Fte-1, a v-fos transformation effector gene, encodes the mammalian homologue of a yeast gene involved in protein import into mitochondria

(Finkel–Biskis–Jenkins murine sarcoma virus/rhodamine 123/insertional mutagenesis)

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ABSTRACT Revertants were isolated from v-fos-transformed rat-1 cells cotransfected with a human cDNA expression library and a selectable marker (pMEX-neo). Molecular analysis of one clone, R2.2, suggested that the revertant phenotype resulted from the disruption of a transformation effector gene by the integration of the pMEX-neo plasmid. Genomic sequences flanking the plasmid integration site were cloned and used as probes in Northern blot analyses. A probe derived from sequences 5' to the integration site hybridized to a unique 1.2-kilobase mRNA and was used to isolate a 0.9-kilobase cDNA clone (fte-1). The open reading frame of the fte-1 cDNA predicts a highly basic protein that shows a remarkable level of similarity with two genes from Saccharomyces cerevisiae. One of these yeast genes contains an unidentified open reading frame and the other, MFT1, is a gene isolated from a yeast mutant that fails to import a fusion protein into mitochondria [Garrett, J. M., Singh, K. K., Vonder Haar, R. A. & Emr, S. D. (1991) Mol. Gen. Genet. 225, 483–491]. Expression of the fte-1 gene was induced ∼5-fold in v-fos-transformed fibroblasts, but expression was reduced in clone R2.2 and in several independent revertant clones. Transfection of R2.2 cells with fte-1 expression vectors resulted in the reacquisition of a transformed phenotype. These results demonstrate that the mammalian homologue of a gene implicated in protein import into yeast mitochondria is a v-fos transformation effector gene.

The p55 v-fos oncogene (1) and its cellular homolog, c-fos, encode nuclear phosphoproteins that can form heterodimeric complexes with the product of the c-jun protooncogene (2). Both c- and v-Fos–Jun heterodimers function as transcriptional regulators that increase transcription from genes whose regulatory regions contain phorbol 12-O-tetradecanoate 13-acetate responsive elements. Constitutive overexpression of either v-fos or c-fos genes induces cell transformation (3). It is therefore reasonable to conjecture that Fos-induced cell transformation results from an aberration in the cell’s pattern of gene expression. This hypothesis is consistent with the observation that all mutant Fos proteins that lose their ability to form heterodimers, their ability to bind to DNA, or their ability to activate transcription of genes containing phorbol 12-O-tetradecanoate 13-acetate responsive elements, concomitantly lose their transforming capability (4). Thus to understand the mechanism of Fos-induced cell transformation, it is necessary to define cellular genes that mediate or modulate Fos-induced alterations in gene expression (5) and genes encoding the proteins of the biochemical pathways of Fos-induced cell transformation.

The approach we have taken to identify these genes involves isolation and molecular analysis of revertant cell lines (nontransformed variants) (6–8). In the present report, we describe the isolation of a revertant that resulted from a mutagenic insertion of a transfected plasmid into a fos transformation effector gene (fte-1). Molecular cloning and sequencing of the fte-1 cDNA7 indicated that the effector gene encodes a protein that has been extremely well conserved during evolution and may play a role in protein import into the mitochondria.

MATERIALS AND METHODS

Isolation and Characterization of Revertants. Isolation of the v-fos-transformed rat-1 cell line 1302-4-1 and revertants (EMS-1-19 and EMS-1-17) was as described (6). A cDNA library was generated with mRNA from the human foreskin fibroblast (HS68, ATCC) by using the eukaryotic expression vector pCDM8 (9). DNA from the pooled cDNA clones was cotransfected into 1302-4-1 cells with the selectable marker plasmid pMEX-neo (10). DNA (10 μg) from the cDNA library was mixed with 10 μg of pMEX-neo plasmid and used to transfect 1 × 10⁶ cells per 100-mm dish (11). Twenty-four hours after transfection, cells were incubated with growth medium containing 200 μg of G418 (GIBCO) per ml and cultured at 37°C for 14 days. Cells were then stained by exposure with rhodamine 123 (Sigma) and washed as described (6). Eighteen hours later, cells were trypsinized, resuspended at 2 × 10⁶ cells per ml, filtered through a 70-μm (pore size) nylon mesh, and sorted using a FACStarPlus fluorescence-activated cell sorter (Becton Dickinson). Viable cells that did not retain rhodamine 123 were collected using previously described parameters (6). Colonies visible after 14 days were again stained with rhodamine and examined directly by epifluorescent illumination using an inverted microscope. Flat colonies that failed to retain the dye were isolated using cloning cylinders and further purified by repeated cloning. The growth of putative revertants in soft agar and tumorigenic potential of revertants were assessed as described (6).

Molecular Analysis of Revertants. Total RNA was prepared using the method of Chomczynski and Sacchi (12). Southern and Northern blot analyses were performed using Hybond-N nylon membranes (Amersham) as described by Sambrook et al. (13). Probes were labeled using the random-priming method of Feinberg and Vogelstein (14). The subgenomic DNA library was constructed by fractionating EcoRI-digested cellular DNA on a 10–40% (wt/vol) sucrose gradient. Fractions containing the DNA of interest were ethanol-precipitated, ligated directly to an EcoRI-digested EMBL4

Abbreviations: neo, neomycin phosphotransferase; ORF, open reading frame.
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1The sequence reported in this paper has been deposited in the GenBank data base (accession no. M84716).
vector (15), and packaged using Gigapack II Gold (Stratagene). The library was screened using a [α-32P]dCTP-labeled neomycin phosphotransferase (neo) gene probe [a 0.92-kilobase (kb) Pst I fragment of the pMEX-neo plasmid] by filter hybridization. Plasmid rescue from genomic DNA was performed by digesting DNA to completion with HindIII and diluting the DNA to a final concentration of 1 μg per 100 μl of reaction mixture before ligation. The ligated DNA was then electroporated (16) into Escherichia coli DH5α and transformants were selected on LB/agar plates containing ampicillin (100 μg/ml). The 1302-4-1 cDNA library was constructed in the Agt10 vector using standard procedures (13) and screened with the isolated [α-32P]dCTP-labeled genomic probe by filter hybridization. The cDNA inserts in purified phage DNA were amplified using PCR primers (Clontech) flanking the EcoRI cloning site in Agt10 and cloned directly into pCR1000 (Invitrogen) (17), and double-stranded plasmid DNA was sequenced using a modified Sequenase protocol (United States Biochemical) (18). Sequence manipulation and homology searches were performed on a VAX computer (DEC) using University of Wisconsin Genetics Computer Group and the National Center for Biotechnology Information BLAST programs.

RESULTS

Isolation and Characterization of Revertants. A rapid protocol (6) was used to isolate revertants from populations of cells that were transfected with plasmid DNA. 1302-4-1 cells were cotransfected with DNA isolated from a human foreskin fibroblast cDNA expression library and the pMEX-neo plasmid. A total of 1 x 10^8 G418-resistant colonies was obtained and sorted on the basis of rhodamine 123 retention within their mitochondria (6). The 0.1–0.5% of the cells that demonstrated the least fluorescence were collected and reexamined by epifluorescence and phase-contrast microscopy. Three putative revertant cell lines, R1.2, R2.2 (Fig. 1A), and R2.3, were selected.

The presence of an intact v-fos oncogene within these three lines was confirmed by Southern blot analysis and by retrovirus rescue experiments as described (6). Northern blot analyses demonstrated that the level of expression of the 3.8-kb v-fos RNA in each of the revertants was comparable to those observed in 1302-4-1 cells (data not shown).

Each of the three revertant lines exhibited a reduced cloning efficiency in soft agar medium (ranging from 0.2% to 0.04%) when compared to G418-resistant 1302-4-1 cells (13%). The small number of colonies formed by the revertants invariably contained <40 cells, whereas G418-resistant 1302-4-1 cells gave rise to colonies of >200 cells. Tumorigenicity of the revertants was assessed by subcutaneous injection of 10^6–10^7 cells into weanling Fisher 344 rats. None of the revertant clones gave rise to tumors over a 20-week observation period, whereas large tumors (10–30 g) were detected in animals injected with 1302-4-1 cells. Samples of 1 x 10^6 cells were similarly injected into weanling athymic nude mice. The revertants did give rise to small tumors, but only after a prolonged latency period in these animals (60–80 days), when compared with 1302-4-1 cells (10 days). The nodules induced by the revertants did not exceed 0.1 g, even 18 weeks after injection. These results indicated that the putative revertants isolated from the DNA transfection experiments had a phenotype that was much less transformed than that of the parental cells.

Molecular Analysis of Revertant R2.2. When a Southern blot was probed with the neo gene fragment from the pMEX-neo plasmid, a single 10-kb EcoRI fragment was detected in clone R2.2 (Fig. 2A). This result suggested that a single copy of the transfected pMEX-neo plasmid had integrated into the genomic DNA of this revertant. No hybridizing bands were detected in DNA from clone R2.2 when sequences specific to the pCDM8 cDNA vector were used as probes (Fig. 2B). The absence of a transfected cDNA plasmid suggested that the phenotype of revertant R2.2 might have resulted from integration of the pMEX-neo plasmid into a v-fos transformation effector gene.

To isolate the putative effector gene, the sequences flanking the plasmid integration site were cloned. A recombinant phage library was constructed by ligating size-fractionated (9–12 kb) EcoRI-digested R2.2 genomic DNA into the phage vector EMBl4 (15). A library containing 400,000 recombinants was screened for the presence of neo sequences, and a single phage containing an insert of 10 kb was purified. Restriction analysis and DNA sequencing indicated that this 10-kb insert contained almost the entire pMEX-neo plasmid, except for 12 base pairs (bp) that had been deleted. The 10-kb insert also contained 3.6 kb of rat cellular sequences that were 3' of the plasmid integration site (Fig. 3A). No cellular sequences 5' to the integration site were present. A plasmid rescue strategy was, therefore, used (19) to clone sequences 5' to the plasmid integration site. Using this approach, we were able to rescue plasmids containing 2.8 kb of cellular sequences 5' of the integration site (Fig. 3A). Southern blot analysis confirmed that these cloned cellular sequences were derived from sequences flanking the pMEX-neo plasmid integration site. Nonrepetitive probes derived from the 5' or the 3' flanking sequences, detected a single band in restricted genomic DNA from rat-1 and 1302-4-1 cells, whereas two bands were seen with R2.2 DNA (Fig. 3B and C). Moreover, we were able to determine that the rat sequences flanking the plasmid integration site are contiguous in the unrearranged allele, ruling out any complex genomic rearrangements around the integration site (data not shown).

To determine whether the integration of pMEX-neo plasmid had disrupted a transcription unit, the nonrepetitive DNA probes from the 5' and 3' flanking regions were used in Northern blot analyses of RNA from rat-1, 1302-4-1, and R2.2 cells. Only one of these probes, a 520-bp Sac I–Nde I fragment (probe 0.52) detected a unique transcript (1.2 kb) in all three cell lines. The level of expression of this mRNA was induced ~5-fold in 1302-4-1 cells when compared to rat-1

Fig. 1. Morphology and proliferation of revertant R2.2 in semisolid medium. (A) Phase-contrast micrographs (×75) of the parental 1302-4-1 (1) and R2.2 (2) cells. (B) Colony growth in soft agar. Approximately 1 x 10^6 cells were seeded in medium containing 0.33% Noble agar. After 5 weeks, cultures were stained with p-iodonitrotetrazolium violet (Sigma) and fixed onto Whatman filter paper.
cells, whereas the level in R2.2 was reduced by a factor of 2 when compared to 1302-4-1 cells (Fig. 4). These results were consistent with the possibility that the phenotype of clone R2.2 cells was due to the disruption of a fos transformation effector gene that is encoded by the 1.2-kb mRNA. Expression of the 1.2-kb transcript was also reduced ∼10-fold in revertant clones EMS-1-17 and EMS-1-19 (Fig. 4), suggesting it may play an important role in v-fos-induced cell transformation.

Isolation and Sequencing of CDNA Clones Encoding the fte-1 Gene. We subsequently screened oligo(dT)-primed Agt10 cDNA libraries prepared with RNA from 1302-4-1 cells or from R2.2 cells with the probe 0.52. Three cDNA clones isolated from the 1302-4-1 cDNA library and one cDNA clone from the R2.2 library were studied in detail. Among the three clones from the 1302-4-1 library, two contained inserts of ∼900 bp and one had an insert of 500 bp. The single clone from the R2.2 library also had a cDNA insert of ∼900 bp.

Several restriction fragments from the cDNA inserts were cloned into a pGEM vector and double-stranded plasmid DNA was subjected to nucleotide sequence analysis. The nucleotide and predicted amino acid sequences are shown in Fig. 5A. The sequence revealed a continuous open reading frame (ORF) capable of encoding 264 amino acids (predicted molecular mass of 30 kDa), beginning from nucleotides 19 to 811 (Fig. 5A). It is likely that this ORF represents the entire coding segment of fte-1, as primer-extension experiments indicated that there were only ∼30 nucleotides upstream of the putative initiator methionine codon (data not shown). The nucleotides surrounding the putative ATG initiator codon (ACCATGG) have most of the features required for initiation of eukaryotic translation (20). The 3′ untranslated region of the cDNA contains the polyadenylation consensus sequence AAUAAA (21), 23 nucleotides upstream of the poly(A) addition site.

The predicted amino acid sequence contains a high percentage of positively charged amino acids (22%), most of which are lysine (14%), and has a predicted isoelectric point of 10.53. The predicted protein sequence also included nine potential phosphorylation sites and a potential nuclear localization sequence, Lys-Lys-Pro-Lys (22), at amino acid residues 219–222.

The nucleotide sequence was compared to all sequences deposited in the GenBank (release 68.0) and found to be highly homologous to two genes from yeast (Saccharomyces cerevisiae). It is 61% identical to both an unknown ORF downstream of the SIR3 (silent information regulator) gene (23) and the MFTI (mitochondrial fusion targeting) gene (24). MFTI is 91% identical to the unidentified ORF and it has been suggested that these two genes could represent a recent gene duplication (24). The predicted amino acid sequences of fte-1 and MFTI were also very similar (63% identical, with an additional 24 conservative amino acid substitutions) in the regions also present in the ORF 3′ of SIR3 (residues 1–178). fte-1 and MFTI were somewhat divergent at their C termini. Amino acids 178–264 were 52% identical if two gaps were introduced into the MFTI sequence (Fig. 5B).

When the fte-1 cDNA was used to probe Southern blots, fte-1-related sequences were detected in DNA from rats, mice, and humans. By using different restriction enzyme digests, several hybridizing bands of moderate intensities were observed (data not shown), suggesting that the fte-1 gene contains a large number of exons or is a member of a small gene family. Northern blot analyses indicated that a 1.2-kb transcript of fte-1 can be detected in human and mouse fibroblasts (Fig. 4D) and in a wide range of tissues (data not shown).

Overexpression of fte-1 RNA in Revertant R2.2. To determine if overexpression of fte-1 can restore the transformed phenotype of R2.2 cells, the full-length cDNA was cloned into the EcoRI restriction site of pMEX-neo (10). R2.2 cells were cotransfected with 1 μg of the selectable marker pY3 (25) and 20 μg of either the pMneo-fte-1 plasmid or the control

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**Fig. 2.** Southern blot analysis of revertant clone R2.2. Genomic DNAs (20 μg) prepared from the parental rat-1 cells, v-fos-transformed rat-1 cells (1302-4-1), and revertant R2.2 were digested with EcoRI and hybridized with 32P-labeled neo probe (0.92-kb Pst I fragment of pMEX-neo) (A) and 32P-labeled cytomegalo virus promoter probe (1.03-kb Pvu II–HindIII fragment of pCDM8) (B). Amounts of the pCDM8 vector DNA that would approximate one or five integrated copies of pCDM8 were included.

**Fig. 3.** Integration of pMEX-neo plasmid DNA into chromosomal DNA in clone R2.2. (A) Restriction map of cloned DNA sequences in the vicinity of the plasmid integration site. Probes 0.52 and C are the nonrepetitive DNA fragments used for hybridization probes. xx indicates presence of high repetitive sequences as determined by Southern blot analysis. (B) Genomic DNA (20 μg) from each indicated cell line was digested with HindIII, separated on a 0.8% agarose gel by electrophoresis, and transferred to HyBond-N (Amersham) nylon membrane. The blot was hybridized with 32P-labeled probe 0.52. (C) Same analysis as in B except that DNA was digested with EcoRI and hybridized with 32P-labeled probe C.
Fig. 4. Expression patterns of fte-1 mRNA. (A) Probe 0.52 hybridized to a unique 1.2-kb mRNA in rat cells. (Upper) Poly(A)+ RNA (1 μg) from each indicated cell line was separated by electrophoresis on a 1.2% agarose/formaldehyde gel and transferred to nylon membrane. (Lower) Hybridization with a GAPDH (glyceraldehyde 3-phosphate dehydrogenase) DNA probe. (B) RNA blot of normal (rat-1), v-fos-transformed (1302-4-1), and revertant (R2.2, EMS-1-19, and EMS-1-17) cell lines hybridized with 32P-labeled fte-1 cDNA. (Upper) Total cellular RNA (20 μg) was separated by electrophoresis on a 1.2% agarose/formaldehyde gel and transferred to nylon membrane. (Lower) Hybridization with GAPDH. (C) Quantitation of the expression levels of fte-1 mRNA. Northern blots similar to those in B were probed with 32P-labeled fte-1 cDNA and quantitated using a PhosphorImage (Molecular Dynamics, Sunnyvale, CA). The blots were then stripped, rehybridized with the GAPDH probe, and quantitated to account for the different amounts of RNA on the blots. Histogram shows the averaged results from three experiments. (D) Total RNA (rat-1, 10 μg; mouse NIH 3T3, 20 μg) and poly(A)+ RNA (human Hs68, 3 μg) were electrophoresed in a 1.2% agarose/formaldehyde gel, blotted, and hybridized to 32P-labeled fte-1 cDNA probe.

pMEX-neo plasmid. Approximately 39% of the hygromycin B-resistant clones transfected with the fte-1 expression plasmid had a transformed morphology similar to that of the parental v-fos-transformed cells, whereas only 5% of the clones transfected with pMEX-neo had a transformed morphology. We isolated and expanded several colonies of the fte-1-transfected lines and analyzed clones for expression of the exogenous fte-1 gene by an RNA PCR (data not shown). Clones were then assayed for their ability to grow in soft agar. The results indicated that fte-1 expression restored the cloning efficiency of R2.2 to the same level observed for the parental 1302-4-1 cells (Table 1). We have also been able to retransform an independent revertant (EMS-1-19) with pMEX-neo-fte1 DNA (data not shown). Thus these results suggest that there is a link between fte-1 expression and cellular transformation by the v-fos oncogene.

Fig. 5. Structure of the fte-1 cDNA. (A) Nucleotide sequence of the fte-1 cDNA and its predicted amino acid sequence. The ORF extends from nucleotides 19 to 811. (B) Comparison of the predicted amino acid sequences of fte-1 protein, MFTI protein, and ORF 3' of SIR3. The ORF 3' of SIR3 is incomplete and contains only 178 amino acid residues. The sequences are aligned for optimal homology. Residues identical to MFT1 are designated by dashes and conserved amino acids are denoted by asterisks. Conserved amino acids are grouped as follows using the single-letter code: (P), (T), (Q), (N), (E), (D), (K), (R), (M), (C), (V), (L), (A), and (F, Y, W, H). Gaps introduced into sequence to maximize homology are denoted by dots.

DISCUSSION

Recently, several groups (25-28) have isolated revertants from transformed cells after DNA-mediated gene transfer. A ras-related gene, Krev-1 (29), and several other transformation suppressor genes have been cloned using this methodology. We have therefore performed analogous experiments to isolate suppressor genes using v-fos-transformed rat-1 cells. However, when applying this methodology, it is plausible that some of the revertants isolated have not acquired a suppressor gene but, rather, have sustained an inactivating mutation in a transformation effector gene. Consistent with this possibility is the observation that we can routinely isolate revertants from v-fos-transformed cells using retrovirus insertional mutagenesis (data not shown).
Table 1. Cloning efficiency of R2.2 cells transfected with fte-l expression vector in soft agar

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>Cloning efficiency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nontransfected</td>
<td>14.6</td>
</tr>
<tr>
<td>1302-4-1</td>
<td>6.8</td>
</tr>
<tr>
<td>R2.2</td>
<td>&lt;0.4</td>
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<td>13.0</td>
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<td>7.6</td>
</tr>
<tr>
<td>R2.2-fte1-4</td>
<td>6.3</td>
</tr>
<tr>
<td>R2.2-fte1-7</td>
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Cloning efficiency is the number of soft agar colonies per number of cells plated.

*Independent hygromycin B-resistant clones.

In this report we show that revertants can be isolated from v-fos-transformed rat-l cells that have been transfected with plasmid DNA. Detailed molecular analysis of one revertant, R2.2, indicated that it did not harbor an exogenous cDNA expression construct but did harbor a single integrated copy of the pMEX-neo plasmid. These findings suggested that the nontransformed phenotype of this clone was induced by a mutagenic insertion of the plasmid into a transformation effector gene.

The integrated plasmid and flanking genomic fragments were, therefore, cloned by screening a genomic library prepared in λ phage and by using a plasmid rescue strategy. Data obtained using hybridization probes derived from the 5' or 3' flanking sequences allowed several conclusions to be drawn. (i) Integration of the plasmid into the genomic DNA did not involve complex intrachromosomal or interchromosomal rearrangements, as the 5' and 3' flanking sequences were contiguous in the wild-type locus (data not shown). (ii) Only one of the two alleles was disrupted in revertant clone R2.2. (iii) Sequences 5' to the integration site detected a 1.2-kb transcript whose level of expression in R2.2 was about half of that seen in the 1302-4-1 cells. These results again were consistent with the disruption of one allele of the putative effector gene. (iv) Northern blot analysis demonstrated that an altered or novel transcript was not produced from the disrupted allele.

The predicted amino acid sequence encoded by the fte-l cDNA has suggested a possible mechanism through which fte-l cooperates with Fos to produce a transformed phenotype. The fte-l gene was found to have a high level of identity with two closely related genes present in yeast. One of these genes, MFT1, was recently identified as the gene mutated in yeast that failed to transport a fusion protein into mitochondria (24). The high degree of identity to the MFT1 gene indicates that fte-l is probably the mammalian homologue of this yeast gene and suggests that fte-l may regulate or play a role in transport of mammalian proteins into mitochondria.

Studies have suggested a role for altered mitochondrial function in production of a transformed phenotype (ref. 30 and references therein). It is therefore likely that alterations in the rate or in the specificity of protein import into mitochondria could have profound effects on the cell. As an example, inactivation of genes controlling import might reduce the levels of proteins such as Bcl-2 (31) in mitochondria and, hence, reduce the ability of Fos to induce cell transformation. A similar effect on cell transformation could be induced by a change in the ratio of different proteins being imported into mitochondria. An imbalance in mitochondrial proteins could be produced if fte-l were a member of a family of genes whose members have various protein uptake specificities. An imbalance in the ratio of proteins imported into mitochondria could also be induced if the transport mechanism becomes limiting, so that proteins must compete with one another for transport. In either case, the results presented in this study suggest a role for mitochondrial protein import in cell transformation. The recent identification of a gene closely related to fte-l as a cell-cycle-regulated gene in plant cells (32), however, suggests that fte-l gene expression may have effects on cell growth. Analysis of fte-l gene function and regulation should, therefore, provide insight into the mechanisms of v-fos-induced cell transformation.

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