Expression of the proto-oncogene p93-c-fes and its associated tyrosine kinase activity is marked in mature granulocytes, monocytes, differentiated HL-60 leukemia cells, and leukemia cell lines KG-1, THP-1, HEL, and U-937, which can be induced to differentiate along the granulocyte/monocyte pathway. Conversely, p93-c-fes expression is absent in the K562 cell line, which is resistant to myeloid differentiation. Upon transfection and clonal selection of K562 cells using a mammalian expression vector containing the 13-kilobase pair c-fes gene, c-fes mRNA was transcribed and p93-c-fes tyrosine activity kinase was expressed. Clones expressing c-fes underwent myeloid differentiation as assessed by the appearance of phagocytic activity, Fe receptors, nitro blue tetrazolium reduction, Mac-1 immunofluorescence, and lysozyme production. These results indicate that the expression of the c-fes proto-oncogene and its associated tyrosine kinase activity plays a major role in the initiation of myeloid differentiation.

Proto-oncogenes can be defined as cellular genes that under normal physiological conditions are thought to exhibit proliferative and developmental functions (1-3). In several instances, proto-oncogenes show strong sequence homology with growth factor receptor protein kinases, suggesting that they play a role in the transduction of proliferation and differentiation signals (4). Expression of the c-fes oncogene is apparently limited to hematopoietic cells, especially granulocytes, monocytes, and macrophages, and it is apparent that p93-c-fes plays some functional role in normal myelopoiesis (5-8).

A 13.2-kb EcoRI fragment was isolated from a human lymphoblast cell line (TK6) and identified as the human c-fes locus based on its homology with the v-fes gene (9). The c-fes gene has been mapped to chromosome 15, and it is comprised of 19 exons, the first of which is noncoding (10, 11). The human 93-kDa c-fes gene product predicted from this sequence is closely related to the v-fes transforming proteins (7). However, overexpression of the human c-fes gene product in rat fibroblasts does not result in malignant transformation, suggesting that its oncogenic potential is restrained within normal cells (8).

The K562 cell line was established from the pleural effusion of a patient with chronic myelogenous leukemia in blast crisis (12). Originally, the cell line was considered to represent a highly undifferentiated cell of the granulocytic lineage (13). Many studies have firmly established that K562 cells synthesize hemoglobin of the embryonic and fetal type (14) and that synthesis is strongly increased by hemin. In addition, cell surface analysis by immunofluorescence has shown that K562 cells may also exhibit some early granulocyte markers (15), but major myeloid differentiation markers, such as phagocytosis, NBT reduction, and lysozyme and myeloperoxidase activities, were absent (13). However, multilineage markers are expressed in this cell line, indicating that it is a highly undifferentiated multipotential leukemia cell line.

In the present study, we have introduced the human c-fes gene, a gene that is normally inactive in K562 cells, into this cell line. Our results indicate that stably transfected cells attain the ability to undergo myeloid differentiation and confirm a causative role for this proto-oncogene in this process.

**EXPERIMENTAL PROCEDURES**

**Materials**—All radioisotopes were obtained from Du Pont-New England Nuclear. Tyrosine-agarose, Me2SO, and poly(Glu,Tyr)1:1, were purchased from Sigma. The v-fes probe (460-base pair PstI-PstI fragment) was purchased from Oncor, Gaithersburg, MD. Rabbit antisera to a recombinant c-fes peptide were generously provided by Dr. Dennis J. Slamon, UCLA School of Medicine, Los Angeles, CA (6). Geneticin (G418) was purchased from Gibco. Plasmids p80 and pSV2neo were obtained from the American Type Culture Collection, Rockville, MD. The Mac-1 monoclonal antibody against the macrophage-specific differentiation antigen was obtained from Hybritech, San Diego, CA. The monoclonal antibody (Ab-1) directed against the c-fes transforming protein common to both the Snyder-Theilen and Gardner strains of feline sarcoma virus was purchased from Oncogene Sciences, Manhasset, NY.

**Cell Culture**—HL-60, K562, and Cos-1 cells were obtained from the American Type Culture Collection. HL-60 and K562 cells were grown in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, 40 mM Hepes, pH 7.4, 1 mM sodium pyruvate, nonessential amino acids, 100 units/ml penicillin, and 100 μg/ml streptomycin. Cos-1 cells were grown in Dulbecco's modified Eagle's medium supplemented as above. All cells were subcultured twice weekly and maintained at a density of 106-107 cells/ml. HL-60 cells were treated with 1.6% Me2SO for 4 days to induce granulocytic differentiation.

**Preparation of Cell Extracts**—Cells (0.5-1.0 x 10⁶) were collected by centrifugation and washed twice in Hank's balanced salt solution containing 20 mM EDTA without Mg²⁺ or Ca²⁺. The cell pellet was sonicated for 5 s in 0.5 ml of 50 mM Tris-Cl (pH 7.5) containing 2 mM EGTA, 10 mM dithiothreitol, 0.1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 50 μg/ml aprotinin, 200 μg/ml leupeptin, and

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400 µg/ml soybean trypsin inhibitor and centrifuged at 15,000 x g at 4°C for 10 min. The supernatant was removed, and the pellet was re-extracted with an identical buffer containing 1% Triton X-100. Protein concentrations were determined using a Coomassie Blue-based reagent (Pierce Chemical Co.) and bovine serum albumin as a standard.

**Nondenaturing Gel Assay for Tyrosine Kinase Activity**—Tyrosine kinase activity present in crude cell extracts and column fractions was assayed by nondenaturing polyacrylamide gel electrophoresis as described (16). Briefly, protein samples were subjected to electrophoresis in 4.5% polyacrylamide mini-gels (Hoefer Scientific Instruments) at 4°C. Following electrophoresis, the gels were incubated with Mg³⁺, Mn⁴⁺, and [γ-³²P]ATP in the presence and absence of poly(Glu,Tyr)₉, a synthetic polymer substrate in which tyrosine kinase activity present in crude cell extracts and column fractions of 400 pg/ml soybean trypsin inhibitor and centrifuged at 15,000 x g was eluted from oligo(dT)-cellulose (New England Biolabs). The fraction eluting from oligo(dT)-cellulose selection was hybridized with 32P-labeled c-fes riboprobe (results not shown). Extracts prepared from Cos-1 cells transfected with pECE/fes expressed a single species of tyrosine kinase activity that was present in the 1.0% Triton X-100 cell lysate (results not shown). Following overnight incubation, the hybridization reaction was digested with RNase, and the protected double-stranded RNA fragments were resolved by polyacrylamide-urea gel electrophoresis and visualized by autoradiography. Complete details of the RNase protection assay will be published elsewhere.

**Histochemical Assays**—Lysozyme activity was measured spectrophotometrically at 450 nm by the lysis of Micrococcus lysodeikticus (26). The ability of cells to reduce NBT to formazan was assayed by the method described by Breitman et al. (27). Fc receptors and immunophagocytosis were determined using sheep erythrocytes coated with anti-erythrocyte antibodies (28). Expression of the macrophage-specific differentiation marker, Mac-1, was examined by immunofluorescence following treatment of cells for 2 days with 100 nM TPA (29).

**RESULTS**

**Transfection of Cos-1 Cells with pECE/fes**—The 13.2-kb EcoRI fragment identified previously as the human c-fes gene (9, 11) was cloned into the SV40-based mammalian expression vector pECE (19) and designated pECE/fes. To test this construct, Cos-1 cells were transfected by calcium phosphate precipitation and, 48 h later, Triton X-100 extracts of cellular proteins were analyzed for immunoreactive p93⁺ and for tyrosine kinase activity (5, 16). Cos-1 cells transfected with pECE/fes expressed a 93-kDa protein that was specifically recognized on Western blots by the c-fes polyclonal antibody 19-30 (results not shown). Extracts prepared from Cos-1 cells transfected with pECE/fes expressed a single species of tyrosine kinase activity that was present in the 1.0% Triton X-100 cell extract as determined by the nondenaturing gel assay (30, 31) (results not shown). These results indicated that Cos-1 cells are capable of expressing the genomic DNA encoding c-fes and transcribing a functional gene product and agree with previous work by Greer et al. (8). However, Cos-1 cells did not acquire characteristics of myeloid cells as a result of c-fes transfection.

**Co-transfection of K562 Cells with pECE/fes and pSV-2/neo**—Since K562 cells do not express p93⁺, they are an ideal cell line for transfection experiments with pECE/fes. K562 cells were co-transfected with pECE/fes and pSV-2/neo by protoplast fusion and were selected by cloning in soft agar containing 2.5 mg/ml G418. After 14 days in culture, G418-resistant colonies were selected and amplified in RPMI 1640 medium. One percent Triton X-100 cell extracts representing the membrane fraction of the cell were prepared from G418-resistant colonies and were screened for tyrosine kinase activity. The membrane fraction of the cell was prepared from G418-resistant colonies and was screened for tyrosine kinase activity. The membrane fraction of the cell was prepared from G418-resistant colonies and was screened for tyrosine kinase activity.
ity with the non-denaturing gel assay (Fig. 1). Stably transfected colonies designated WS-1, WS-5, WS-6, and WD-7 had high levels of tyrosine kinase activity. Colony WS-1 expressed a level of tyrosine kinase activity comparable to that present in HL-60 cells treated with 1.6% Me2SO (Fig. 2), a treatment that produces granulocytic differentiation (32). p93<sup>c-fes</sup> tyrosine kinase activity was not present in either parental or pSV2/neo-transfected K562 cells (Fig. 2).

A Southern blot of the DNA prepared from several colonies of stably transfected K562 cells indicated varying levels of integration of the c-fes gene (Fig. 3). The most dramatic example is seen in transfected clone WS-1, in which the level of the c-fes gene is more than 30 times higher than that of the K562 wild type cells. The intensity of the hybridization signal was similar to the level of tyrosine kinase activity expressed by the various clones (Fig. 1). Digestion of WS-1 cell DNA with EcoRI and XhoI generated the expected 13.2- and 4.4-kb fragments that were identical to those present in p80 following hybridization with the v-fes DNA probe (Fig. 4).

Analysis of c-fes Transcript Levels, mRNA Processing, and p93<sup>c-fes</sup> Protein Synthesis in K562/fes Clones—Steady-state levels of c-fes mRNA were determined in transfected K562 clones using an RNase protection assay. The probe used in

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**Fig. 1.** Nondenaturing gel assay for p93<sup>c-fes</sup> tyrosine kinase activity in colonies of K562 cells stably transfected with pECE/fes. K562 cells were co-transfected with pECE/fes and pSV2/neo as a selectable marker, and G418-resistant colonies were selected and screened for p93<sup>c-fes</sup> tyrosine kinase activity. Aliquots of membrane proteins (15 µg) present in 1.0% Triton X-100 cell extracts were assayed for tyrosine kinase activity using the nondenaturing gel assay as described under “Experimental Procedures.”

**Fig. 2.** Comparison of tyrosine kinase activity in colony WS-1 with differentiated HL-60 cells. One percent Triton X-100 extracts were prepared from either wild type K562 cells, pSV2/neo-transfected K562 cells (K562/neo), colony WS-1 (K562/fes), or HL-60 cells treated for 4 days with 1.6% Me2SO (HL-60 + DMSO), and p93<sup>c-fes</sup> tyrosine kinase was partially purified by tyrosine-agarose chromatography. Eluates (3 µg of protein) were assayed for tyrosine kinase activity using the nondenaturing gel assay as described under “Experimental Procedures.”

**Fig. 3.** Southern blot analysis of colonies of K562 cells stably transfected with pECE/fes. DNA (10 µg) was prepared from wild type K562 cells and selected colonies of cells transfected with c-fes (designated WS-1, WS-5, WS-6, WD-1, WD-2, WD-3, WD-4, and WD-7) and digested with EcoRI. Plasmid p80 DNA containing the 13.2-kb c-fes gene served as a control. After electrophoresis in 1% agarose gels, Southern blots were prepared and hybridized with a v-fes probe as described under “Experimental Procedures.” Levels of c-fes integration relative to wild type K562 cells were determined by laser densitometry of the 13.2-kb EcoRI fragment. The endogenous K562 c-fes gene is not visible in the exposure shown (12 h); determination of c-fes levels in wild type cells required longer autoradiographic exposure (>48 h; data not shown).

**Fig. 4.** Southern blot analysis of a restriction digest of DNA prepared from colony WS-1. DNA (10 µg) was prepared from wild type K562 cells and colony WS-1 (K562/fes) and digested with EcoRI and XhoI. Plasmid p80 DNA containing the 13.2-kb c-fes gene served as a control. Southern blots and hybridization were carried out as described under “Experimental Procedures.”
this assay is an antisense RNA transcript 498 nucleotides in length containing the 222-nucleotide sequence complementary to c-fes exon 2. The remainder of the probe is made up of 5' and 3' sequences complementary to c-fes introns 2 and 3, respectively, and 37 nucleotides transcribed from the vector template (see "Experimental Procedures"). Poly(A+)RNA was prepared from K562/fes clones WS-1, WS-5, and WS-6 and hybridized to the c-fes riboprobe overnight. Following RNase digestion, polyacrylamide-urea gel electrophoresis revealed a major protected fragment 222 nucleotides in length in each of the transfected clones, which corresponds to c-fes exon 2 (Fig. 5). The intensity of this band is proportional to the level of c-fes genomic integration (Fig. 3), p93c-fes tyrosine kinase activity (Fig. 1), and immunoreactive p93c-fes protein levels (see below). Note that an identical protected fragment is present following the RNase protection assay of poly(A+)RNA from HL-60 cells, a cell line that normally expresses p93c-fes. By contrast, no protected fragments were observed with RNA from untransfected K562 cells.

In addition to the major band of 222 nucleotides, K562/fes clones WS-1 and WS-5 also exhibited a protected fragment of 460 nucleotides (Fig. 5), which corresponds to the size of the c-fes genomic fragment contained within the probe (i.e. intron and exon sequences). This indicates that a significant fraction of the c-fes mRNA from transfected cells contains intron sequences and suggests that c-fes mRNA is less efficiently processed in the transfected clones than in HL-60 cells, which do not exhibit this band. Minor protected fragments approximately 320 and 370 nucleotides in length are also visible in transfected clones WS-1 and WS-5, as well as in HL-60 cells. These fragments may arise from alternate processing of the primary c-fes transcript that occurs 5' to exon 2, as several alternate splice acceptor sites have been proposed in intron 2 of the c-fes genomic sequence (11).

Translation of c-fes mRNA into p93c-fes protein appears to proceed normally in transfected K562 cells. This conclusion is based on immunoprecipitation experiments using an anti-v-fes monoclonal antibody. As shown in Fig. 6, transfected K562 clones WS-1 and WS-5 express an immunoreactive 93-kDa protein not seen in the K562 wild type cells. Note that an immunoreactive protein of identical electrophoretic mobility is also seen in immunoprecipitates of HL-60 cells, which are enriched in p93c-fes (6).

K562 clones WS-1 and WS-5 express an immunoreactive 93-kDa protein not seen in the K562 wild type cells. Note that an immunoreactive protein of identical electrophoretic mobility is also seen in immunoprecipitates of HL-60 cells, which are enriched in p93c-fes (6).

Phenotypic Alterations in Colonies of K562 Cells Transfected with c-fes—Clones WS-1, WS-5, and WS-6 were selected for further study of the changes in maturation that accompanied selection of these cell lines. After 2–3 passages, WS-1 cells grew at a slower rate than wild type K562 cells (Fig. 7), a property that may be indicative of differentiation. In addition, all clones adhered loosely to the culture flask, a property that was not seen with parental or pSV2/neo transfected cells (Table I). Most notable was the response of WS-1 and WS-5 cells to TPA, a treatment that produced approximately 50% macrophage-like cells (Fig. 8, A and B). TPA treatment of transfected K562 cells also resulted in expression of the macrophage-specific differentiation antigen Mac-1 (33), whereas TPA-treated wild type cells displayed almost no
The maturation and proliferation of hematopoietic cells is a complex but orderly process involving growth factor-controlled programs that lead to terminal differentiation or self-renewal. The human c-fes gene, unlike the macrophage CSF with 100 mM hemin for 5 days resulted in 64, 49, and 63% benzidine-positive cells, respectively (results not shown).

**DISCUSSION**

The differentiation-associated 93-kDa tyrosine kinase purified previously from HL-60 cells treated with the granulocytic differentiation inducer MeSO is the product of the human c-fes gene (5, 6). Expression of p93c-fes is especially high in mature peripheral monocytes and granulocytes, in acute and chronic myelogenous leukemias, and in leukemia cell lines capable of myeloid differentiation (5-7, 36, 27). Conversely, in cell lines resistant to myeloid differentiation, such as K562 and KG-1a, p93c-fes expression is either very low or absent (6, 7, 37). These findings suggest that p93c-fes may play a role during the process of maturation of myeloid cells. The K562 leukemia cell line provided a convenient model to study the function of the human c-fes gene and its role in myeloid differentiation. This cell line was characterized previously as a highly undifferentiated leukemia cell line with some erythroid properties and a few early markers of myeloid cells (12, 13). This cell line does not express p93c-fes and cannot be induced to differentiate along the granulocyte/monocyte pathway by a variety of differentiating agents (35). Therefore, we utilized this cell line for transfection with the human c-fes gene in order to identify the role of c-fes in the differentiation process. In the present work, we observed that K562 cells transfected with the c-fes gene expressed an active p93c-fes tyrosine kinase that coincided with the expression of phenotypic markers indicative of a more differentiated cell type, such as increased phagocytosis (38), Fc receptors (35), NBT reduction (38), and lysozyme activity (39). The latter activity in clone WS-1 was comparable to levels found in mature leukocytes (40). This clonal cell line also responded dramatically to the phorbol ester TPA, resulting in its morphologic transformation to a macrophage-like cell and expression of the macrophage surface antigen Mac-1. Thus, these results demonstrate that an active c-fes gene is imperative for the ultimate expression of the mature myeloid phenotype. This hypothesis is consistent with previous studies that showed that inhibition of p93c-fes activity in HL-60 cells could block, to a large extent, the ability of these cells to undergo differentiation and, conversely, that MeSO-resistant HL-60 cells did not express p93c-fes tyrosine kinase activity (41).

The mechanism by which p93c-fes induces myeloid differentiation is less clear. Some tyrosine kinases are involved in relaying mitogenic and hormonal signals from the plasma membrane (42, 43). Carmier et al. (44) used retroviruses containing the v-fps oncogene to infect chicken myeloid stem cells and found that infected progenitor cells developed macrophage colonies in vitro without exogenous macrophage CSF. They suggested that the tyrosine kinase associated with the v-fps gene product initiated the transduction signals that occur after activation of the macrophage CSF receptor, resulting in a perpetually activated stem cell. Transfection of K-562 cells with the p93c-fes tyrosine kinase may produce a similar effect, viz. the unrestrained expression of a kinase normally activated by growth or differentiation factors, which in turn results in a sustained mimicry of their effects. The present study strongly supports this hypothesis, as the introduction of the c-fes proto-oncogene into the undifferentiated blast line K562 results in the rapid acquisition of myelomonocytic characteristics.

### Table I

<table>
<thead>
<tr>
<th>Phenotype of parental K562 cells and colonies transfected with c-fes</th>
<th>HL-60</th>
<th>K562</th>
<th>K562/WS-1</th>
<th>K562/WS-5</th>
<th>K562/WS-6</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Phenotype</strong></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Phagocytosis</td>
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<td>1</td>
<td>65</td>
<td>56</td>
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<tr>
<td>Fc receptors</td>
<td>68%</td>
<td>52</td>
<td>48</td>
<td>94</td>
<td>85</td>
</tr>
<tr>
<td>NBT reduction</td>
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<td>1</td>
<td>3</td>
<td>64</td>
<td>43</td>
</tr>
<tr>
<td>Lysozyme activity</td>
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<tr>
<td>Adherence</td>
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<tr>
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<tr>
<td>Adherence</td>
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<td>MAC-1</td>
<td>88</td>
<td>2</td>
<td>3</td>
<td>70</td>
<td>73</td>
</tr>
</tbody>
</table>

* The values for phagocytosis, NBT reduction, and lysozyme activity are the average of duplicate determinations. Other values represent single determinations.
* From Ref. 32. HL-60 cells were treated for 5 days with 1.25% MeSO.
* Micrograms of lysozyme/10^6 cells. Normal leukocytes range from 3.6-8.4 (40).
* ND, not determined.
* From Ref. 34.

**Fig. 8. Photomicrographs of parental K562 cells and K562/c-fes clone WS-1.** Parental K562 cells (A, C, and E) and c-fes-transfected clone WS-1 (B, D, and F) were tested for their response to TPA, for their ability to reduce NBT (C and D), or for their capacity to phagocyte sheep erythrocytes (E and F).
receptor/c-fms gene, does not appear to contain a transmembrane domain (45), although it is predominantly a membrane-associated protein. However, previous studies have demonstrated that treatment of HL-60 cells with MeSO results in parallel increases in both granulocyte-macrophage-CSF binding (46) and c-fes expression (6). A number of variant cell lines resistant to differentiation did not express p93 and also did not respond to granulocyte-macrophage-CSF (6, 46). It is possible that the receptor for granulocyte-macrophage-CSF or some other colony-stimulating factor is coupled to the p93 tyrosine kinase, which serves as an intracellular component to evoke a functional response to growth factors.

REFERENCES