To identify the functional domains of the human thrombopoietin (TPO) receptor essential for proliferation and megakaryocytic differentiation, we introduced human wild type c-mpl cDNA and deletion mutants of c-mpl cDNA into the human erythropoietin (EPO)-dependent cell line UT-7/EPO that does not express endogenous c-Mpl. TPO induced the proliferation and megakaryocytic differentiation of UT-7/EPO expressing wild type c-Mpl, as evidenced by increased levels of the CD41 antigen specific for cells of the megakaryocytic lineage and by changes in morphology. Mutational analysis of the cytoplasmic domain of c-Mpl identified four functional regions: (a) two C-terminal regions (amino acids 575–586 and 615–630) containing a domain essential for cell proliferation and megakaryocytic differentiation but not for DNA synthesis; (b) a region (amino acids 587–614) containing a negative domain for TPO-tiation but not for DNA synthesis; (c) a region (amino acids 565–574) including a box2 motif that is required for DNA synthesis. These deletion mutants will provide useful materials for analyzing the signals specific for TPO-induced proliferation and megakaryocytic differentiation.
the signals for proliferation and megakaryocytic differentiation of the cells (Fig. 2A). We selected neomycin-resistant clones expressing high levels of c-Mpl on the surface of the cells by flow cytometry with a polyclonal antibody against the extracellular domain of c-Mpl (Fig. 2B) and designated them UT-7/EPO-MplWT.

Effect of TPO on the Proliferative Response of UT-7/EPO-MplWT—Thymidine incorporation assay revealed that the growth activity of TPO toward UT-7/EPO-MplWT in short-term culture was almost similar to that of EPO (Fig. 3A). However, when the cell number was assessed over longer periods, the peak of growth was slightly reduced in the presence of TPO (Fig. 3B). UT-7/EPO-MplWT cells could be maintained in the presence of TPO alone for at least 3 months, whereas the parent cells could not (data not shown).

Effect of TPO on the Megakaryocytic Differentiation of UT-7/EPO-MplWT—We examined whether or not TPO induces megakaryocytic differentiation of UT-7/EPO-MplWT cells. As shown in Fig. 4, the mean intensity of GPIIb-IIIa antigens on the surface was increased after an incubation with TPO for 7 days. Consistent with this, some cells of TPO-stimulated UT-7/EPO-MplWT became much larger than EPO-stimulated UT-7/EPO-MplWT cells (Fig. 5). These observations indicated that c-Mpl protein expressed exogenously in UT-7/EPO can transduce signals for not only proliferation but also for megakaryocytic differentiation. Therefore, this system would be useful for identifying the functional domains of cytoplasmic c-Mpl.

Preparation of c-Mpl Deletion Mutants—To identify the regions of the receptor required for proliferation and megakaryocytic differentiation, a series of deletion mutants of the intracellular domain of c-Mpl were prepared as shown in Fig. 2A and introduced into UT-7/EPO cells by electroporation. Stable transfectants were isolated after neomycin selection and designated UT-7/EPO-Mpl∆5, UT-7/EPO-Mpl∆21, UT-7/EPO-Mpl∆49, UT-7/EPO-Mpl∆61, UT-7/EPO-Mpl∆box2, UT-7/EPO-Mpl∆81, UT-7/EPO-Mpl∆95, UT-7/EPO-Mpl∆box1, and UT-7/EPO-Mpl∆120. We picked up a single colony from a methyl cellulose semisolid medium (18) and transferred it to liquid medium containing 1 unit of EPO/ml. In the following experiments, we analyzed at least three independent clones. FACs analysis using anti-c-Mpl antibody revealed that each clone expressed significant levels of exogenous c-Mpl on the surface of the cells (Fig. 2B).

Effect of TPO on the Proliferative Response of the Transfectants—We examined whether or not TPO stimulates the proliferative response of the transfectants. The thymidine incorporation assay revealed that TPO induced DNA synthesis in UT-7/EPO-Mpl∆5, UT-7/EPO-Mpl∆21, UT-7/EPO-Mpl∆49, and UT-7/EPO-Mpl∆61 cells in a dose-dependent manner (Fig. 3A). In contrast, UT-7/EPO-Mpl∆box2, UT-7/EPO-Mpl∆81, UT-7/EPO-Mpl∆95, UT-7/EPO-Mpl∆box1, and UT-7/EPO-Mpl∆120 did not respond to TPO at even high concentrations (Fig. 3A). These results indicate that the region (amino acids 565–574) including the box2 motif is required for EPO DNA synthesis. This finding is in accord with other published results (11).

To examine whether or not TPO-induced DNA synthesis leads to cell proliferation, we cultured the transfectants in the presence of TPO in liquid culture for several days. TPO alone sustained the survival and long-term proliferation of UT-7/EPO-Mpl∆5 and UT-7/EPO-Mpl∆49, but not of the other transfectants (Fig. 3B and data not shown). These results indicate that two C-terminal regions (amino acids 575–586 and 615–630) contain a domain essential for cell proliferation but not for DNA synthesis. This finding suggests that DNA synthesis does not always lead to cell proliferation. Alternatively, additional
events besides DNA synthesis may be required to induce cell proliferation.

**Effect of TPO on the Megakaryocytic Differentiation of Transfectants**—To identify the functional domain(s) involved in megakaryocytic differentiation, we examined whether or not TPO can induce an increase in the intensity of the megakaryocytic markers in the transfectants. This was assessed by flow cytometry with an anti-GPIIb-IIIa antibody (CD41). When UT-7/EPO-MplΔ5 and UT-7/EPO-MplΔ49 cells were cultured with TPO (10 ng/ml) for 7 days, these cells increased the expression of CD41 antigens (Fig. 4). However, when others were cultured with TPO alone, most of the cells died within a few days.

To sustain the survival of these transfectants, we cultured the transfectants in medium containing both TPO (10 ng/ml) and EPO (1 unit/ml) for 7 days, then harvested the transfectant cells for flow cytometry with the CD41 antibody. The transfectant cells cultured with 1 unit/ml of EPO alone served as the negative control. The expression of CD41 antigens significantly

![Diagram of Functional Domains of the Human Thrombopoietin Receptor]

**FIG. 2.** Introduction of human c-mpl wild type and its deletion mutants into UT-7/EPO cells. A, a schematic illustration of human c-mpl wild type cDNA and its deletion mutants. B, flow cytometric analysis of the c-Mpl expressed on the surface of the cells transfected with c-mpl cDNAs described above. Thin lines correspond to staining with fluorescein isothiocyanate-labeled second antibody alone. The results are representative of three independent clones.
increased on UT-7/EPO-Mpl WT and UT-7/EPO-MplΔ49, indicating that these transfectants were induced to megakaryocytic differentiation by TPO even in the presence of EPO. In contrast, TPO did not increase the intensity of GPIIb-IIIa antigens on UT-7/EPO-MplΔ21, UT-7/EPO-MplΔ61, UT-7/EPO-MplΔbox2, UT-7/EPO-MplΔ81, UT-7/EPO-MplΔ95, UT-7/EPO-MplΔbox1, and UT-7/EPO-MplΔ120 in the presence of EPO (Fig. 4). These results indicate that the C-terminal regions (amino acids 575–586 and 615–630) of the c-Mpl cytoplasmic domain play an important role in megakaryocytic differentiation, suggesting that the cytoplasmic domain of c-Mpl critical for megakaryocytic differentiation is identical to that for cell growth. In addition, these results indicate that the region (amino acids 587–614) contains a negative domain for the TPO-
induced cell proliferation and megakaryocytic differentiation. EPO inhibited the TPO-induced increase in GPIIb-IIIa antigens on UT-7/EPO-MplΔ5 but not on UT-7/EPO-MplWT or UT-7/EPO-MplΔ49 (Fig. 4). The results were similar in other UT-7/EPO transfectants expressing c-MplΔ5 (data not shown). Porteu et al. (12) reported that although TPO did not induce the megakaryocytic differentiation of UT-7 cells expressing murine mutant c-Mpl with a deletion of 24 amino acids distal to the box2 region (residues 586–609; corresponding to the human c-Mpl region at residues 604–626 by homology), this effect could be restored by TPO plus EPO. These findings suggested that there is a physical association between the TPO and EPO receptors, as in the case for the EPO receptor and c-kit (19). However, in our system EPO did not promote the TPO-induced megakaryocytic differentiation of any transfectant including UT-7/EPO-MplΔ49 (lacking residues 587–635) and UT-7/EPO-MplΔ21 (lacking residues 615–635). Rather, EPO negatively acted on the TPO-induced megakaryocytic differentiation in UT-7/EPO-MplΔ5. Although this discrepancy may be due to a structural difference between human and murine c-Mpl, the possibility that endogenous c-Mpl expressed on UT-7 cells had affected the interaction of EPO and TPO signaling pathways cannot be completely excluded in their system (8, 13).

We summarize the results in Table I. These transfectants could serve as a model system for analyzing TPO signals for cellular proliferation and megakaryocytic differentiation. These projects are in progress in our laboratory.

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