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Reconstituted Human Granulocyte-Macrophage Colony-Stimulating Factor Receptor Transduces Growth-Promoting Signals in Mouse NIH 3T3 Cells: Comparison with Signalling in BA/F3 Pro-B Cells

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Granulocyte-macrophage colony-stimulating factor (GM-CSF) plays a critical role in growth and differentiation of myeloid cells. We previously reconstituted high-affinity human GM-CSF receptor (hGM-CSFR) in a pro-B cell line, BA/F3, by cotransfecting α- and β-chain cDNA clones and showed that the reconstituted receptor could transduce growth-promoting signals. The high-affinity hGM-CSFR was also reconstituted in mouse NIH 3T3 cells, but its ability to transduce signals in fibroblasts remained undetermined. In the present study, we further characterized signal transduction by the reconstituted hGM-CSFR in both NIH 3T3 cells and BA/F3 cells. We found that the reconstituted hGM-CSFR transduces signals in NIH 3T3 fibroblasts and BA/F3 cells in response to hGM-CSF to activate transcription of the c-*fos*, c-*jun*, and c-*myc* proto-oncogenes. hGM-CSF also induces protein tyrosine phosphorylation and DNA synthesis in both cell types. These results indicated that hGM-CSFR is functional in fibroblasts, that signal transduction via hGM-CSFR in fibroblasts involves tyrosine kinase(s), and that association of hGM-CSFR with a factor(s) specific to hematopoietic cell lineage is not essential to transduce growth-promoting signals.

Cytokines play a pivotal role in regulating proliferation, differentiation, and functions of hematopoietic cells. Interleukin-3 (IL-3) is a lymphokine found in activated T cells and mast cells that stimulates growth and differentiation of pluripotent hematopoietic stem cells (1, 5, 28, 38). Since this stem cell gives rise to all the different blood cell types, IL-3, either directly or indirectly, regulates the functions of many types of cells. Granulocyte-macrophage colony-stimulating factor (GM-CSF) has a spectrum of biological activity similar, but not identical, to that of IL-3. GM-CSF is produced by activated T cells and mast cells, but unlike IL-3, it is also produced by a variety of other cell types such as stromal cells and macrophages. Both IL-3 and GM-CSF act in a species-specific manner between mice and humans (25, 47).

Molecular cloning of cDNAs encoding IL-3 receptor (IL-3R) or GM-CSF receptor (GM-CSFR) had revealed that these two receptor systems have an α chain specific to each and a common β chain in both human and mouse systems (12, 15, 18, 22). Furthermore, it was shown that the same β chain associates with interleukin-5 receptor (IL-5R) α chain to form the high-affinity IL-5R (7, 42, 43). Both the α subunits of IL-3R, GM-CSFR, and IL-5R as well as the β subunit are members of the cytokine receptor superfamily which have common structural features within the extracellular domain (30). Neither the α nor the β subunit of IL-3R and GM-CSFR has structural features characteristic of a tyrosine kinase or a serine/threonine kinase domain even though IL-3 or GM-CSF induces rapid tyrosine phosphorylation of several proteins including proteins p140, p90, and p60 (17, 19, 32, 33). These results indicated that the tyrosine kinase(s) activated by IL-3 or GM-CSF is distinct from IL-3R or GM-CSFR. Recently, *lyn*, a member of the *src* family tyrosine kinase specific to lymphocytes, has been suggested to be involved in signal transduction from the IL-3R (44).

Previous works have indicated that the expression of the high-affinity and functional IL-3R and GM-CSFR is mainly restricted to hematopoietic cells (4, 8, 36, 37). We have shown that the high-affinity GM-CSFR or IL-3R could be reconstituted by transfecting pro-B cells (BA/F3) or T cells (CTLL2) with α- and β-chain cDNAs (20–22). The reconstituted GM-CSFR in T cells as well as pro-B cells transduced growth-promoting signals. By the same approach, we have also shown that the high-affinity form of the human GM-CSFR (hGM-CSFR) is reconstituted in fibroblasts by introducing cDNA clones encoding the α and β chains (15). This indicated that a molecule(s) specific to hematopoietic cells does not contribute to form a high-affinity GM-CSFR. Furthermore, certain cells of nonhematopoietic origin can respond to GM-CSF and thus presumably express GM-CSFR. For example, hGM-CSF stimulates the migration and proliferation of human endothelial cells (3). This raises the interesting question whether hematopoietic cells and nonhematopoietic cells such as fibroblasts share the signal transduction components associated with GM-CSFR. We have previously reported that the human epidermal growth factor (hEGF) receptor, introduced into the IL-3-dependent immature mast cell line IC2, induces c-*fos* and c-*myc* mRNAs in response to EGF (46). However, EGF supported only short-term proliferation of IC2 cells expressing EGF receptor and failed to support long-term growth. These data indicated that the EGF receptor which is normally expressed in fibroblasts is functional and transmits certain growth signals in hematopoietic cells.

In this study, we addressed the question of whether hematopoietic cell-specific factors are essential for signal

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transduction by GM-CSFR. For this purpose, we examined the functions of the reconstituted hGM-CSFR in nonhematopoietic cells, i.e., mouse NIH 3T3 fibroblasts and mouse embryonic teratocarcinoma cell (EC) line P19, by either transient or stable transfection systems. Our results indicated that the reconstituted high-affinity GM-CSFR is functional in fibroblasts and transduces growth-promoting signals such as the induction of c-*fos* mRNA, protein tyrosine phosphorylation, and DNA synthesis in response to the ligand. Thus, the occurrence of a signal-transducing molecule(s) associated with GM-CSFR may not be restricted to hematopoietic cells. Both α and β chains are essential to transduce signals, but hGM-CSF did not induce internalization of the reconstituted receptor.

MATERIALS AND METHODS

Chemicals, media, antibody, and cytokines. [^{3}H]thymidine and ^{125}I-hGM-CSF were from Amersham Japan Co. Ltd. G418 was a gift from T. Nagabhushan, Schering-Plough Corp. Antiphosphotyrosine antibody 4G10 was obtained from Upstate Biotechnology Inc. Opti MEM was from GIBCO Co. Ltd. Fetal calf serum (FCS) was from Biocell Laboratories Co. Ltd. Dulbecco's modified Eagle's medium (DMEM) and RPMI 1640 medium were from Nikken Bio Medical Laboratories Co. Ltd. Noble agar was from Difco Co. Ltd. Recombinant hGM-CSF produced in *Escherichia coli* was provided by R. Kastelein, DNAX Research Institute. Mouse IL-3 (mIL-3) produced by silkworms (*Bombyx mori*) was purified as described previously (31).

Cell lines and culture methods. An mIL-3-dependent pro-B-cell line, BA/F3 (35), was maintained in RPMI 1640 medium containing 10% FCS, 1 ng of recombinant mIL-3 per ml, 100 U of penicillin per ml, and 100 μg of streptomycin per ml. Transfected BA/F3 cells expressing hGM-CSFR α and β chains were grown in the same medium supplemented with 500 μg of G418 per ml. A mouse NIH 3T3 fibroblast line and a mouse embryonic carcinoma cell line, P19 (27), were maintained in DMEM containing 10% FCS, 100 U of penicillin per ml, and 100 μg of streptomycin per ml. For growth assays, NIH 3T3 cells were maintained in Opti MEM with or without various amounts of recombinant hGM-CSF. A human factor-dependent myeloid cell line, TF-1 (24), was maintained in RPMI 1640 medium containing 10% FCS, 1 ng of recombinant hGM-CSF per ml, 100 U of penicillin per ml, and 100 μg of streptomycin per ml.

Plasmids and genes. hGM-CSFR α chain (10) and KH97 (β chain) were cloned into pCEV4 (18) and pME18, respectively, as described previously (15). The c-*fos* promoter-luciferase gene fusion plasmid was constructed from the c-*fos* promoter fragment (−404 to +41) of the human c-*fos* promoter-chloramphenicol acetyltransferase gene (kindly provided by I. M. Verma, Salk Institute) (6) and the luciferase fragment of the SRα-luciferase plasmid. Rat c-*jun* and mouse c-*fos* cDNA plasmids were kindly provided by T. Curran (Roche Institute of Molecular Biology). Mouse c-*myc* was provided by N. Arai (DNAX Research Institute).

Transient transfection of DNA. Individual plasmid DNA or a combination of multiple plasmid DNAs (5 μg) was transfected into semiconfluent mouse NIH 3T3 cells by electroporation, BA/F3 cells by the DEAE-dextran method, and P19 cells by the calcium phosphate method (2). Briefly, NIH 3T3 cells (4×10^6) resuspended in 0.4 ml of ice-cold Dulbecco's phosphate-buffered saline (PBS) were transferred to a 0.4-cm electroporation cuvette and mixed with DNA. After 5 min of incubation on ice, cells shocked at 250 μF and 300 V by a Gene Pulser (Bio-Rad Co. Ltd.) and kept on ice for 10 min were transferred to several 6-cm culture plates supplemented with 2.5 ml of normal medium. After 14 h, culture medium was changed to DMEM with 0.5% FCS, and cells were incubated under the same conditions. After 24 h, either FCS or hGM-CSF was added to the culture medium as specified, and the cells were harvested after 5 h. BA/F3 cells (3×10^6) mixed with DNA and 500 μg of DEAE-dextran per ml in 3 ml of Tris-buffered saline were incubated at room temperature for 30 min, washed, and plated onto several 6-cm culture plates. One day after transfection, BA/F3 cells, cultured for 5 h in IL-3-free medium containing 10% FCS, were stimulated by either mIL-3 or hGM-CSF for 5 h. P19 cells were incubated for 24 h in medium containing 10% FCS and calcium phosphate DNA precipitate (2) and then for 12 h in the medium containing 10% FCS. Cells were stimulated with hGM-CSF for 5 h and subjected to a luciferase assay. In cotransfected analysis of c-*fos* promoter-luciferase gene and hGM-CSFR α/β cDNAs, the total amount of transfected DNA was adjusted by the addition of a control vector plasmid without hGM-CSFR α or β cDNA.

Radioligands and internalization assay. Internalization of bound hGM-CSF was measured by the method described previously (39). TF-1 cells were incubated in complete medium containing 5 ng of recombinant IL-1α per ml for 12 h to upregulate high-affinity hGM-CSFR (23). TF-1 cells or NIH 3T3GMRα/β cells were incubated with 200 pM of ^{125}I-hGM-CSF for 30 min at 4°C in the binding buffer (RPMI 1640 containing 5 mg of bovine serum albumin (BSA) per ml, 100 μM chloroquine, and 20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES], pH 7.4). The cells were washed twice with the chilled binding buffer and then incubated at 37°C in the binding buffer with or without 0.2% sodium azide. Duplicate 50-μl aliquots (5×10^5 cells) were removed at various time points, added to 0.6 ml of ice-cold buffer, and immediately microcentrifuged for 30 s. The radioactivity in the supernatant was determined as a measure of dissociated hGM-CSF. The cells were then resuspended in 150 μl of 10 mM citrate (pH 3.0) containing 0.14 M sodium chloride and 50 μg of BSA per ml. After 30 s of incubation at room temperature, the cells were centrifuged through a phthalate oil layer (3:2,di-octyl phthalate/di-*n*-butyl phthalate). The radioactivity in the cell pellet and that in the supernatant were measured as the internalized and membrane-bound GM-CSF, respectively.

Northern (RNA) blot analysis. Total cellular RNA was extracted by the guanidinium thiocyanate extraction method (26). Total RNA (10 μg), suspended in 15 μl of running buffer containing 0.1 M 3-[*N*-morpholino]propanesulfonic acid (MOPS) (pH 7.0) buffer, 40 mM sodium acetate, 5 mM EDTA, 6% formaldehyde, and 50% formamide, was denatured by incubation at 65°C for 10 min and then separated by electrophoresis through 1.2% agarose gels containing 6% formaldehyde at 2 to 3 V/cm. After the electrophoretic separation, the RNA was transferred to a nylon membrane by capillary blotting with 20× SSC (150 mM sodium chloride, 15 mM sodium citrate) for 12 h and then immobilized by soaking the filter in 0.05 M sodium hydroxide for 5 min. RNA blots were hybridized with ^{32}P-labeled cDNA probes (c-*fos*, c-*myc*, c-*jun*, and β-actin genes) labeled by random priming (specific activity, 3×10^8 to 7×10^8 cpm/μg). Hybridization was performed for 12 to 16 h at 42°C with a buffer containing 5× Denhardt's solution, 6× SSPE (150 mM sodium chloride, 1 mM sodium dihydrogen phosphate, 1 mM EDTA), 0.5% sodium dodecyl sulfate (SDS), 100 μg of salmon sperm DNA per ml, and 50% formamide. After

hybridization, blots were washed twice for 15 min with 2× SSC–0.1% SDS at room temperature and once for 15 min with 0.1× SSC–0.1% SDS at room temperature and once for 30 min with 0.1× SSC–0.1% SDS at 65°C. They were exposed to imaging plates for 2 h and analyzed with the FUJI image analyzer (model BAS-2000).

[^{3}H]thymidine incorporation. DNA synthesis was measured by the extent of [^{3}H]thymidine incorporation. Aliquots (100 μl) containing approximately 1.2×10^4 cells suspended in DMEM without FCS were transferred to a 96-well microtiter plate. After depletion of FCS for 24 h, either FCS or hGM-CSF was added and the cells were incubated for an additional 22 h. [^{3}H]thymidine (70 Ci/mmol) at 1 μCi for each well was added and incubated for 2 h. Cells were harvested by a cell harvester (model 1295-001; LKB Co. Ltd.) and ^{3}H radioactivity incorporated was determined by a liquid scintillation spectrophotometer (model LOS-1000; Aloka Co. Ltd.). All the data represent the average amount of radioactivity in eight wells.

Soft agar assay. Cultures were prepared as follows. Base layers of 2 ml of medium containing 0.5% Difco Noble agar were set in 60-mm plastic dishes before the addition of a second layer with 1.5×10^4 cells in 3 ml of medium containing 0.33% Noble agar. The plates were incubated for 4 weeks at 37°C in a humidified atmosphere of 5% CO_2 in air. Formation of anchorage-independent colonies was examined periodically with a low-power microscope. As a positive control, NIH 3T3 cells expressing *erbB2* (provided by T. Yamamoto, Institute of Medical Science, University of Tokyo) were used. The growth factor(s) used was 10 to 100 ng of hGM-CSF per ml, or 50 ng of hEGF per ml, or a combination of 50 ng each of hGM-CSF and hEGF per ml in DMEM–10% FCS or Opti MEM.

Luciferase assay. Proteins were extracted from cells transfected with c-*fos* promoter-luciferase DNA by three cycles of freezing and thawing. Each sample containing approximately 15 μg of total protein was mixed with 0.35 ml of 50 mM HEPES buffer containing 5 mM ATP and 15 mM $MgSO_4$ and subjected to a luminescence assay. D-Luciferin (100 μl of 1 mM concentration) was automatically injected into the sample in the luminometer (model LE9501; Berthold Lumat Co. Ltd.), and the intensity of luminescence was measured. Luciferase activity was expressed in terms of the luminescence intensity (relative light units per minute per microgram of total protein).

Polymerase chain reaction. RNA samples for polymerase chain reaction analysis were prepared by the guanidinium thiocyanate method (26). Polymerase chain reaction analysis of reverse-transcribed RNA was done in a 25-μl reaction mixture for 30 cycles (94°C for 1 min, 55°C for 2 min, 72°C for 3 min) with primer nucleotide pairs corresponding to the following nucleotide positions: mouse c-*fyn*, 1546 to 1565 (sense strand), 1777 to 1796 (antisense strand); mouse c-*lyn*, 1475 to 1494 (sense strand), 1740 to 1759 (antisense); mouse c-*fgr*, 1336 to 1354 (sense strand), 1704 to 1725 (antisense strand); mouse c-*src*, 1292 to 1312 (sense strand), 1549 to 1568 (antisense strand); human c-*lck*, 1556 to 1577 (sense strand), 1199 to 1218 (antisense strand); mouse c-*hck*, 1367 to 1386 (sense strand), 1654 to 1673 (antisense strand); and mouse c-*yes*, 1384 to 1404 (sense strand), 1801 to 1820 (antisense strand). A control reaction in which reverse transcriptase was omitted was performed for each RNA sample. Amplified fragments were detected by staining with ethidium bromide in a 5% polyacrylamide gel.

Analysis of protein tyrosine phosphorylation. After FCS depletion for 16 h, parental NIH 3T3 cells or NIH 3T3GMRα/β cells were detached with 2 mM EDTA in PBS, washed twice with DMEM, and resuspended at 10^6 cells per ml in DMEM containing 0.1% BSA. The cells (2×10^5 cells per sample) were then stimulated with 6-ng/ml human basic fibroblast growth factor (hbFGF), 10-ng/ml hGM-CSF, or control buffer at 37°C for 5 min; this incubation was terminated by the addition of 75 volumes of ice-cold PBS. The cells were precipitated and solubilized for 45 min at 4°C in 200 μl of 0.5% Nonident P-40–50 mM HEPES (pH 7.4)–100 mM sodium fluoride–10 mM sodium PP_i–2 mM sodium orthovanadate–4 mM EDTA–2 mM phenylmethylsulfonyl fluoride–100 kIU of aprotinin per ml–2 μg of leupeptin per ml. The cell lysates were microcentrifuged for 15 min at 15,000 rpm, and 100 μl of the resulting supernatant was made 4% in SDS, 5% in 2-mercaptoethanol, and 10% in glycerol and boiled for 2 min. The proteins were separated on an SDS-7.5% polyacrylamide gel and electrotransferred to Immobilon polyvinylidene difluoride membranes (Millipore) for 2 h at 4°C in 10 mM 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS) (pH 10)–10% methanol. The membrane was blocked for 2 h in 5% BSA–20 mM Tris-HCl, (pH 7.5)–0.15 M sodium chloride (TBST) and incubated at 2°C for 2 h with 1 μg of 4G10 antiphosphotyrosine per ml in TBST. The blot was washed at 3°C for 5 min with TBST before incubation with 1:2,000 horseradish peroxidase-conjugated goat anti-mouse immunoglobulin (Amersham) for 1 h. After being washed as before, the blot was developed with the enhanced chemiluminescence system (Amersham) according to the manufacturer's instructions.

RESULTS

Activation of c-*fos* promoter by hGM-CSF in NIH 3T3 fibroblasts expressing hGM-CSFR α/β chains. The action of GM-CSF is species specific between mice and humans (25). This species-specific nature of the GM-CSF system was used to assess the function of GM-CSFR reconstituted in various cell types. We reported that the cotransfection of BA/F3 cells with hGM-CSFR cDNAs encoding α (low-affinity) chain and β chain (KH97) yielded hGM-CSFR with both high (120 pM)- and low (6.6 nM)-affinity binding sites (20). The hGM-CSFR reconstituted in BA/F3 cells was able to transduce growth signals. Similarly, hGM-CSF supported the proliferation of mouse CTLL2 cells expressing high-affinity hGM-CSFR (20). Also, we previously showed that introduction of hGM-CSFR α- and β-chain cDNAs in mouse NIH 3T3 cells resulted in the exhibition of the high (170 pM)-affinity GM-CSF binding site (NIH 3T3GMRα/β) (15). However, it was not clear whether GM-CSFR reconstituted in fibroblasts requires additional components which are present in cells of hematopoietic lineage or T lymphocytes to transduce growth signals. The c-*fos* mRNA is rapidly and transiently induced by IL-3 in the mIL-3-dependent cell lines IC2 (46) and BA/F3 (13). To monitor the function of the reconstituted receptor, we performed a transient transfection assay of the c-*fos* promoter with luciferase as a reporter gene. BA/F3 cells expressing hGM-CSFR α and β chains (BA/F3GMRα/β) were transfected with the c-*fos* promoter-luciferase plasmid and incubated for 24 h with complete medium. After 5 h of depletion of IL-3, cells were stimulated for a further 5 h with either hGM-CSF or mIL-3, harvested, and assayed for luciferase activity. As expected, mIL-3 (20 ng/ml) activated the c-*fos* promoter approximately fourfold (329 ± 20 relative to a luciferase activity of 100 with no stimulus [n = 3 ± standard deviation]) as assessed by the increase of luciferase activity. Consistent with the previous

TABLE 1. Activation of c-*fos* promoter and stimulation of [^{3}H]thymidine incorporation by hGM-CSF in NIH 3T3GMRα/β cells[a]

Stimulus	Relative luciferase activity[b]	[^{3}H]thymidine incorporation (cpm)[c]
None	100	6,927 ± 1,058
FCS[d]	265 ± 19	136,545 ± 7,813
hGM-CSF[e]	459 ± 65	25,565 ± 3,990
hEGF[f]	204 ± 48	51,165 ± 7,694
hGM-CSF + FCS	574 ± 16	109,150 ± 10,096
hGM-CSF + hEGF	530 ± 51	48,748 ± 3,132

[a] These experiments were performed three times, giving essentially the same results.
[b] Luciferase activity is expressed relative to that of unstimulated samples, and values represent the average of three independent experiments with standard deviation.
[c] The amount of ^{3}H radioactivity incorporated is the average result of eight samples with standard deviation.
[d] 15%.
[e] At 10 ng/ml.
[f] At 20 ng/ml.

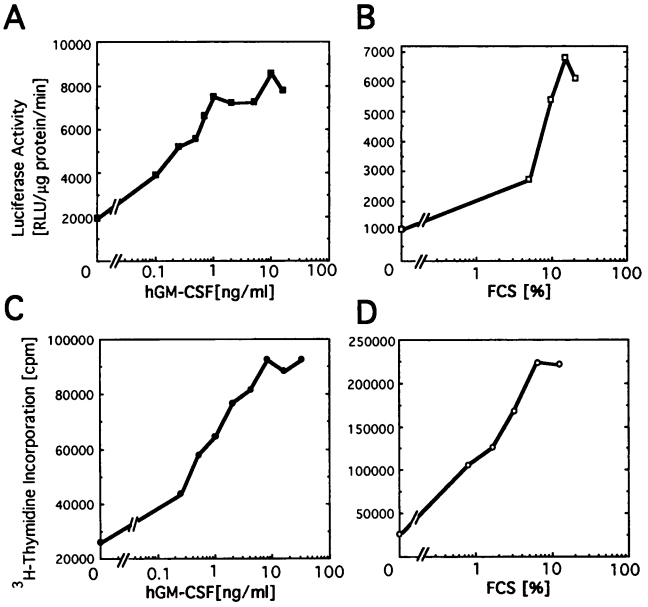

FIG. 1. Effects of hGM-CSF or FCS on c-*fos* promoter activity and DNA synthesis of NIH 3T3 cells expressing high-affinity hGM-CSFR. Activation of c-*fos* promoter by hGM-CSF (A) or FCS (B) in NIH 3T3GMRα/β cells. c-*fos* promoter-luciferase plasmid (5 μg) was transfected into NIH 3T3GMRα/β cells (4×10^6) by electroporation as described in Materials and Methods. After overnight incubation in medium containing 10% FCS, cells were first depleted of FCS for 24 h. Following incubation for 5 h in the presence of various concentrations of either FCS or hGM-CSF, cells were harvested and subjected to the luciferase assay. These experiments were performed twice and yielded essentially the same results. Luciferase activity, represented as luminescence intensity (RLU is relative light unit), is the average of duplicate samples. Initiation of DNA synthesis by hGM-CSF (C) or FCS (D) in NIH 3T3GMRα/β cells was measured by the amount of [^{3}H]thymidine incorporated as described in Materials and Methods. After depletion of FCS, either hGM-CSF (final concentration, 10 ng/ml) (C) or FCS (final concentration, 10%) (D) was added, and incubation was continued for an additional 24 h. The amount of [^{3}H]thymidine radioactivity incorporated is the average result of eight samples.

data that hGM-CSFR is functional in BA/F3 cells, hGM-CSF (20 ng/ml) also stimulated the c-*fos* promoter about fourfold (386 ± 17 relative to a luciferase activity of 100 with no stimulus [n = 3 ± standard deviation]). A control plasmid without the c-*fos* promoter did not respond to hGM-CSF (data not shown). Likewise, the activity of the c-*fos* promoter was monitored by transfecting NIH 3T3GMRα/β cells with the c-*fos* promoter-luciferase plasmid. Transfected NIH 3T3GMRα/β cells were depleted of FCS for 24 h and stimulated with FCS, hGM-CSF, or hEGF. As shown in Table 1, the c-*fos* promoter in NIH 3T3 cells was activated by these stimuli. hGM-CSF stimulation of c-*fos* promoter activity was neither additive nor synergistic with FCS or hEGF stimulation. To determine whether this activity is dependent on the c-*fos* promoter, we transfected cells with a luciferase plasmid lacking the c-*fos* promoter. Neither hGM-CSF nor FCS stimulated the luciferase activity of this control plasmid (data not shown). Activation of the c-*fos* promoter is dependent on the dose of either hGM-CSF (Fig. 1A) or FCS (Fig. 1B). These results indicated that hGM-CSFR is functional in fibroblasts. The maximum activity was achieved at 1 ng of GM-CSF per ml (~50 pM), suggesting that the high-affinity form of GM-CSFR is responsible for activation of the c-*fos* promoter.

Both α and β chains of hGM-CSFR are required to activate c-*fos* promoter. In the mouse system, two distinct but closely related IL-3R β chains, AIC2A and AIC2B, have been found (11, 18). AIC2A associates with the IL-3R α chain only, whereas AIC2B is able to interact with the α chains of IL-3R, GM-CSFR, and IL-5R (7, 12, 20, 42). We previously reported that proliferation of BA/F3 cells transfected with hGM-CSFR α chain alone was stimulated with at high dose (>10 ng/ml) of hGM-CSF (20). In contrast, hGM-CSF failed to stimulate CTLL2 expressing the hGM-CSFR α chain. It was hypothesized that AIC2B, which is expressed in BA/F3 cells but not in CTLL2, interacted with hGM-CSFR α chain and transduced signals in response to high concentrations of hGM-CSF. To examine whether both α and β chains of hGM-CSFR are required to transduce signals, the α or β chain of the receptor cDNA clone either alone or in combination and c-*fos* promoter-luciferase were introduced into NIH 3T3 cells by a transient cotransfection protocol (Table 2). Luciferase activity from the c-*fos* promoter was markedly stimulated upon the addition of hGM-CSF when both α and β chains of hGM-CSFR were used. However, luciferase activity cotransfected with either α or β chain alone showed no detectable change by the addition of GM-CSF compared to the basal level. Luciferase activity was increased dose dependently on both α- and β-chain cDNAs. When excess α-chain cDNA (10 μg) was cotransfected, the β-chain cDNA requirement for half-maximum activity was less than 0.2 μg/ml and almost saturated at 1 μg/ml (Fig. 2A). Similarly, when the saturating level of β-chain DNA (10 μg) was used, half-maximum activity was achieved by the α-chain cDNA at about 0.2 μg/ml (Fig. 2B). These results clearly indicated that both the α and β chains of the receptor are required to activate the c-*fos* promoter in response to hGM-CSF in NIH 3T3 cells. By using a transient transfection system, we examined whether the c-*fos* promoter was activated by hGM-CSF in mouse P19 embryonic carcinoma cells. As shown in Table 2, the c-*fos* promoter was also activated by hGM-CSF in P19 cells transfected with hGM-CSFR α- and β-chain cDNAs. Both α- and β-chain cDNAs were also required in P19 cells. The c-*fos* promoter was markedly stimulated by hGM-CSF when both α and β chains of the

TABLE 2. Activation of c-*fos* promoter by hGM-CSF via transiently expressed hGM-CSFR α and β chains on NIH 3T3 and P19 cells[a]

Plasmid[b]	hGM-CSF[c]	Relative luciferase activity	
		NIH 3T3	P19
pME18 + pCEV4	−	100	100
pME18 + pCEV4	+	84 ± 12	84 ± 13
hGM-CSFR α + pME18	+	117 ± 11	101 ± 19
hGM-CSFR β + pCEV4	+	92 ± 12	91 ± 32
hGM-CSFR α,β	+	698 ± 37	500 ± 116

[a] Cells were cotransfected with the c-*fos* promoter-luciferase plasmid and the hGM-CSFR α and/or β cDNA plasmid or vectors without a cDNA insert (pME18, pCEV4) (18) as control plasmids. Experiments were performed three times with essentially the same results. Luciferase activity is expressed relative to that of unstimulated sample. Values represent the average of three independent experiments with standard deviation.
[b] DNA cotransfected with the c-*fos* promoter-luciferase plasmid.
[c] At 20 ng/ml.

hGM-CSFR were transfected. However, luciferase activity was not increased with either α or β chain alone by the addition of hGM-CSF (Table 2).

Effects of hGM-CSF on activation of endogenous immediate-early response genes in NIH 3T3GMRα/β fibroblasts. To examine whether hGM-CSFR can also induce the endogenous c-*fos* gene and other immediate-early response genes, we performed Northern blot analysis. To deplete either mIL-3 or FCS, we incubated cells expressing the high-affinity hGM-CSFR for 5 h without mIL-3 (for BA/F3GMRα/β) or with 0.5% FCS for 24 h (for NIH 3T3GMRα/β cells), respectively. After the addition of mIL-3, hGM-CSF, or FCS, total RNA was extracted at various time points and mRNAs of immediate-early response genes were analyzed (Fig. 3). In NIH 3T3 cells, c-*fos* mRNA was not detectable, but basal levels of c-*jun* and c-*myc* mRNAs were detectable without stimulation. After the addition of hGM-CSF, c-*fos* and c-*jun* transcripts appeared immediately and reached the maximum within 30 min and then declined quickly (Fig. 3A). In contrast, c-*myc* transcript reached the maximum after 60 min and was present at least for 2 h. The kinetics of induction by hGM-CSF in fibroblasts was very similar to that in 3T3GMRα/β cells by FCS or in BA/F3GMRα/β cells by mIL-3 or hGM-CSF (Fig. 3B). Induction of these genes was not observed in parental NIH 3T3 cells treated with hGM-CSF (data not shown).

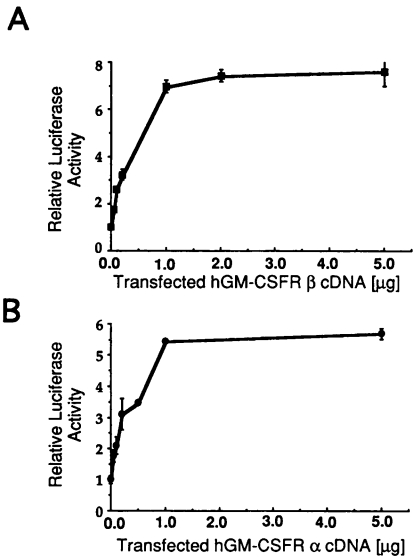

FIG. 2. Dependence of c-*fos* promoter activation by hGM-CSF on the expression of the amount of hGM-CSFR α and β chains. (A) To titrate hGM-CSFR β chain, we transfected NIH 3T3 cells (4×10^6) with 5 μg of c-*fos* promoter-luciferase plasmid, 10 μg of hGM-CSF α-chain DNA, and various amounts of hGM-CSFR β-chain DNA as described in Materials and Methods. (B) Titration of hGM-CSFR α chain was performed in the same way except that 10 μg of hGM-CSF β-chain DNA and various amounts of hGM-CSFR α-chain DNA were used. After 5 h of stimulation, cells were harvested and luciferase activity was determined. These experiments were performed twice and gave essentially the same results. Luciferase activity expressed relative to that of either hGM-CSF α-chain DNA alone (A) or hGM-CSFR β-chain DNA alone (B) is the average of duplicate samples. Standard deviation is shown as error bars.

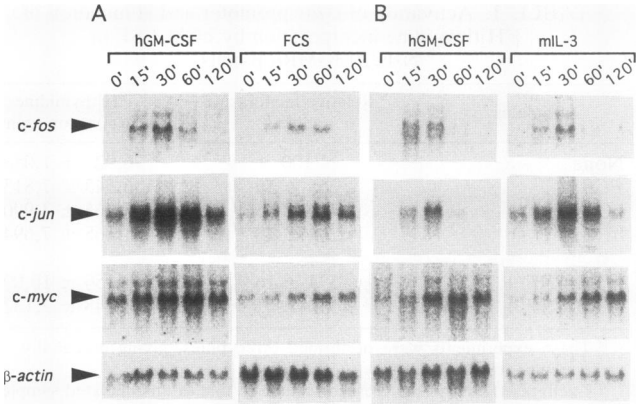

FIG. 3. Factor-dependent activation of endogenous immediate-early response genes in BA/F3 or NIH 3T3 cells expressing high-affinity hGM-CSFR. FCS-depleted NIH 3T3GMRα/β cells (A) or factor-depleted BA/F3GMRα/β cells (B) (prepared as described in Materials and Methods) were stimulated with mIL-3 (10 ng/ml), hGM-CSF (10 ng/ml), or FCS (10%). At the times indicated (minutes), cells were harvested and total RNA was analyzed by Northern blotting as described in Materials and Methods. The positions of c-*fos*, c-*jun*, c-*myc*, and β-actin mRNAs are indicated.

Tyrosine phosphorylation induced by hGM-CSF in NIH 3T3GMRα/β fibroblasts. Both IL-3 and GM-CSF stimulate tyrosine phosphorylation of several cellular proteins in hematopoietic cells. We examined the pattern of tyrosine phosphorylation following treatments with hGM-CSF by Western blotting (immunoblotting) analysis using antiphosphotyrosine antibody (Fig. 4). hGM-CSF stimulated tyrosine phosphorylation of several proteins (140, 90, and 50 kDa) in NIH 3T3GMRα/β cells but not in parental NIH 3T3 cells. In contrast, hbFGF induced phosphorylation of several cellular proteins in both cell types regardless of the expression of hGM-CSFR.

Effect of hGM-CSF on initiation of DNA synthesis in NIH 3T3GMRα/β fibroblasts. The effect of hGM-CSF on DNA synthesis in NIH 3T3 cells carrying the high-affinity hGM-CSFR was determined (Table 1; Fig. 1C and D). Cells plated in microtiter plates were incubated in DMEM without FCS. After 24 h, various amounts of hGM-CSF or FCS were added and incubation was continued for an additional 24 h. During the last 2 h, the cells were pulsed with [^{3}H]thymidine. hGM-CSF, FCS, or hEGF stimulated [^{3}H]thymidine incorporation, and neither additive nor synergistic effects were

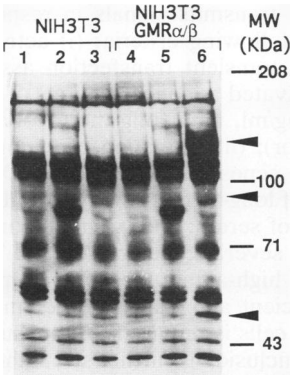

FIG. 4. Protein tyrosine phosphorylation induced by hGM-CSF in fibroblasts. NIH 3T3 (parental) cells or NIH 3T3GMRα/β (GMR) cells depleted for FCS were stimulated by hGM-CSF and analyzed by Western blotting with antiphosphotyrosine antibody as described in Materials and Methods. Stimuli were as follows: lanes 1 and 4, none; lanes 2 and 5, hbFGF; lanes 3 and 6, hGM-CSF. Arrowheads indicate the tyrosine-phosphorylated proteins specific to hGM-CSF stimulation.

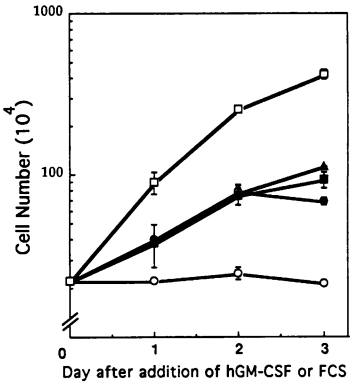

FIG. 5. Growth of NIH 3T3GMRα/β cells in response to hGM-CSF. NIH 3T3GMRα/β cells incubated for 24 h in Opti MEM were plated with various concentrations of hGM-CSF as indicated by the symbols: none (○), 5 ng/ml (●), 50 ng/ml (▲), or 150 ng/ml (■). As a control, cells were plated with 10% FCS (□). Cells were cultured and numbers of viable cells were counted every 24 h as indicated. These experiments were performed twice with essentially the same results. Cell numbers are the average of duplicate samples. Standard deviation is shown as error bars.

observed by combining hGM-CSF with other stimuli (Table 1). As shown in Fig. 1C, hGM-CSF stimulated [^{3}H]thymidine incorporation in a dose-dependent manner. Maximum and half-maximum activities were achieved at around 8 and 0.5 ng of GM-CSF per ml, respectively, levels slightly higher than those required for activation of the c-*fos* promoter. FCS also stimulated DNA synthesis in a dose-dependent manner (Fig. 1D). However, in contrast to GM-CSF, the amount of FCS required to induce DNA synthesis was much lower than that for activation of the c-*fos* promoter.

Long-term growth of NIH 3T3GMRα/β cells by hGM-CSF. NIH 3T3 cells survive but do not proliferate in DMEM containing low concentrations of FCS (<0.5%) or essential media such as Opti MEM. We examined whether hGM-CSF not only induces DNA synthesis but also supports long-term cell growth. NIH 3T3GMRα/β cells transferred to Opti MEM for 24 h were incubated with various concentrations of hGM-CSF or 10% FCS, and the number of cells was counted every 24 h (Fig. 5). NIH 3T3GMRα/β cells in Opti MEM only were unable to proliferate but survived under this condition. hGM-CSF supported long-term proliferation and increased the cell number of NIH 3T3GMRα/β cells, but the extent of proliferation was much less than that supported by FCS. hGM-CSF required for long-term proliferation was 5 ng/ml, a value similar to that was determined for DNA synthesis, and excess hGM-CSF (either 50 or 150 ng/ml) did not augment cell growth further. In contrast, hGM-CSF at 300 ng/ml in DMEM alone failed to support survival or proliferation of NIH 3T3GMRα/β cells (data not shown), whereas the same concentration of hGM-CSF supported cell proliferation for more than 4 days in DMEM containing 0.5% FCS (data not shown). These results indicated that, beside hGM-CSF, additional components (such as transferrin and insulin) present in low concentrations of FCS are required to support the proliferation of NIH 3T3GMRα/β cells.

Internalization of reconstituted high-affinity GM-CSFR. In the IL-2R system, the high-affinity IL-2R or the β chain of the IL-2R is rapidly internalized after binding of IL-2 (9). Similarly, GM-CSF and IL-3 were known to be internalized after binding to their high-affinity receptors in hematopoietic cells. We examined whether the high-affinity hGM-CSFR reconstituted in fibroblasts could be internalized after the interaction with its ligand. As a control, we used TF-1, a factor-dependent human myeloid cell line from which the α and β chains of the GM-CSFR and IL-3R cDNAs were cloned. As shown in Fig. 6A, ^{125}I-hGM-CSF on TF-1 was

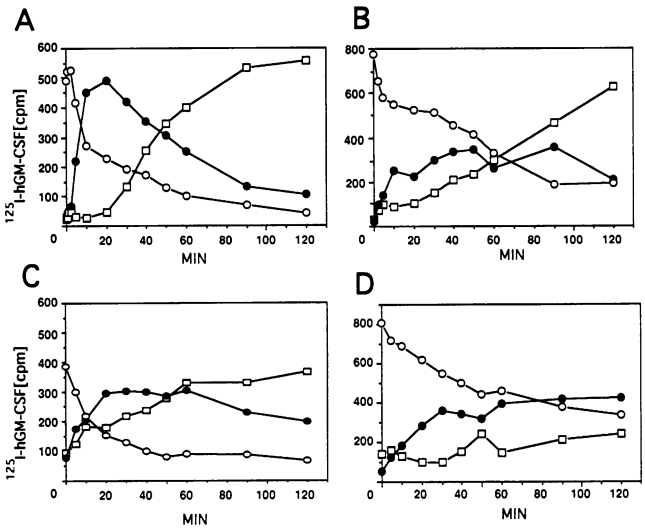

FIG. 6. Internalization of hGM-CSF bound to TF-1 or NIH 3T3GMRα/β cells. Internalization of ^{125}I-hGM-CSF bound to GM-CSF-dependent human TF-1 cells in the absence (A) or presence (C) of 0.2% sodium azide or NIH 3T3GMRα/β cells in the absence (B) or presence (D) of 0.2% sodium azide was measured as described in Materials and Methods. Symbols: ●, ^{125}I-hGM-CSF internalized; ○, membrane-bound ^{125}I-hGM-CSF; □, ^{125}I-hGM-CSF dissociated from the cells. These experiments were performed three times with essentially the same results. The amount of ^{125}I radioactivity in counts per minute is the average of duplicate samples and represents the specific binding which is inhibited by excess nonradioactive hGM-CSF. Control experiments showed that total as well as specific binding of radioactive hGM-CSF to NIH 3T3 cells expressing GM-CSFR β chain alone is negligible (data not shown). Likewise, the amount of membrane-bound and acid-resistant radioactivity was undetectable (data not shown).

internalized within 10 min following binding of hGM-CSF. In contrast, ^{125}I-hGM-CSF binding to the high-affinity hGM-CSFR reconstituted on NIH 3T3GMRα/β cells became gradually acid resistant, albeit at a lower level than that of TF-1 cells (Fig. 6B). Binding of radioactive hGM-CSF to either TF-1 or NIH 3T3GMRα/β cells is specific and receptor mediated because the addition of excess nonradioactive hGM-CSF abolished the binding (data not shown). The internalization process has been known to be energy dependent and is blocked by sodium azide, an inhibitor of cytochrome *c* oxidase and mitochondrial H^+-ATPase. To determine whether the event observed in NIH 3T3GMRα/β cells is truely an internalization or due to another process such as endocytosis, we used sodium azide to block energy metabolism of TF-1 and NIH 3T3GMRα/β cells. As expected, sodium azide inhibited rapid internalization of GM-CSFR in TF-1 cells (Fig. 6A and C), whereas the patterns of the internalization process were not affected by sodium azide in NIH 3T3GMRα/β cells (Fig. 6B and D). In TF-1 cells, 65% (Fig. 6A) and 45% (Fig. 6C) of total counts were internalized at 20 min in the absence or presence of sodium azide, respectively. These results indicated that in TF-1 cells, some component of the internalization process is sensitive to sodium azide and a separate component is azide resistant. In NIH 3T3GMRα/β cells, however, 27% (Fig. 6B, without sodium azide) and 29% (Fig. 6D, with sodium azide) of total counts were internalized. These results suggested that the apparent and slow internalization of hGM-CSF in NIH 3T3GMRα/β cells and the azide-resistant component in TF-1 cells is not a true internalization process and is probably due to nonspecific endocytosis of the ligand complexed with GM-CSFR. Furthermore, it also argues against the role of internalization in signal transduction via GM-CSFR.

DISCUSSION

Proliferation of mammalian cells is controlled by various growth factors which exert their functions via their specific receptors. Fibroblasts or epithelial cells grow in response to EGF, platelet-derived growth factor, or FGF, whereas growth of hematopoietic cells is supported by various cytokines or colony-stimulating factors such as IL-2, IL-3, and GM-CSF. In general, a functional receptor is divided into several components: a ligand binding unit, a transducer, an effector, and a regulatory unit (30). In contrast to growth factor receptor, the ligand binding unit of several cytokine receptors is composed of multiple subunits and their cytoplasmic domains have no tyrosine kinase activity. The distinct growth requirements among cells of different lineages or differentiation stages, in many instances, are due to the difference in the cell's ability to respond to particular growth factors. This ability is determined primarily by the expression of the ligand binding unit or the signal-transducing components of the growth factor receptors. Hematopoietic cells and fibroblasts selectively express different receptors such as GM-CSFR and EGF receptor. However, it was not clear whether these cellular systems share the same, if not identical, signal-transducing components associated with the receptors. This can be tested by forced expression of the ligand binding unit of the receptor in cells of different lineages which normally do not express this receptor. In this study, we examined the functions of GM-CSFR in fibroblasts which normally do not express this receptor. The results described in this report unequivocally demonstrated that the high-affinity hGM-CSFR reconstituted on mouse fibroblasts by transfection of α- and β-chain cDNA clones is functional and transmits signals in response to hGM-CSF based on the following criteria: (i) activation of the c-*fos* promoter in a transient transfection assay (the c-*fos* promoter was activated at concentrations of hGM-CSF of even less than 0.2 ng/ml, indicating the involvement of the high-affinity receptor); (ii) activation of endogenous immediate-early response genes; (iii) induction of DNA synthesis; (iv) maintenance of long-term growth in combination with a low concentration of serum; and (v) induction of tyrosine phosphorylation of several cellular proteins. These results indicated that the high-affinity receptor composed of α and β chains is sufficient and that no other molecule specific to hematopoietic cells is required to transduce signals in fibroblasts. This conclusion is further strengthened by the finding that hGM-CSFR is also functional in mouse P19 embryonic carcinoma cells. By using soft agar assays, we also examined whether or not hGM-CSF can support anchorage-independent proliferation of NIH 3T3GMRα/β cells. Cells were cultured in 0.33% soft agar in the presence of various concentrations of hGM-CSF (10 to 100 ng/ml) in either DMEM containing 10% FCS or Opti MEM, and colony formation was monitored for 4 weeks. Under these conditions, unlike NIH 3T3 cells carrying *erbB2*, NIH 3T3GMRα/β cells failed to form any detectable colonies in soft agar. The combination of hGM-CSF and hEGF also did not support colony formation of NIH 3T3GMRα/β cells (data not shown). These observations raise several interesting questions regarding the mechanisms by which GM-CSFR transduces signals in hematopoietic cells and in other types of cells such as fibroblasts. (i) Does GM-CSFR in fibroblasts and in hematopoietic cells utilize the same signal transduction pathway or does it operate through a different mechanism? (ii) Does GM-CSFR in fibroblasts utilize a common signal transduction pathway normally used by growth factor receptors such as EGF or platelet-derived growth factor receptors or does it utilize a different pathway?

In BA/F3GMRα/β cells, mIL-3 and hGM-CSF induced several immediate-early response genes in a manner and with kinetics similar to hGM-CSF-dependent induction of the same genes in NIH 3T3GMRα/β cells. These observations suggest that the signal transduction pathway to which GM-CSFR is linked in BA/F3GMRα/β cells is similar to that in NIH 3T3GMRα/β cells, including the involvement of tyrosine kinase. There are several possibilities concerning the nature of the tyrosine kinase associated with GM-CSFR signalling. (i) GM-CSFR cooperates with a specific tyrosine kinase common to many GM-CSF-dependent cells regardless of cell type. (ii) GM-CSFR cooperates with several tyrosine kinases in a cell-type specific manner, i.e., it interacts with a unique kinase in one cell type yet is capable of interacting with another unique kinase in another cell type. (iii) Cooperation of GM-CSFR and tyrosine kinase is neither unique nor cell-type specific, i.e., the combination of GM-CSFR and a tyrosine kinase is versatile and differs from cell to cell. Several lines of evidence indicated that the first possibility is unlikely and that tyrosine kinases involved in GM-CSFR or IL-3R signalling of BA/F3 and NIH 3T3 cells appear not to be identical. First, polymerase chain reaction analysis indicated that no *src* family kinase was detected common to IL-3/GM-CSF-dependent hematopoietic cell lines (such as BA/F3, IC2, MC9, and TF-1), P19, and NIH 3T3 cells (data not shown). This may be analogous to the situation with IL-2-mediated signal transduction in which involvement of *lck*, the lymphoid-specific kinase, has been implicated in one cell (14, 16), whereas *lyn* (45) has been suggested in another cell line. It is also possible that GM-

CSFR cooperates with a tyrosine kinase other than *src* family kinase. Second, the pattern of protein tyrosine phosphorylation induced by hGM-CSF is different between BA/F3 and NIH 3T3 cells. In NIH 3T3GMRα/β cells, major tyrosine phosphorylation occurred at three proteins with apparent molecular masses of 140, 90, and 50 kDa. The most prominent 120-kDa protein is likely hGM-CSFR β chain itself. In BA/F3GMRα/β cells, the major tyrosine phosphorylation induced by mIL-3 or hGM-CSF (40) occurred at a 95-kDa protein. It is not clear at present whether the difference in tyrosine phosphorylation pattern between NIH 3T3 and BA/F3 cells is due to the involvement of different tyrosine kinases or substrates, or both. The indication that GM-CSFR in NIH 3T3 and BA/F3 cells operates through different mechanisms also comes from internalization experiments. Previous works revealed that tyrosine phosphorylation occurred in the absence of receptor internalization in M-CSF and GM-CSF receptor systems (34, 41). Our data with NIH 3T3 cells suggest that energy-dependent internalization is not essential not only for tyrosine phosphorylation but also for signal transduction via GM-CSFR leading to induction of early response genes and cell proliferation.

In the present study, in addition to the cell lines which stably express the transfected GM-CSFR cDNA, we used transient transfection assays of the α and β chains of GM-CSFR in NIH 3T3 cells. The advantage of this system over the stable transfection protocol is that one can monitor the function of transfected receptor cDNA clones relatively free from secondary phenotypic changes caused during establishment of stable cell lines. Furthermore, it is easy to manipulate the ratio of α and β chains by controlling the amounts of DNA transfected, and many receptor cDNA mutants may be quickly and easily analyzed. By using the transient transfection assay in COS7 and NIH 3T3 cells, we have shown that both α and β chains are required to form a high-affinity receptor (15). In this report, we extended these observations and showed that both α and β chains of GM-CSFR are required for activation of the c-*fos* promoter. With this system, requirements for cytoplasmic domains of α and β chains were examined in NIH 3T3 fibroblasts. The mutant β-receptor cDNA which almost completely lacks the cytoplasmic domain failed to stimulate the c-*fos* promoter as expected (data not shown). Interestingly, a similar deletion mutant of the α-chain receptor was also inactive, indicating that cytoplasmic domains of both α and β chains contributed to form a functional receptor capable of activating the c-*fos* promoter.

In the IL-2R system, the high-affinity receptor established by transfecting α- and β-chain cDNAs in fibroblasts failed to transduce growth-promoting signals (14, 29). This led to the proposal that the high-affinity IL-2R must associate with an additional component(s) specific to hematopoietic cells to transduce signals to the downstream area. In this report, we showed that the reconstituted high-affinity hGM-CSFR on fibroblasts transduces growth-promoting signal and induce several early response genes in response to GM-CSF. Further work is necessary to characterize the structure and function of each component of GM-CSFR and the mechanism by which GM-CSFR transduces its signals. The fibroblast assay system described in this report should help to shed more light on the mode of action of GM-CSFR.

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