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### Research Article

Interleukin 6 (IL-6), a cytokine produced by skeletal cells, stimulates osteoclast recruitment. The IL-6 soluble receptor (sIL-6R) increases IL-6 activity, and IL-6 and sIL-6R levels are increased in conditions of increased bone resorption. We examined the production of IL-6 by primary rat osteoblasts (Ob cells) cultured in the presence of IL-6 and sIL-6R. IL-6 alone did not induce IL-6 transcripts, but IL-6 was stimulatory in the presence of sIL-6R. Furthermore, sIL-6R by itself increased IL-6 transcripts. Cycloheximide superinduced IL-6 transcripts and did not prevent the effect of IL-6 and sIL-6R. IL-6 in the presence of sIL-6R stimulated IL-6 rates of transcription and the activity of IL-6 promoter fragments in transiently transfected Ob cells. 5' deletions of the IL-6 promoter and targeted mutations of the multiple response element (MRE)/cAMP responsive element (CRE), the nuclear factor for IL-6 (NF-IL-6), and the nuclear factor-kappaB (NF-kappaB) binding sites indicated that NF-IL-6 and NF-kappaB, in combination with MRE/CRE, binding sites are required for the induction of the IL-6 promoter by IL-6. In conclusion, IL-6 induces its own synthesis in osteoblasts by transcriptional mechanisms. This positive feedback may be important in conditions of increased bone resorption.

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# Interleukin 6 Is Autoregulated by Transcriptional Mechanisms in Cultures of Rat Osteoblastic Cells

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## Abstract

Interleukin 6 (IL-6), a cytokine produced by skeletal cells, stimulates osteoclast recruitment. The IL-6 soluble receptor (sIL-6R) increases IL-6 activity, and IL-6 and sIL-6R levels are increased in conditions of increased bone resorption. We examined the production of IL-6 by primary rat osteoblasts (Ob cells) cultured in the presence of IL-6 and sIL-6R. IL-6 alone did not induce IL-6 transcripts, but IL-6 was stimulatory in the presence of sIL-6R. Furthermore, sIL-6R by itself increased IL-6 transcripts. Cycloheximide superinduced IL-6 transcripts and did not prevent the effect of IL-6 and sIL-6R. IL-6 in the presence of sIL-6R stimulated IL-6 rates of transcription and the activity of IL-6 promoter fragments in transiently transfected Ob cells. 5' deletions of the IL-6 promoter and targeted mutations of the multiple response element (MRE)/cAMP responsive element (CRE), the nuclear factor for IL-6 (NF-IL-6), and the nuclear factor- $\kappa$ B (NF- $\kappa$ B) binding sites indicated that NF-IL-6 and NF- $\kappa$ B, in combination with MRE/CRE, binding sites are required for the induction of the IL-6 promoter by IL-6. In conclusion, IL-6 induces its own synthesis in osteoblasts by transcriptional mechanisms. This positive feedback may be important in conditions of increased bone resorption. (*J. Clin. Invest.* 1997. 100:1797–1803.) Key words: bone remodeling • osteoporosis • cytokines • DNA-binding sites • transcription factors

## Introduction

Interleukin 6 (IL-6), a pleiotropic cytokine with osteotropic activities, is produced by multiple cells in the skeletal tissue, including cells of the osteoblast and osteoclast lineage (1, 2). IL-6 is considered an autocrine or paracrine mediator of bone resorption since it stimulates the development of osteoclasts from precursor cells (2, 3). IL-6 production in skeletal cells is stimulated by hormones, such as parathyroid hormone and 1,25-dihydroxyvitamin D<sub>3</sub>, and by local cytokines, such as TNF and IL-1 (4–7). IL-6 may be an important intermediary in the

effects of these agents on bone resorption, and it appears to mediate the bone loss observed in the estrogen-deficient state (8). Myeloma cells are a source of IL-6, and thus, this cytokine may also be responsible for the increased bone resorption observed in patients with multiple myeloma (9–11).

Although IL-6 appears to play a pivotal role in bone resorption, its mechanism of action is poorly understood. In coculture systems of mouse osteoblasts and bone marrow cells, IL-6 stimulates the formation of multinucleated osteoclast-like cells in the presence but not in the absence of the IL-6 soluble receptor (sIL-6R)<sup>1</sup> (12). sIL-6R can be generated by proteolytic cleavage of the IL-6 membrane-bound receptor, an 80-kD glycoprotein (gp-80), or by translation of an alternatively spliced RNA (13–15). Signal transduction occurs after the binding of IL-6 to gp-80 and the association of the complex with two 130-kD glycoproteins (gp-130). The cytoplasmic domain of gp-80 is not required for IL-6 signaling, and the sIL-6R can mediate IL-6 signals since it retains ligand-binding activity (16).

sIL-6R has been detected in biological fluids such as plasma, urine, and synovial fluid of healthy humans, and its levels, like those of IL-6, are elevated in conditions of increased bone resorption, such as multiple myeloma and estrogen deficiency (17–19). This suggests that not only IL-6 but also its soluble receptor play a role in bone resorption.

In this study, we examined whether IL-6 stimulates IL-6 production in cultures of osteoblast-enriched cells from 22-d fetal rat calvariae (Ob cells). We also postulated that the sIL-6R potentiates this autoregulation. Since IL-6 and sIL-6R levels are increased in conditions of increased bone resorption, IL-6 autoregulation in osteoblasts may be critical for IL-6 paracrine effects on osteoclast development and bone resorption. We determined the mechanisms involved and studied the regulatory elements of the rat IL-6 gene promoter responsible for the stimulatory effects of IL-6 and sIL-6R on IL-6 expression.

## Methods

**Cell culture.** The culture method used was described in detail previously (20, 21). Parietal bones were obtained from 22-d-old fetal rats immediately after the mothers were killed by blunt trauma to the nuchal area. This project was approved by the Animal Care and Use Committee of Saint Francis Hospital and Medical Center. Cells were obtained by five sequential digestions of the parietal bone using bacterial collagenase (CLS II; Worthington Biochemical Corp., Freehold, NJ). Cell populations harvested from the third to fifth digestions were cultured as a pool. These cells were shown previously to express osteoblastic characteristics, including the production of type I collagen, high levels of alkaline phosphatase activity and osteocalcin,

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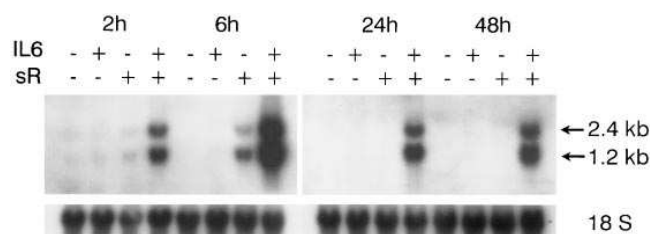
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1. Abbreviations used in this paper: AP-1, activation protein-1; CMV, cytomegalovirus; CRE, cAMP responsive element; gp-80 and gp-130, 80- and 130-kD glycoproteins; MRE, multiple response element; NF-IL-6, nuclear factor for IL-6; NF- $\kappa$ B, nuclear factor- $\kappa$ B; Ob cells, primary rat osteoblastic cells; sIL-6R, IL-6 soluble receptor.



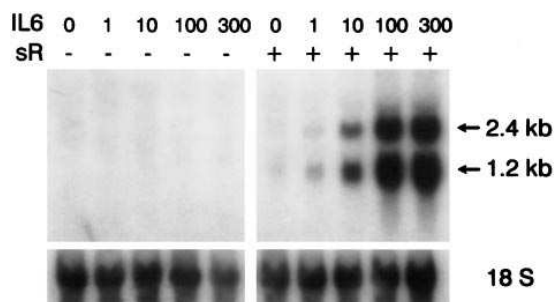


**Figure 2.** Effect of IL-6 at 100 and sIL-6R (sR) at 125 ng/ml, singly and in combination, on IL-6 mRNA levels in cultures of Ob cells treated for 2–48 h. Total RNA from control or treated cells was subjected to Northern blot analysis and hybridized with an [ $\alpha$ - $^{32}$ P]-labeled IL-6 cDNA. IL-6 transcripts of 1.2 and 2.4 kb were visualized by autoradiography. Blots were stripped and rehybridized with an [ $\alpha$ - $^{32}$ P]-labeled 18S rRNA cDNA. *Upper panel*, IL-6 transcripts. *Lower panel*, 18S rRNA transcripts.

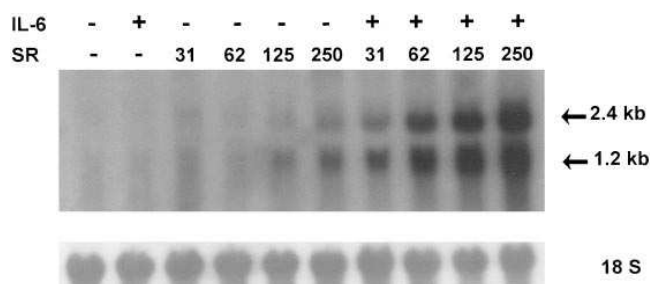
NF-IL-6 or MRE/CRE and NF-IL-6 mutated constructs as a template. Newly synthesized wild-type and target-mutated IL-6 promoter fragments were cloned into pGL-3 Basic (Promega Corp.) containing a luciferase reporter gene. Sequences of the wild-type and mutated constructs generated by either the Morph Mutagenesis Kit or PCR were confirmed by DNA sequence analysis using the Sequenase Version 2.0 DNA sequencing kit (United States Biochemical Corp., Cleveland, OH).

## Results

Northern blot analysis of total RNA from serum-deprived confluent Ob cells revealed limited expression of a 1.2- and a 2.4-kb IL-6 transcript. IL-6 by itself did not induce IL-6 transcripts, but in the presence of sIL-6R, IL-6 caused a time- and dose-dependent stimulation of IL-6 mRNA levels (Figs. 2 and 3). IL-6 at 100 ng/ml in the presence of sIL-6R at 125 ng/ml induced IL-6 mRNA levels after 2 h; the effect was maximal after 6 h, and was sustained for 24–48 h (Fig. 2). In the presence of sIL-6R, the effect of IL-6 was observed at concentrations of 1–300 ng/ml (Fig. 3). sIL-6R at 31–250 ng/ml also caused a dose-dependent induction of IL-6 mRNA, and this effect was ob-



**Figure 3.** Effect of IL-6 at 1–300 ng/ml in the presence (+) and absence (–) of sIL-6R (sR) at 125 ng/ml on IL-6 mRNA levels in cultures of Ob cells treated for 6 h. Total RNA from control, IL-6–, or sIL-6R–treated cells was subjected to Northern blot analysis and hybridized with an [ $\alpha$ - $^{32}$ P]-labeled IL-6 cDNA. IL-6 transcripts of 1.2 and 2.4 kb were visualized by autoradiography. Blots were stripped and rehybridized with an [ $\alpha$ - $^{32}$ P]-labeled 18S rRNA cDNA. *Upper panel*, IL-6 transcripts. *Lower panel*, 18S rRNA transcripts.

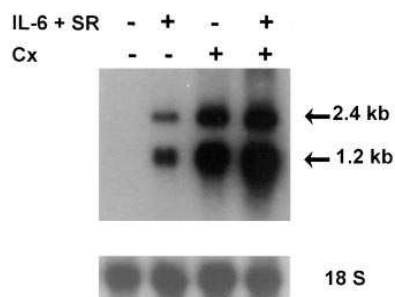


**Figure 4.** Effect of sIL-6R (SR) at 31–250 ng/ml in the presence (+) or absence (–) of IL-6 on IL-6 mRNA levels in cultures of Ob cells treated for 6 h. Total RNA from control, IL-6–, or sIL-6R–treated cells was subjected to Northern blot analysis and hybridized with an [ $\alpha$ - $^{32}$ P]-labeled IL-6 cDNA. IL-6 transcripts of 1.2 and 2.4 kb were visualized by autoradiography. Blots were stripped and rehybridized with an [ $\alpha$ - $^{32}$ P]-labeled 18S rRNA cDNA. *Upper panel*, IL-6 transcripts. *Lower panel*, 18S rRNA transcripts.

served in the presence and absence of IL-6 at 100 ng/ml (Fig. 4). To determine whether the autoregulation of IL-6 was dependent on protein synthesis, Ob cells were treated for 6 h with IL-6 at 100 and sIL-6R at 125 ng/ml in the presence or absence of the protein synthesis inhibitor cycloheximide at 3.6  $\mu$ M. Cycloheximide superinduced IL-6 mRNA levels and did not prevent the effect of IL-6 and sIL-6R (Fig. 5).

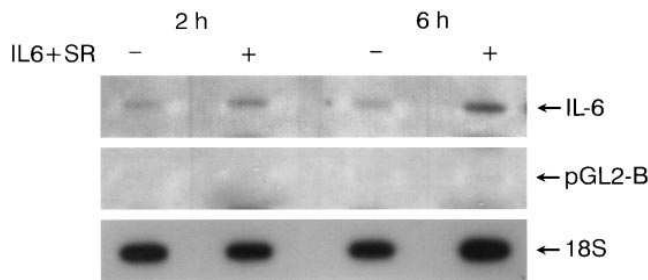
To analyze the mechanisms involved in the autoregulation of IL-6, we examined the effects of IL-6 and sIL-6R on the rate of transcription of the IL-6 gene by a nuclear run-on assay performed on nuclei from Ob cells exposed to control medium or to IL-6 at 100 and sIL-6R at 50 ng/ml for 2 and 6 h. After 6 h, IL-6 in the presence of sIL-6R increased IL-6 transcription rates 2–2.5-fold, demonstrating a transcriptional effect (Fig. 6).

To confirm that IL-6 autoregulation occurs by transcriptional mechanisms, and to define gene elements responsible for the effect, Ob cells were transfected transiently with chimeric constructs containing fragments of the IL-6 promoter linked to the reporter gene luciferase. The effects of IL-6 at 100 and sIL-6R at 125 ng/ml alone or in combination were tested for 6–24 h on a bp –2906 to +20 fragment of the rat IL-6 promoter. IL-6 or sIL-6R alone caused a small, for the most part not statistically significant, stimulatory effect on promoter



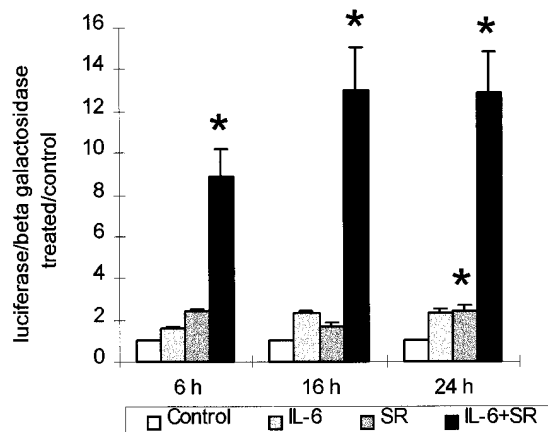
**Figure 5.** Effect of IL-6 at 100 and sIL-6R (SR) at 125 ng/ml in the presence (+) or absence (–) of cycloheximide (Cx) at 3.6  $\mu$ M on IL-6 mRNA levels in cultures of Ob cells treated for 6 h. Total RNA from control, cycloheximide–, or IL-6 + sIL-6R–treated cells was

subjected to Northern blot analysis and hybridized with an [ $\alpha$ - $^{32}$ P]-labeled IL-6 cDNA. IL-6 transcripts of 1.2 and 2.4 kb were visualized by autoradiography. Blots were stripped and rehybridized with an [ $\alpha$ - $^{32}$ P]-labeled 18S rRNA cDNA. *Upper panel*, IL-6 transcripts. *Lower panel*, 18S rRNA transcripts.

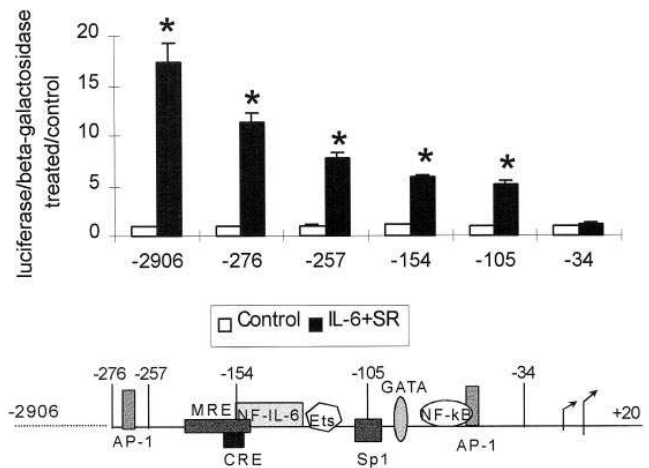


**Figure 6.** Effect of IL-6 at 100 ng/ml in the presence of sIL-6R (SR) at 50 ng/ml on IL-6 transcription rates in cultures of Ob cells treated for 2 and 6 h. Nascent transcripts from control (–) or IL-6- and sIL-6R (+)-treated cultures were labeled *in vitro* with [ $\alpha$ - $^{32}$ P]UTP, and the labeled RNA was hybridized to immobilized cDNA for IL-6. 18S rRNA cDNA was used to demonstrate loading, and pGL2-Basic vector DNA was used as a control for nonspecific hybridization.

activity (Fig. 7). However, IL-6 at 100 ng/ml, in the presence of sIL-6R at 125 ng/ml, caused an eightfold increase in promoter activity after 6 h, and a 13–16-fold stimulation after 16 and 24 h (Figs. 7 and 8). To characterize the regulatory elements involved, 5' deletion constructs of the IL-6 promoter ranging from bp –2906 to +20 to bp –34 to +20 were tested in six different experiments. 5' deletions from bp –2906 to –257 of the IL-6 promoter resulted in a decrease in the basal activity of the IL-6 promoter (not shown), but did not preclude the response to IL-6 in the presence of sIL-6R. IL-6 with sIL-6R induced the bp –276 to +20 and the bp –257 to +20 of the IL-6 pro-



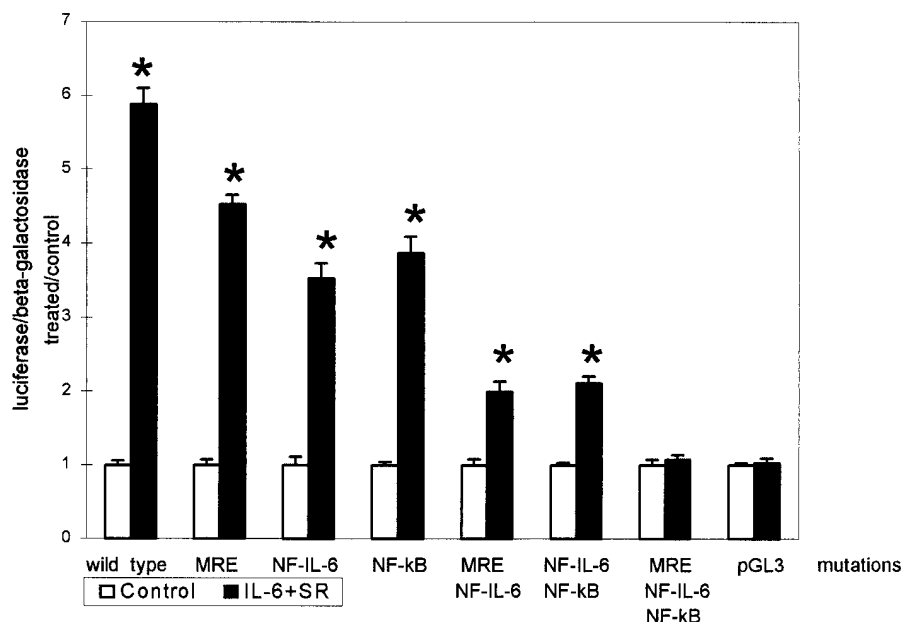
**Figure 7.** Effect of IL-6 at 100 and sIL-6R (SR) at 125 ng/ml, singly and in combination, on IL-6 promoter activity in transiently transfected Ob cells. Ob cells were transfected with 35  $\mu$ g DNA of a chimeric construct containing the rat IL-6 promoter sequence of bp –2906 to +20 linked to a luciferase reporter gene by calcium phosphate–DNA coprecipitation. To control for transfection efficiency, 10  $\mu$ g of a CMV- $\beta$ -galactosidase expression vector was cotransfected. 24 h after transfection, Ob cells were serum-deprived for 20–24 h and exposed to IL-6 or sIL-6R singly and in combination for the indicated periods of time. Cells were harvested, and luciferase activity was determined, corrected for  $\beta$ -galactosidase activity, and expressed as treated/control ratios for each individual time period studied. Values are means  $\pm$  SEM of 18–24 observations pooled from four independent experiments; SEM not shown were too small to be depicted. \*Significantly different from control ( $P < 0.05$ ).



**Figure 8.** Effect of IL-6 at 100 ng/ml, in the presence of sIL-6R (SR) at 125 ng/ml, on IL-6 promoter activity in transiently transfected Ob cells. Ob cells were transfected with 35  $\mu$ g DNA of chimeric constructs containing fragments of the IL-6 promoter linked to a luciferase reporter gene by calcium phosphate–DNA coprecipitation. The 5' deletion endpoints of the IL-6 promoter are indicated (*bottom*). To control for transfection efficiency, 10  $\mu$ g of a CMV- $\beta$ -galactosidase expression vector was cotransfected. 24 h after transfection, Ob cells were serum-deprived for 20–24 h and exposed to IL-6 in the presence of sIL-6R for 16 h. Cells were harvested and analyzed, and luciferase activity was determined, corrected for  $\beta$ -galactosidase activity, and expressed as treated/control ratios for each construct tested. Values are means  $\pm$  SEM of 22–33 observations, pooled from six independent experiments; SEM not shown were too small to be depicted. \*Significantly different from respective control ( $P < 0.05$ ). (*Bottom*) Putative *cis*-regulatory elements of the rat IL-6 promoter with their approximate locations relative to the major start site. Bars, 5' deletion points of the truncated constructs used.

motor construct by 11- and 8-fold, respectively. In individual experiments, we were unable to detect consistent differences in the response of these two constructs. Deletion to bp –154 and to bp –105 resulted in a partial loss of the stimulatory effect of IL-6 and its soluble receptor, and together they increased the activity of the two constructs five- to sixfold (Fig. 8). The basal activity of the bp –34 to +20 IL-6 promoter fragment was decreased dramatically (not shown), and IL-6, with sIL-6R, did not modify the activity of this construct. These results suggest that MRE/CRE, NF-IL-6, and NF- $\kappa$ B binding sites contained in the bp –257 to +20 region of the IL-6 gene are potentially necessary for the response to IL-6 and sIL-6R.

To define the elements responsible for the autoregulation of IL-6, a bp –257 to +20 fragment of the IL-6 promoter was cloned into pGL-3 Basic, and targeted mutations of the known consensus sequences of MRE/CRE, NF-IL-6, and NF- $\kappa$ B binding sites were made singly or in combination and tested in two separate experiments, which revealed analogous results. In the experiment shown in Fig. 9, IL-6 and sIL-6R increased the activity of the bp –257 to +20 wild-type IL-6 construct sixfold. Targeted single mutations of NF-IL-6, NF- $\kappa$ B, or MRE/CRE binding sites resulted in a decrease in the response to IL-6 and its soluble receptor (all  $P < 0.05$  vs. wild-type). The combined NF-IL-6 and NF- $\kappa$ B or MRE/CRE and NF-IL-6 mutations resulted in a comparable decrease in the response to IL-6



**Figure 9.** Effect of IL-6 at 100 ng/ml, in the presence of sIL-6R (SR) at 125 ng/ml, on IL-6 promoter activity in transiently transfected Ob cells. Ob cells were transfected with 35  $\mu$ g of a chimeric construct containing a bp  $-257$  to  $+20$  fragment of the IL-6 promoter, with or without mutations of MRE, NF-IL-6, and NF- $\kappa$ B alone or mutations of the combinations MRE and NF-IL-6, NF-IL-6 and NF- $\kappa$ B, and MRE, NF-IL-6, and NF- $\kappa$ B. Constructs were linked to a luciferase reporter gene and transfected by calcium phosphate-DNA coprecipitation. To control for transfection efficiency, 10  $\mu$ g of a CMV- $\beta$ -galactosidase expression vector was cotransfected. 24 h after transfection, Ob cells were serum-deprived for 20–24 h and exposed to IL-6 in the presence of sIL-6R for 16 h. Cells were harvested, and luciferase activity was determined, corrected for  $\beta$ -galactosidase activity, and expressed as treated/control ratios for each construct tested. Values are means  $\pm$  SEM of six observations. \*Significantly different from control ( $P < 0.05$ ).

and sIL-6R, which was more pronounced than the decrease observed with single mutations ( $P < 0.05$ ). The triple mutation of MRE/CRE, NF-IL-6, and NF- $\kappa$ B resulted in a total loss of the IL-6 promoter response to IL-6 and sIL-6R ( $P < 0.05$  vs. wild-type, and all constructs with single or double mutations).

## Discussion

In these studies, we have demonstrated that IL-6 in the presence of its soluble receptor induces IL-6 mRNA in Ob cells. Although IL-6 has been shown to induce IL-6 mRNA in the osteoblastic cells MC3T3-E1, the effect was small and only detected by reverse transcription-PCR (33). The lack of an IL-6 effect when tested alone is probably due to the low level of expression of the membrane-bound IL-6 receptor in Ob cells or due to receptor downregulation. IL-6 binds an 80-kD surface glycoprotein, and the IL-6/surface receptor complex associates with gp-130 molecules, which activate the JAK/STAT signal transduction pathway (34). A soluble form of the IL-6 receptor is present in human and mouse serum and was found to enhance the effects of IL-6 in various cell types, including osteoclasts (12, 17, 35). Similarly, we showed that the effects of IL-6 on IL-6 transcripts in Ob cells were observed in the presence of its soluble receptor. Moreover, increasing concentrations of sIL-6R induced IL-6 transcripts in the absence of exogenous IL-6, suggesting that endogenous IL-6 is produced in sufficient amounts to induce its own synthesis in the presence of its soluble receptor.

The sIL-6R is a 55-kD protein generated by proteolytic cleavage of the membrane-bound receptor at a site adjacent to the transmembrane domain, or by translation of an alternatively spliced RNA (13–15, 36, 37). The sIL-6R binds IL-6 with similar affinity as the membrane-bound receptor, and the complex can elicit IL-6 signaling in cells that do not express cell surface-associated receptor, provided that they express gp-130

(14). This indicates that the sIL-6R is sufficient to mediate IL-6 action. sIL-6R can be measured in human serum, and elevated levels are associated with conditions of increased bone resorption, such as multiple myeloma or estrogen deficiency (18, 19). However, it is not known whether or not osteoblasts or other cells present in the bone environment produce sIL-6R. The concentrations of sIL-6R used in these studies are similar to those found in serum, suggesting that the amplification of the IL-6 effect by its soluble receptor is physiologically or pathologically relevant.

To study the mechanisms of IL-6 stimulation of its own synthesis, we tested the effect of the protein synthesis inhibitor cycloheximide. As previously described, cycloheximide super-induced IL-6 transcripts, suggesting stabilization of IL-6 mRNA (38). In the presence of cycloheximide, IL-6 and sIL-6R had an additive effect on IL-6 mRNA levels, demonstrating that IL-6 autoregulation is not protein synthesis dependent. In addition, we have shown that the autoregulation of IL-6 occurs at the transcriptional level, since IL-6 in the presence of sIL-6R enhanced IL-6 rates of transcription and promoter activity. Using 5' deletion constructs, we demonstrated that a bp  $-257$  to  $+20$  fragment of the IL-6 promoter is responsive to IL-6 and sIL-6R. The  $-257$  to  $+20$  region of the IL-6 gene contains important sequences, including MRE/CRE, NF-IL-6, and NF- $\kappa$ B binding sites, and these sequences are essential for the response of IL-6 to IL-1 and TNF- $\alpha$  (39, 40). In hepatocytes, IL-6 increases NF-IL-6 mRNA levels and NF-IL-6 binding activity (40, 41). In this study, we demonstrated that an NF-IL-6 binding site is necessary for the response of the IL-6 promoter to IL-6, but mutation of the NF-IL-6 binding site was not sufficient to abolish the response to IL-6. Even though experiments using 5' deletion constructs indicated that MRE/CRE or NF- $\kappa$ B binding sites are involved in the response to IL-6 and its soluble receptor, single targeted mutations of MRE/CRE or NF- $\kappa$ B did not abrogate the response of the IL-6 promoter. When NF-

IL-6 and NF- $\kappa$ B binding sites were mutated in combination, the response of the IL-6 promoter to IL-6 and sIL-6R was decreased further, and when all three binding sites were mutated, no response to IL-6 and sIL-6R was observed. These results indicate that MRE/CRE, NF-IL-6, and NF- $\kappa$ B could operate synergistically, an observation supported by previous work demonstrating that both NF-IL-6 and NF- $\kappa$ B binding sites are required for the regulation of immune and acute-phase response genes such as the IL-6 gene (42). Similar to our results, prostaglandin and cAMP activation of the IL-6 gene require MRE, NF-IL-6, and NF- $\kappa$ B binding sites as well as an activation protein-1 (AP-1) binding site (43). In this study, we were unable to detect consistent differences in the response of the bp -276 to +20 and bp -257 to +20 construct to IL-6 and sIL-6R, indicating that the AP-1 site located at bp -263 to -269 of the IL-6 promoter probably does not play a central role in the autoregulation of IL-6 in osteoblasts. Additional regulatory binding sites upstream of bp -276 may be required for the IL-6 promoter response to IL-6 and sIL-6R, since deletions from bp -2906 to -276 resulted in a decrease in the response to IL-6 and sIL-6R.

In conclusion, IL-6 synthesis is autoinduced in osteoblastic cells by transcriptional mechanisms, an effect that requires the presence of sIL-6R. IL-6 autoregulation may play a central role in the effects of IL-6 on bone resorption.

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