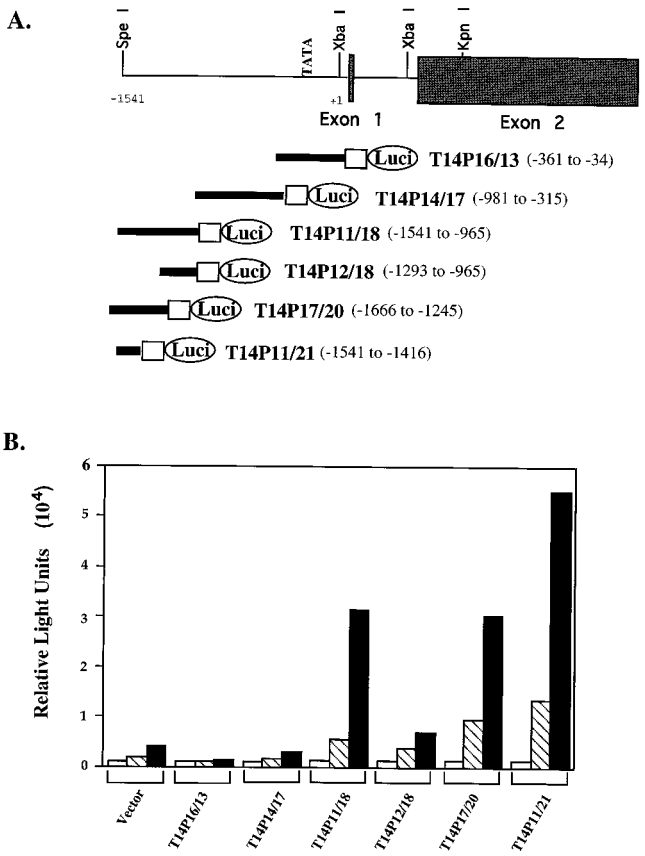

FIG. 6. Identification of a βc-specific response element in the *DUB-1* promoter region. (A) The indicated various DNA fragments of the *DUB-1* promoter region were subcloned into the pGL2Promoter vector and tested for enhancer activity. Open boxes, simian virus 40 basal promoter. Luci, luciferase. (B) Luciferase activity was assayed in cells transfected with the constructs shown in panel A. The cells were starved and restimulated with no growth factor (open bars), 1 pM IL-3 (hatched bars), or 10 pM IL-3 (filled bars). Luciferase assays were performed after 12 h.

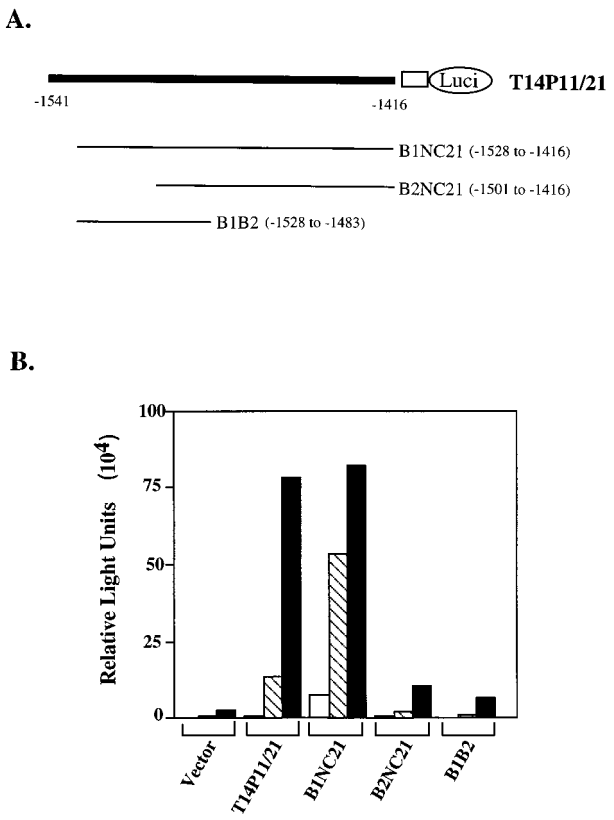

FIG. 7. Identification of a minimal βc-specific response element. (A) The indicated various DNA fragments were generated by PCR and subcloned into the pGL2Promoter vector, as was done for the experiment shown in Fig. 6. Luci, luciferase. (B) Luciferase activity was assayed in cells transfected with the constructs shown in panel A. The cells were starved and restimulated with no growth factor (open bars), 1 pM IL-3 (hatched bars), or 10 pM IL-3 (filled bars). Luciferase assays were performed after 12 h.

In order to assess the importance of these base pair changes in the binding of the specific protein complexes, we tested the activity of mutant probe 1 or probe 3 in gel shift assays. IL-3 induced the formation of two protein complexes that bind to ^{32}P-labeled probe 1 (Fig. 9C, lane 3). Interestingly, these complexes were inhibited by wild-type (Fig. 9C, lane 4) but not mutant (lane 5) probe 1. In contrast, the specific protein complex that binds to probe 3 (Fig. 9D, lanes 2 and 3) was inhibited by both wild-type (lane 4) and mutant (lane 5) probe 3. These data demonstrated that residue G-−1523 is required for the binding of the IL-3-inducible complexes. This residue falls within an ETS-like consensus sequence (Fig. 9A) (29). In accordance with these data, the 112-bp element which contains the mutation of G-−1523 to T, *DUB-1*(Mut), ablates βc-responsive enhancer activity (Fig. 9B).

The membrane-proximal region of the βc subunit is required for activation of the *DUB-1* enhancer. In order to identify regions of the βc cytoplasmic tail required for *DUB-1* induction, we next assayed various truncated mutants of the hβc subunit (Fig. 10). Initially, we generated a Ba/F3 subclone stably expressing the hGM-CSF-Rα subunit. These cells grow in murine IL-3 but do not grow in hGM-CSF. When these cells were next transfected with the cDNA encoding the wild-type hβc subunit, the resulting cells (Ba/F3–hGM-CSF-Rα–hβc cells) grew in either murine IL-3 or hGM-CSF. These data are in accordance with previous studies (15) that report a functional interaction between the hGM-CSF-R-α chain and the hβc chain.

In addition, Ba/F3–hGM-CSF-R-α cells were stably transfected with cDNAs encoding a truncated βc mutant terminating at amino acid 544, βc(544), or a truncated βc mutant terminating at amino acid 455, βc(455) (45, 46) (Fig. 10A). To ensure that the βc mutant polypeptides were expressed, we performed an immunoblot analysis with an anti-hβc antibody (Fig. 10B). Wild-type, βc(544), and βc(455) polypeptides were also expressed on the surfaces of the respective Ba/F3 subclones, as demonstrated by fluorescence-activated cell sorter analysis (data not shown).

The respective Ba/F3 clones were transfected with the B1NC21-luc construct, which contains the minimal enhancer region, and assayed for cytokine-inducible luciferase activity (Fig. 10C). As a positive control for each Ba/F3 subclone, luciferase activity induced by murine IL-3 was determined. Luciferase activity induced by hGM-CSF via the wild-type βc subunit was similar to activity induced by murine IL-3. Luciferase activity induced by the βc(544) mutant was 50% of the activity induced by murine IL-3. Luciferase activity induced by the βc(455) mutant was 2% of the activity induced by murine IL-3 and was equivalent to that of the unstimulated control. Taken together, these results confirm that the hβc subunit, like

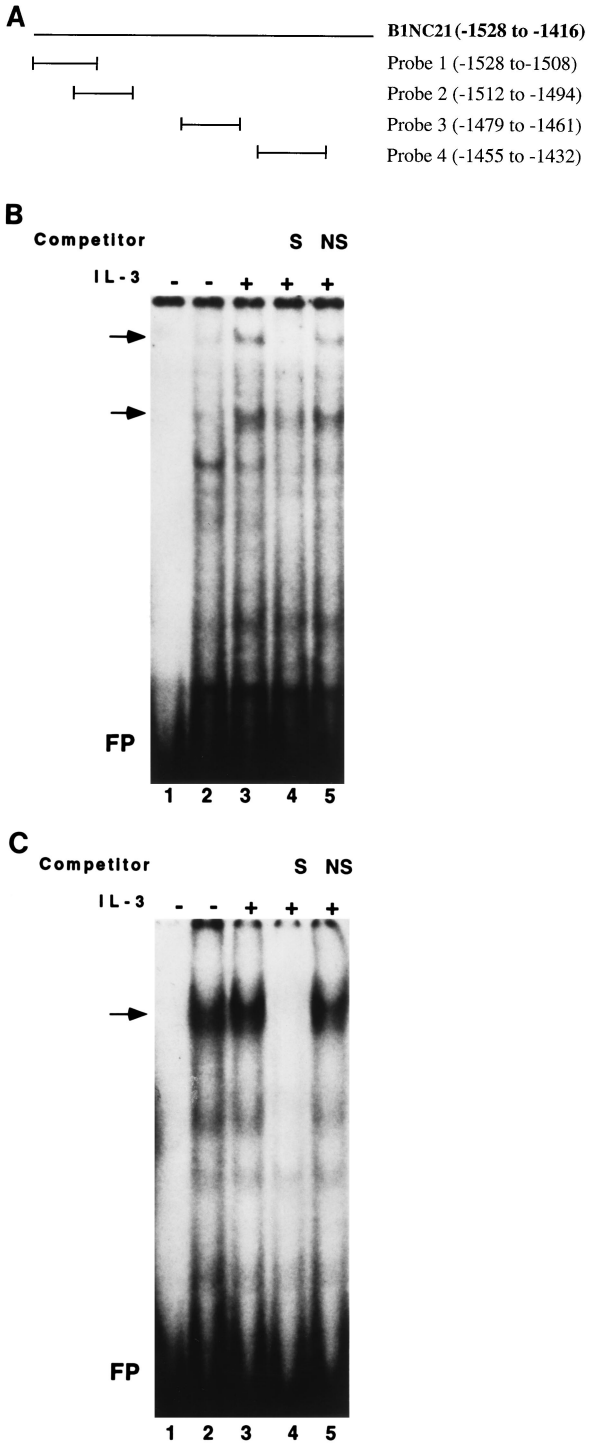

FIG. 8. Identification of inducible and noninducible protein complexes that bind to the *DUB-1* enhancer. (A) Schematic representation of B1NC21 (the minimal *DUB-1* enhancer region) and probes used for gel mobility shift assays. (B) Nuclear extracts were prepared from either unstimulated (−) or IL-3 (10 pM)-stimulated (+) Ba/F3 cells, and a gel shift assay was performed with radiolabeled probe 1. In lane 1, no extract was used. In lanes 4 and 5, 500-fold molar excesses of unlabeled probe 1 (S) and double-stranded nonspecific oligonucleotide (NS), respectively, were used for competition in the binding reactions. (C) Nuclear extracts were prepared from either unstimulated or stimulated Ba/F3 cells, and gel shift was performed with radiolabeled probe 3. In lane 1, no extract was used. In lanes 4 and 5, 500-fold molar excesses of unlabeled probe 3 (S) and double-stranded nonspecific oligonucleotide (NS), respectively, were used for competition. Specific protein complexes are indicated with arrows. FP, free (unbound) probe.

the murine βc subunit, is required for *DUB-1* induction and that amino acids 455 to 544 of hβc are required for this activity.

DISCUSSION

We have isolated an immediate-early gene, *DUB-1*, that is specifically induced by the cytokines IL-3, IL-5, and GM-CSF but not by EPO and IL-4. Since IL-3R, IL-5R, and GM-CSF-R share the βc subunit, we hypothesize that βc generates a specific signal for *DUB-1* induction. In addition, transient transfection experiments utilizing *DUB-1* promoter-luciferase plasmids delineated a 112-bp minimal enhancer region required for βc responsiveness. To further confirm the importance of the βc subunit, we utilized truncated forms of hβc. The proximal membrane region of βc was necessary and sufficient for *DUB-1* transcriptional activation. Taken together, these data demonstrate that a specific signaling mechanism, activated by the βc subunit, can induce transcription of *DUB-1*.

DUB-1 is the first example of an immediate-early gene that is specifically induced by the βc subunit. Previous studies have identified immediate-early genes inducible by IL-3 (49, 52), but these genes were induced by other cytokines as well. Recently, Beadling et al. (4) identified immediate-early genes that were specifically induced by IL-2. Otherwise, there are few examples of cytokine-specific, immediate-early genes.

The possible cellular function of a βc-specific inducible gene remains unclear. IL-3, IL-5, and GM-CSF, on the one hand, induce cellular proliferation of early hematopoietic progenitor cells. EPO, on the other hand, is a lineage-specific growth factor that induces both cellular proliferation and differentiation (25). Our results suggest that *DUB-1* expression may play a role in the sustained proliferation of IL-3-, GM-CSF-, or IL-5-responsive cells. The *DUB-1* gene product is selectively expressed in the early G_1 phase of the cell cycle, following βc stimulation. Misregulated expression of *DUB-1* results in G_1 phase arrest (53). These results suggest that the *DUB-1* protein couples βc stimulation to cell cycle progression, probably via the ubiquitin-mediated proteolytic pathway.

The *DUB-1* enhancer is the first example of an enhancer that is specifically activated by a specific subunit of a cytokine receptor. Deletion analysis identified a minimal enhancer region of 112 bp. Attempts to further truncate the 112-bp enhancer region resulted in significant loss of activity (Fig. 7). Consistent with these observations, the 112-bp region demonstrates at least two specific protein complexes, one at its 5′ end and one at its 3′ end (Fig. 8). Interestingly, the 5′ complex is inducible by a βc signaling pathway. These data suggest that efficient enhancer activity requires both binding complexes in vivo. Other examples of complex enhancers containing multiple regulatory elements have been described (7, 33). Binding of the two protein complexes to the *DUB-1* enhancer correlates with transcription of *DUB-1*. For instance, 32D cells do not exhibit the protein complexes (data not shown) and do not express *DUB-1* mRNA (Fig. 1B).

In order to identify specific base pairs within the *DUB-1* enhancer required for βc responsiveness, we have compared the *DUB-1* enhancer with an analogous region of the *DUB-3* gene which is not βc inducible. A similar approach of comparing inducible elements of mouse interferon-inducible genes has previously been reported (13). The *DUB-1* enhancer and the analogous *DUB-3* sequence differed by only 6 bp. Interestingly, one of these base pair alterations, G-−1523 to T, ablated binding of the IL-3-inducible protein complex and eliminated IL-3-inducible enhancer activity.

The specific DNA-binding proteins constituting the two protein complexes of the *DUB-1* enhancer remain unknown. The

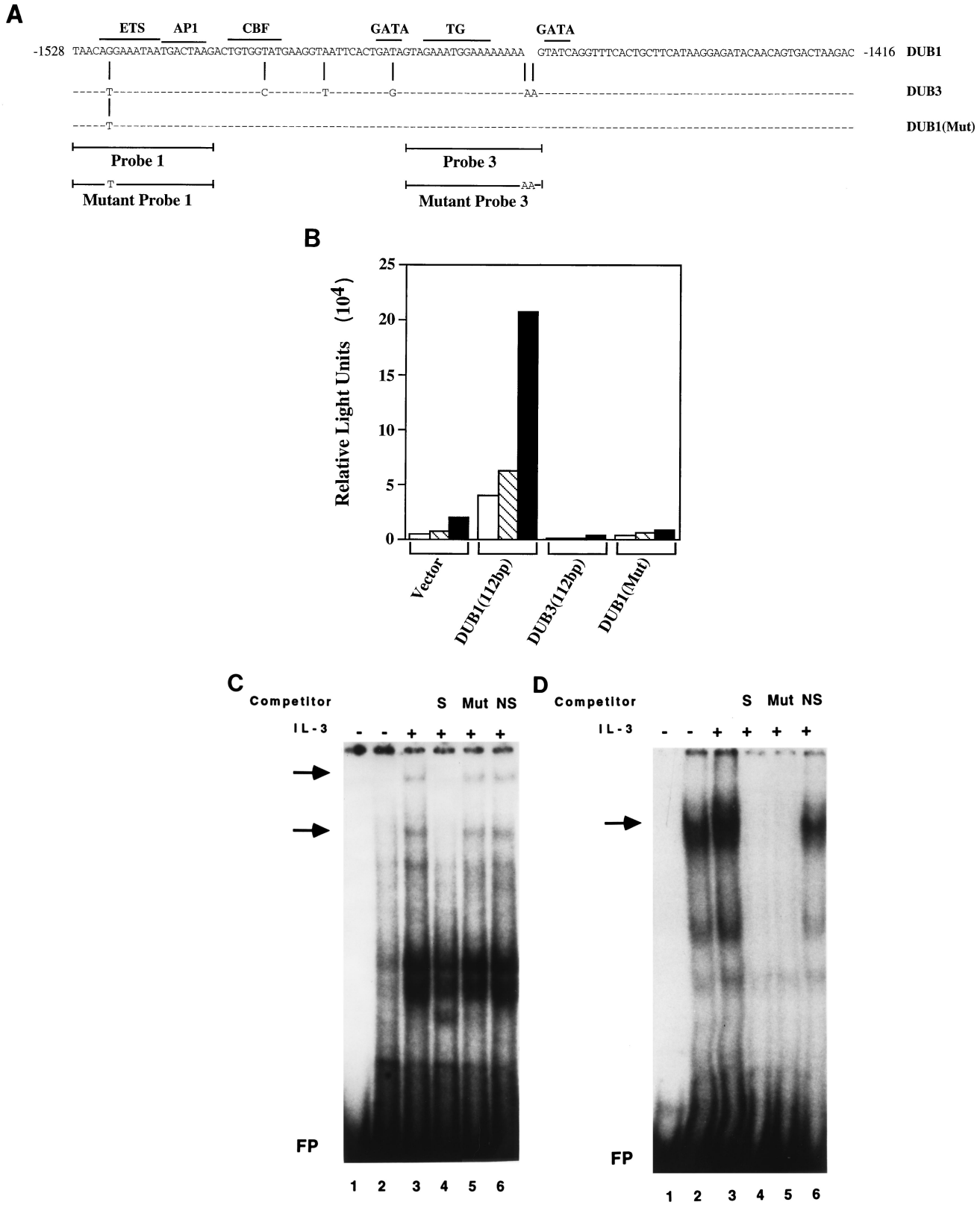

FIG. 9. Mutations in the *DUB-1* enhancer region ablate βc subunit-inducible activity. (A) Comparison of the minimal enhancer regions of *DUB-1*, *DUB-3*, and *DUB-1*(Mut). Probe 1, mutant probe 1, probe 3, and mutant probe 3 are indicated. These enhancer regions contain an ETS protein consensus sequence, an AP1 site, two GATA sites, a CBF site, and a TG protein binding site as indicated. (B) Luciferase activity was assayed in Ba/F3 cells transfected with the indicated constructs. The cells were starved and restimulated with no growth factor (open bars), 1 pM IL-3 (hatched bars), or 10 pM IL-3 (filled bars). Luciferase assays were performed after 12 h. (C) Nuclear extracts were prepared from unstimulated (−) or IL-3-stimulated (+) Ba/F3 cells, and a gel shift assay was performed with radiolabeled probe 1. In lane 1, no extract was used. In lanes 4 to 6, 500-fold molar excesses of unlabeled probe 1 (S), mutant probe 1 (Mut), and nonspecific oligonucleotide (NS), respectively, were used for competition. Mutant probe 1 contains the G−−1523-to-T mutation indicated in panel A. (D) Nuclear extracts were prepared from unstimulated or IL-3-stimulated Ba/F3 cells, and a gel shift assay was performed with radiolabeled probe 3. In lane 1, no extract was used. In lanes 4 to 6, 500-fold molar excesses of unlabeled probe 3 (S), mutant probe 3 (Mut), and nonspecific oligonucleotide (NS), respectively, were used for competition. The mutant probe 3 contains the mutation indicated in panel A. FP, free (unbound) probe.

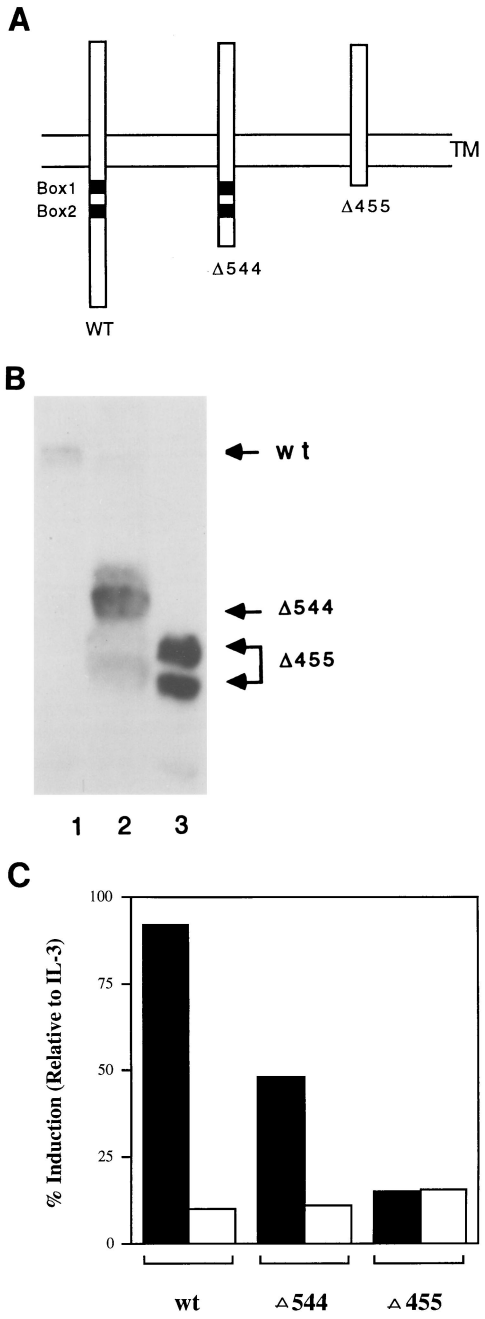

FIG. 10. The membrane-proximal region of the hβc subunit is required for activation of the *DUB-1* enhancer. (A) Schematic diagram showing wild-type (WT) and two carboxy-terminally truncated mutants of the hβc subunit (Δ544 and Δ455). TM, transmembrane region. (B) The cDNAs encoding the βc polypeptides shown in panel A were transfected into Ba/F3 cells. Stable expression of the full-length wild-type βc (wt), βc(544) (Δ544), and βc(455) (Δ455) polypeptides is demonstrated by immunoblot analysis with an anti-hβc antiserum. (C) Ba/F3 subclones were transfected with the B1NC21-luc construct containing the minimal enhancer region. After 48 h, the transfected cells were starved and restimulated with either no growth factor (open bars), hGM-CSF (10 ng/ml) (filled bars), or murine IL-3 (10 pM). Luciferase activity was normalized as a percentage of murine IL-3-induced activity for each Ba/F3 subclone. The results are representative of three independent experiments.

DUB-1 112-bp minimal enhancer contains candidate binding sequences (Fig. 9A). The sequence contains an ETS protein consensus (29, 47) (nt −1524 to −1516), an AP-1 site (29) (nt −1515 to −1509), two GATA sites (43) (nt −1483 to −1480 and −1461 to −1458), a CBF site (34) (nt −1505 to −1498), and a TG protein binding site (13) (nt −1476 to −1467). The CBF site located on probe 2 does not demonstrate a significant gel shift. Whether any of these proteins contributes to the complexes remains unknown. Interestingly, the ETS consensus sequence is disrupted by the G-−1523-to-T mutation, suggesting the possibility that the inducible protein complex contains an ETS family member. No members of the ETS family of transcription factors are known to be cytokine inducible.

Finally, we have demonstrated that the membrane-proximal region of βc, containing amino acids 455 to 544, is required for induction of the *DUB-1* gene. This region of βc has previously been shown to activate the JAK-STAT pathway and the c-*myc* induction pathway (45, 46), suggesting that these pathways may play a role in *DUB-1* induction. In contrast, the Ras-Raf-MAP kinase pathway is activated by the distal region of the βc receptor. This region is therefore not required for full induction of *DUB-1* transcription but may partially contribute to *DUB-1* induction.

Although activation of the JAK-STAT pathway by the proximal domain of βc correlates with *DUB-1* induction, the JAK-STAT pathway alone cannot account for the specificity of *DUB-1* induction. For instance, both IL-3 and EPO activate STAT5A and STAT5B yet EPO does not induce *DUB-1*. In addition, no STAT binding elements (10) are found in the 112-bp enhancer region of the *DUB-1* gene. Taken together, these observations suggest that additional levels of specificity must be activated by the βc subunit. For instance, the βc subunit may activate an additional unique signaling pathway, such as one recently described (39). Further analysis of the *DUB-1* enhancer and its DNA-binding proteins may reveal specific determinants of a βc-specific signaling pathway.

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