Elevated Expression of Ets2 or Distinct Portions of Ets2 Can
Reverse Ras-mediated Cellular Transformation*

(Received for publication, January 26, 1998, and in revised form, May 12, 1998)

Gabriele Foos, José Javier García-Ramírez, Christina K. Galang, and Craig A. Hauser†‡
From the La Jolla Cancer Research Center, The Burnham Institute, La Jolla, California 92037

Ets transcription factors are important downstream targets of oncogenic Ras. The transcriptional activity of several Ets family members is regulated by Ras, and interfering with Ets-dependent transcription by expression of just the Ets2 DNA binding domain can inhibit or reverse Ras-mediated cellular transformation. To better understand the role of Ets proteins in Ras transformation, we have now analyzed the effects of stably expressing a variety of Ets2 constructs in Ras-transformed NIH3T3 (DT) cells. Expression of only the Ets2 transactivation domains, which also inhibits Ras or Neu/ErbB-2-mediated activation of Ets-dependent transcription, strongly inhibited anchorage-independent growth, but did not revert the transformed DT cell morphology. Unexpectedly, high expression of full-length Ets2, a transcriptional activator, broadly reversed the transformed properties of DT cells, including anchorage-independent growth, transformed morphology, and tumorigenicity, but did not impair attached cell growth. Increasing full-length Ets2 transcriptional activity by fusing it to the VP16 transactivation domain enhanced its ability to reverse DT cell transformation. Mutational analysis revealed that the mitogen-activated protein kinase phosphorylation site required for Ras-mediated activation, Ets2(T72), was not essential for Ets2 reversion activity. The distinct reversion activities of the highly expressed Ets2 transactivation domains or full-length Ets2, along with the specific reversion activity by Ets2 constructs that either inhibit or activate Ets-dependent transcription, suggests multiple roles for Ets factors in cellular transformation. These results indicate that several distinct approaches for modulating Ets activity may be useful for intervention in human cancers.

Ets2 is one of the founding members of the Ets transcription factor family, which are characterized by a conserved DNA binding motif called the ETS domain (1). There are now over 30 known members of the Ets family, found in species ranging from humans to Caenorhabditis elegans (2). The mouse and human Ets2 genes were initially identified through extended similarity to chicken v-ets genes (3, 4). c-ets2 gene is ubiquitously expressed in mouse embryonic and adult tissues (5). One evolutionarily conserved role of many Ets proteins is their function as downstream mediators of the Ras signal transduction pathway. The Drosophila Ets proteins Pointed P2, a transcriptional activator, and Yan, a transcriptional repressor, are targets of Ras signaling (6–8). Ets2 and several other mammalian Ets proteins contain a region of homology with Drosophila Pointed P2 distinct from the Ets domain, called the Pointed domain (9). Ras-mediated signaling through Pointed P2 requires a threonine residue within a MAPK kinase recognition site in the Pointed domain (6, 7). A corresponding MAPK kinase recognition sequence is also found in the Ets2 pointed domain. We previously showed that phosphorylation of this corresponding Ets2(T72) residue is essential for Ras- or Neu/ErbB-2-mediated activation of Ets2 transactivation activity (10, 11), and that Ras signaling to Ets2 takes place through the Ras/Raf/MEK/MAPK pathway (12).

Several lines of evidence suggest that not only are Ets proteins targets of Ras signaling, but that Ets factors play an important role in mediating cellular transformation. Initial characterization of several oncogene-responsive promoter elements revealed that adjacent Ets and AP-1 binding sites mediate their oncogene responsiveness, and that both the Ets and AP-1 binding sites are essential for induction by the activated non-nuclear oncogenes (13, 14). Similar elements have since been found in the promoters of many genes, and where it has been tested, Ets2 can cooperate with AP-1 family members and Ras to activate their transcription. The products of these Ras-induced genes have a wide spectrum of functions, including roles in cellular metabolism, growth control, and metastasis (15–17). There is a good correlation between the ability of various non-nuclear oncogenes to activate the transcription of reporter genes containing adjacent Ets and AP-1 binding sites, and their ability to transform rodent fibroblasts (2, 14, 15, 18). Although Ets binding sites in oncogene-responsive promoter elements are often found adjacent to binding sites for the Ras activated AP-1 family of transcription factors, Ets2 is a distinct target of Ras signaling. We previously showed that two adjacent Ets2 consensus binding sites, similar to those found in the stromelysin promoter (13), are sufficient to confer both Ets2 and Ras responsiveness to a minimal promoter (10, 19). Despite initial reports indicating that high level expression of Ets1 or Ets2 in NIH3T3 cells could give rise to infrequent transformed colonies (20, 21), there has been little subsequent evidence to suggest that simply increasing the amount of Ets1 or Ets2 proteins is sufficient for cellular transformation. However, the necessary role of Ets proteins in fibroblast transformation has been revealed by inhibition experiments, where expression of the Ets2 DNA binding domain alone was found to inhibit oncogenic Ras- or Neu-mediated cellular transformation (11, 22). In addition, expression of the Ets1, Ets2, or PU.1 DNA binding

* This work was supported by National Institutes of Health Grant CA63130 (to C. A. H.) and postdoctoral fellowships (to G. F. and J. G.-R.) from the Deutsche Forschungsgemeinschaft and the Spanish Ministry of Education, respectively. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† To whom correspondence should be addressed: La Jolla Cancer Research Center, The Burnham Institute, 10901 N. Torrey Pines Rd., La Jolla, CA 92037. Tel.: 619-646-3119; Fax: 619-646-3192; E-mail: chauser@ljcrc.edu.

‡ The abbreviations used are: MAP, mitogen-activated protein; MAPK, mitogen-activated protein kinase; MEK, MAPK kinase; TAD, transactivation domains; DBD, DNA binding domain; ERK, extracellular signal-regulated kinase; PBS, phosphate-buffered saline; IRES, internal ribosome entry site.
domains was shown to reverse the transformed phenotype of stably Ras-transformed fibroblasts (23). Importantly, the expression of these presumptive dominant inhibitory Ets mutants did not impair normal cell growth in these studies, suggesting that altering Ets activity may be an approach to specifically reverse the transformed phenotype.

Because Ets family proteins bind to similar DNA sequences centered about a conserved GGAA/T core (2), it is likely that high level expression of the ETS domain from Ets2 inhibits the binding and function of many members of the Ets family. This idea was supported by the finding that expression of the ETS domain of either of two of the most divergent Ets family members, Ets1 or PU.1, could reverse Ras transformation (23). Thus, studies utilizing high expression of ETS domains do not identify which individual Ets family members are actually important in cellular transformation. In addition, although most Ets factors are associated with transcriptional activation (2), several widely expressed Ets proteins including ERF, SAP2/NET, and TEL have been reported to be negative regulators of transcription (24–26). Thus, it is possible that the dominant inhibitory mutants do not inhibit transformation by blocking Ets-dependent transcriptional activation, but instead may be overcoming transcription inhibition by Ets family repressors.

To further analyze the role of Ets proteins in cellular transformation, we have generated a number of Ets2 expression constructs, which can inhibit or activate Ets-dependent transcription, and tested their ability to reverse several criteria of the transformed phenotype when stably expressed in Ras-transformed mouse fibroblasts. To more specifically target Ets2, which is expressed in the Ras-transformed DT cells used in this analysis (27), we have used an expression construct for the Ets2 transactivation domains without the ETS domain. We previously showed that expression of the Ets2 transactivation domains inhibits activation of Ets-dependent transcription by either oncopgenic Ras or Neu/ErbB-2 (11). This Ets2 construct contains extensive homology only with Ets1, as well as more limited homology with other members of the Pointed domain subfamily of Ets proteins. We show here that expression of either the Ets2 DNA binding domain or the Ets2 transactivation domains can inhibit Ras-mediated anchorage-independent growth, but these two constructs do not behave identically in reversing other features of the transformed phenotype. Somewhat unexpectedly, we also found that elevated expression of full-length Ets2 phenotypically reverts DT cell transformation, and that expression of a construct that artificially increases the full-length Ets2 phenotypically reverts DT cell transformation.

MATERIALS AND METHODS

Cell Growth and Selection—The DT cell line is a Ras-transformed NIH 3T3 cell line originally transformed by integration of two separate copies of the Kirsten murine sarcoma virus carrying the v-Ki-Ras gene (28). The DT cells were kindly provided by Dr. Y. S. Cho-Chung (National Cancer Institute, National Institutes of Health). DT cells were grown in DMEM supplemented with 10% fetal calf serum, and NIH 3T3 cells were grown in DMEM supplemented with 10% calf serum. Stable DT cell lines were generated by transfection using the calcium phosphate precipitation method we previously described (56), except that, for a 60-mm dish, 3 μg of pcIN-derived expression plasmid DNA and 12 μg of high molecular weight herring sperm carrier DNA were used. Stable colonies were selected 3 days after transfection using medium containing 400 μg/ml active G418 (Calbiochem). Multiple isolated DT cell clones derived from each expression plasmid were randomly picked, then expanded and passaged in the presence of 400 μg/ml G418 for subsequent analysis.

Plasmids—The pcIN4 mammalian expression vector used for all of the constructs is a derivative of the pCIN plasmid (30), and was kindly provided by Steve Rees (Glaxo Wellcome). This vector is based on pcDNA3 (Invitrogen) and utilizes the CMV enhancer/promoter to express an inserted coding sequence, which is linked by a viral internal ribosome entry site (IRES) to the neomycin phosphotransferase gene. We have previously described the construction of pcDNA3-based mouse Ets2 expression constructs (FN-Ets2) for full-length wild type Ets2 (27), Ets2(A72), Ets2TAD, Ets2DBD, and the empty FNpcDNA3 (11). These constructs all derive from the FN vector containing the pCIN4 CMV enhancer/promoter up to the NotI site in the pCIN4 polynucleotide. The resulting pcIN-Ets2 constructs (from 5' to 3') contained a consensus translational start, the FLAG and SV40 NLS sequences fused to the Ets2 coding sequences, a splice site, an IRES, the neomycin phosphotransferase gene, and a poly(A) signal. The VP-16-containing constructs were generated using standard polymerase chain reaction methods to insert VP16 residues 410–479 in-frame as a BamHI-BglII fragment into the BamHI site at the junction of the FLAG/NLS and the Ets2 coding sequences in FN-Ets2, or into the BamHI site of empty FNPCDNA3 vector. These sequences were then transferred to pcIN4 as described above.

The reporter plasmid Δ56FosdE-luc is the same as the modified minimal 56Fos promoter–containing Δ56FosdE-CAT previously described (19), except that the coding sequence of CAT was replaced by that of luciferase. The CAT coding sequence of Δ56 FosdE-CAT was removed by digestion with Nael and EcoRI and replaced with a HindIII-SmaI fragment containing the luciferase coding sequence from L.56 FosdE-CAT (57). Both the vector (EcoRI) and insert (HindIII) sites were blunted prior to ligation. Similarly, the E.18-luc and Py-luc reporters were constructed as described previously (10), except that the E.18pal oligonucleotide (two adjacent Ets2 binding sites in inverted orientation) or two copies of the Py oligonucleotide (the adjacent Ets and AP-1 binding sites from the polyoma enhancer) were inserted into Δ56FosdE-luc instead of Δ56FosdE-CAT.

Soft Agar Assay—Attached Growth Assay—Soft agar assays were essentially performed as described previously (31). Either 500 or 1000 cells were plated in 60-mm dishes in 1.5 ml of medium containing 0.33% agar, which was overlaid onto 7 ml of solidified medium containing 0.5% agar. The medium used for soft agar assays was DMEM + 10% fetal calf serum, and did not contain G418. The soft agar plates were fed with 0.5 ml of medium every 5–7 days, and after 14 days, the cells were stained overnight (at 37° and 5% CO2) with the vital dye p-iodonitrotetrazolium violet (Sigma) that was prepared as a 10 mg/ml stock solution in ethanol, then diluted with PBS to 1 mg/ml, and 0.5 ml of this diluted solution was added to each dish. In parallel to each soft agar assay, an attached growth assay was performed, where 100 cells from the same cell dilutions were plated on standard tissue culture dishes and grown for 7 days. The resulting attached colonies were stained with crystal violet and counted. The ratio of attached colonies for each cell line was then normalized by multiplying by the ratio (attached colonies from DT cells/attached colonies from the cell line) in the same experiment.
The average tumor volume (in mm$^3$) was estimated to be the average of the tumor length and width. At 2-day intervals, the tumors were measured externally by caliper, and the statistical analysis of tumor growth was performed by comparing the individual volumes of the 6–8 tumors derived from each cell line relative to that of tumors from DT cells treated with the empty expression vector pCIN#5, using a two-tailed paired t-test.

**RESULTS**

**Generation of Stable Ets-expressing DT Cell Lines**—The findings that high expression of the Ets2 DNA binding domain can block oncogene-mediated cellular transformation (11, 22) and can also reverse the transformed phenotype of Ras-transformed fibroblasts (23) indicate that Ets protein function is important in cellular transformation. To better characterize the role of Ets proteins in Ras-mediated cellular transformation, a variety of Ets2 expression constructs were generated, including expression constructs for full-length Ets2, the Ets2 transactivation domains (Ets2TAD), and the Ets2 DNA binding domain (Ets2DBD). A schematic diagram of these constructs is shown in Fig. 1. The ability of these Ets2 expression constructs to reverse Ras-mediated transformation was analyzed in stable clones generated in the DT cell line, a v-Ki-Ras-transformed derivative of mouse NIH3T3 fibroblasts (28). This doubly transformed (DT) cell line is well suited for reversion analysis due to its low rate of spontaneous reversion (29). To improve the frequency of individual stable drug-resistant colonies expressing high levels of the Ets2 constructs, we used the pCIN4 vector (30). This plasmid contains an IRES and generates a bicistronic message with the inserted upstream (Ets2) coding sequences at the 5′ side of the mRNA linked by the IRES to the 3′ neomycin phosphotransferase gene. Because both genes are present on a single mRNA, any G418-resistant colonies derived with this vector should express moderate to high levels of the upstream Ets2 coding sequences. Indeed, as was reported for the serotonin receptor (30), we found a dramatic increase in the number of pCIN-derived stable colonies detectably expressing Ets2 constructs, relative to that seen using the parental pcDNA3 vector (data not shown), and nearly all of the cell lines had clearly visible expression of the introduced gene products (see below). This high frequency of cell lines expressing the introduced Ets2 constructs allowed us to directly analyze the phenotype of stable clones, without preselecting for adherent revertants as was described previously for the Ets2 DNA binding domain (23). For the analysis described below, multiple individual stable DT cell clones generated with each Ets2 expression construct or control plasmid were randomly picked, expanded, and assayed for alterations in the transformed phenotype and for expression of the introduced gene product.

**Expression of Full-length Ets2, Ets2TAD, or Ets2DBD Inhibits Anchorage-independent Growth of DT Cells**—The ability of fibroblasts to grow in an anchorage-independent manner is a hallmark of cellular transformation, and is the best in vitro assay for predicting malignant tumorigenicity in vivo (31). NIH3T3 cells are unable to grow in soft agar, whereas the Ras-transformed DT-3T3 cells plate quite efficiently in soft agar. This assay was used to analyze the capacity for anchorage-independent growth of individual DT cell clones transfected with either empty pcIN vector or the pcIN-Ets2 expression constructs schematically shown in Fig. 1. The results of this analysis are shown in Fig. 2A. None of the five independent cell lines derived with the empty pcIN expression vector displayed significantly reduced growth in soft agar relative to the parental DT cells, but surprisingly, 7/11 of the cell lines derived with full-length Ets2 showed a greater than 4-fold reduction in soft agar growth. This high frequency of reversion seen in randomly picked Ets2-expressing cell lines virtually excludes the possibility that a secondary mutation is required for reversion. The majority of clones derived with the Ets2TAD, which lacks the Ets2 DNA binding domain, also showed significantly reduced anchorage-independent growth. Finally, similar to a previous report (23), several Ets2DBD cell lines also showed significant reductions in anchorage-independent growth (Fig. 2A).

To examine the anchorage-independent growth of several individual clones in more detail, and to establish the reproducibility of the soft agar assay, several frozen cell lines were reestablished and reassayed for growth in soft agar. The small standard deviations of duplicate samples between at least two different plating experiments (Fig. 2B) shows that the soft agar plating assay was highly reproducible. In addition, these data show that anchorage-independent growth in some cell lines, such as Ets2full#2 or Ets2TAD#52, is reduced more than 10-fold.

**Ets2 Expression Constructs Do Not Inhibit Attached Cell Growth**—To determine that reduced soft agar growth reflected a specific loss of anchorage-independent growth and not impaired attached cell growth or errors in cell counting, cells from the same dilution used for the soft agar assay were plated in parallel onto normal tissue culture dishes. The results of a
typical parallel soft agar and attached growth assay are shown in Fig. 3A, where the Ets2-expressing lines show a clear reduction in soft agar growth but not in attached growth. The number of attached colonies obtained in each assay was used to normalize the number of soft agar colonies (see "Materials and Methods"), but this normalization caused only minor changes in the results. Although no clear differences were seen in cell growth rate during expansion or attached colony growth of the various cell lines, the attached growth rates of representative cell lines were examined in more detail. We found that the growth rates of cell lines expressing full-length Ets2 or the Ets2TAD were similar to that of pCIN only lines or the parental DT cells during exponential growth, but consistent with a more reverted phenotype, the cell lines generated with Ets2 expression constructs exhibited a reduced saturation density (Fig. 3B). Overall, analysis of attached colony growth and cell growth rates (Fig. 3, A and B, and data not shown) revealed that the cell lines expressing the Ets2 constructs were not impaired in attached cell growth. Together, the data in Figs. 2 and 3 show that expression of either full-length Ets2 or the Ets2 transactivation domains can strongly and specifically inhibit anchorage-independent growth of DT cells.

Morphological Reversion of DT Cells by Expression of Full-length Ets2 or Ets2DBD, but Not Ets2TAD—The cell morphology of stable cell lines found to exhibit significantly reduced anchorage-independent growth was examined using phase-contrast microscopy of live cells. Normal NIH3T3 cells exhibit a flat, non-refractile morphology, whereas the Ras-transformed DT cells containing empty pCIN expression vector are spindly and refractile (Fig. 4), as are the parental DT cells (data not shown). However, we found that all of the full-length Ets2 or the Ets22DBD DT cell lines which exhibited significantly reduced soft agar growth were flat and non-refractile. Interestingly, this apparent morphological reversion was not observed in any of the cell lines expressing the Ets2TAD, despite the fact that they showed reduced growth in soft agar. The morphology of representative cell lines is shown in Fig. 4. In addition, we analyzed the cytoskeletal organization of the various cell lines after staining with rhodamine-conjugated phalloidin. As previously observed, NIH3T3 cells contained well organized actin stress fibers, which are not present in Ras-transformed DT cells (Fig. 4). Similar to the changes in overall cell morphology, the actin stress fibers reappeared in DT cell lines expressing full-length Ets2 or the Ets22DBD, but not in cells expressing the Ets2TAD (Fig. 4). Thus, unlike expression of full-length Ets2 or

| DT cells transfected with: | Number of stable lines with indicated soft agar colony formation (relative to DT) |
|---------------------------|----------------------------------------------------------------------------------|
|                          | >50%  | 25-50% | 10-25% | <10% |
| pCIN                     | 5     | 0      | 0      | 0    |
| Ets2full                 | 2     | 1      | 4      | 4    |
| Ets2DBD                  | 5     | 1      | 5      | 3    |
| EtsTAD                   | 1     | 0      | 4      | 2    |
| E2TAD(A72)               | 1     | 2      | 2      | 1    |
| E2Full(A72)              | 0     | 2      | 2      | 1    |
| VP16-E2full              | 0     | 0      | 0      | 10   |
| VP16 only                | 6     | 1      | 0      | 2    |
the Ets2DBD, expression of the Ets2 TAD is unable to cause reversion of cell morphology or actin filament reorganization in DT cells, indicating that there are differences in the targets of these Ets2 constructs.

The Ets2 Thr-72 Residue Is Not Required for Reversion Activity—We previously showed that phosphorylation of the Ets2(T72) residue is essential for the large Ras pathway-mediated increase in Ets2 transactivation activity, and that an Ets2(A72) mutant retained basal transcriptional activity, but lost Ras responsiveness (10, 11). To test the connection between Ras signaling to Ets2 and the ability of expressed Ets2 to revert transformation, we determined whether expression of Ets2TAD(A72) or full-length Ets2(A72) could reverse the anchorage-independent growth or transformed morphology of DT cells. Schematic diagrams of the pCIN expression constructs used for this analysis are shown in Fig. 1. As described above for the wild type Ets2 constructs, multiple independent cell lines were derived after transfecting these expression constructs into DT cells. The ability of individual stable cell lines to grow in soft agar is shown in Fig. 2A. Although introduction of the (A72) mutation caused a modest reduction in apparent reversion activity, both the full-length Ets2(A72) and the Ets2TAD(A72) constructs still clearly caused significant decreases in anchorage-independent growth. The soft agar growth analysis of three cell lines containing either the Ets2TAD(A72) or the Ets2(A72) expression constructs (Fig. 5B) revealed an over 4-fold reduction in anchorage-independent growth, indicating that phosphorylation of Ets2(T72) was not essential for their reversion activity. As described above, parallel plating experiments on tissue culture dishes and growth rate analysis demonstrated that the reduced soft agar colony formation in these cell lines was not due to a reduced cell growth rate or altered plating efficiency (data not shown). Analysis of the effects of full-length Ets2(A72) or the Ets2TAD(A72) on cell morphology and actin stress fibers revealed that these Ala-72 constructs had the same effect as their wild type counterparts; the full-length Ets2(A72) cell lines appeared reverted, and the Ets2TAD(A72) cell lines did not (data not shown).

Reversion Activity of Full-length Ets2 Constructs Correlates with their Transactivation Activity—The surprising result that expression of full-length Ets2 could inhibit DT cell anchorage-independent growth and reverse-transformed cell morphology, and that the growth and transcriptionally active Ets2(A72) mutant was less efficient at reversion (Fig. 2A), suggested that the observed reversion was not simply due to inhibition of Ets-dependent transactivation. To analyze the role of Ets2-mediated transcription activation in reversion activity, we generated a full-length Ets2 construct with increased transactivation activity. The powerful viral transcription activation domain from VP16 was fused to the N terminus of full-length Ets2. Cotransfection of the resulting pCIN-VP16-Ets2 expression construct was found to transactivate the expression of the Ets-dependent reporter gene E.18pal by over 100-fold in NIH3T3 cells (data not shown), a substantial increase over the maximal 10-fold activation we observed with normal full-length Ets2 (10, 19). To confirm that full-length Ets2 and VP16-Ets2 also activate Ets-dependent transcription in the context of the Ras-transformed DT cells, we assayed the effects of transiently cotransferring the various Ets2 expression constructs with several reporter genes in DT cells. As previously reported (23), expression of the Ets2DBD inhibited Ets-dependent transcription (data not shown). In DT cells, the Ets2 expression constructs did not significantly alter expression of the minimal promoter-containing reporter Δ56FosD4 (Fig. 5A). Although only a small amount of Ets-dependent reporter gene activation was seen with Ets2(A72), wild type Ets2 expression activated the expression of the E.18 reporter, which contains two Ets binding sites, by 3.1-fold (±0.5) and Pβ2 reporter, which contains two copies of adjacent Ets and AP-1 binding sites, by 2.3-fold (±0.1). Expression of VP16-Ets2 activated E.18 and Pβ2 transcription by 31.5-fold (+7.3) and 8.6-fold (+3.1), respectively (Fig. 5A). Preliminary analysis of the stable clones has shown the same pattern of E.18 reporter gene transactivation. Thus, as assayed by these reporter genes in DT cells, expression of full-length Ets2 does indeed activate Ets-dependent transcription, and VP16-Ets2 is a significantly stronger transcriptional activator.

Stable DT cell lines were derived both with the VP16-Ets2 construct, and a VP16-only construct as a control. Soft agar growth of all 10 of the individual VP16-Ets2 clones examined was inhibited more than 4-fold (Fig. 2A). Several of these clones almost completely lost their ability to grow in soft agar (Fig. 5B). Morphological analysis revealed that, like full-length Ets2-expressing cell lines, the VP16-Ets2 lines had reverted to a flat morphology with clearly visible actin stress fibers (data not shown). The expression of VP16 alone did not cause impaired growth in soft agar for most of the stable clones (Fig. 2A). However, two of nine clones showed significant inhibition of soft agar growth, consistent with a previously reported observation that very high levels of VP16 expression inhibits Ras transformation (32). As described for the other Ets2 constructs above, parallel plating experiments and growth curve analysis showed that the almost complete loss of anchorage-independent growth of the VP16-Ets2 cell lines was not due to reduction of attached growth rate (data not shown).

Expression Levels of Ets2 Constructs in Reverted Cell Lines—Because DT cells already express full-length Ets2, reversion of the transformed phenotype by the introduced full-length Ets2

---

2 G. Foos and C. A. Hauser, unpublished data.
expression construct is likely due to the elevated levels of Ets2 present in the reverted cell lines. To measure the amount of introduced full-length Ets2 mRNA relative to the endogenous Ets2 levels, RNase protection assays were performed with a probe that could distinguish between the endogenous Ets2 and the FN-tagged introduced Ets2 (Fig. 6A). To determine how much overexpression was required for reversion, we tested a full-length Ets2 cell line that was only moderately reverted (Ets2full#2), and two others that showed strongly reduced growth in soft agar (Ets2full#5 and Ets2full#6, see Fig. 2B).

Quantitative analysis of the data shown in Fig. 6A (see "Materials and Methods") revealed that the introduced Ets2 mRNA was expressed at an 8-, 12-, and 17-fold higher level than the endogenous Ets2 mRNA in Ets2full#2, Ets2full#5, and Ets2full#6, respectively. The introduced Ets2 did not appear to influence endogenous Ets2 expression, as normalization of the introduced Ets2 to L32 message (Fig. 6A) gave the same ratios of relative expression. Thus, we estimate that approximately 10-fold overexpression of Ets2 is required for strong reversion activity in DT cells. Consistent with this idea, the last lane in Fig. 6A shows that in a tumor that arose after a significant delay following injection of the Ets2#6 line into a nude mouse, introduced Ets2 mRNA had dropped from 17- to only 5-fold greater levels than endogenous Ets2.
Ets2 Reverts Ras-mediated Transformation

5 × 10^5 cells were subcutaneously injected into each dorsal flank of nude mice, and the tumors were measured by caliper on the indicated days. Tumor volumes were estimated as described under “Materials and Methods,” and the average volume for 6–8 separate tumors derived from each cell line is shown. Statistically significant (p < 0.05) reductions in tumor growth, relative to tumors from DT cells containing empty vector (pCIN#5), were determined using Student’s t-test, and significantly reduced tumor volumes are indicated in bold text.

### TABLE I

| Cell line | Day 4 | Day 6 | Day 8 | Day 10 |
|-----------|-------|-------|-------|--------|
| pCIN5     | 19 ± 7 | 263 ± 159 | 1,331 ± 649 | 2,816 ± 1,272 |
| full#5    | 4 ± 7  | 51 ± 39  | 390 ± 246  | 1,223 ± 661  |
| full#6    | 8 ± 16 | 103 ± 62 | 399 ± 379  | 1,467 ± 949  |
| DBD#10    | 16 ± 11| 147 ± 96 | 470 ± 228  | 1,736 ± 1,136 |
| TAD#53    | 8 ± 16 | 49 ± 51  | 357 ± 277  | 1,618 ± 1,004 |
| VP16full#1 | 22 ± 13 | 76 ± 62 | 630 ± 516  | 1,553 ± 973  |
| VP16full#7 | 7 ± 8  | 93 ± 87  | 255 ± 245  | 1,197 ± 1,056 |

### An assumption in the above assays comparing the apparent reversion strength of individual cell lines is that the various pCIN-Ets2 expression constructs are expressed over similar ranges, and that for each construct, the apparent reversion correlates to the expression level of the introduced protein. Six randomly selected Ets2DBD cell lines were assayed both for reduced growth in soft agar (reversion) and for Ets2DBD protein expression (Fig. 6B). These results show that, as postulated from the bicistronic message encoding Ets2DBD and G418 resistance, all of these G418-resistant cell lines express levels of the Ets2DBD protein detectable by Western blotting. The blotted Ets2DBD protein was detected using the anti-FLAG antibody directed against the N-terminal FLAG epitope tag of Ets2DBD. Comparison of the Ets2DBD expression levels and the reduction of soft agar growth in these cell lines (Fig. 6B) shows that there is indeed a correlation between Ets2DBD expression and apparent reversion in these cell lines.

The steady state protein levels of other Ets2 constructs in individual cell lines were similarly analyzed by Western blot. The relative differences in introduced full-length Ets2 mRNA levels (Fig. 6A) were reflected in the steady-state Ets2full protein levels (Fig. 6C). VP16Ets2full protein levels were also analyzed in several clones, and there was about 4-fold more VP16Ets2full#7 than Ets2full#5. Because the reversed VP16-Ets2full clones all had higher stable levels of protein expression than the Ets2full clones, we cannot exclude the possibility that the enhanced apparent reversion activity of the strongly trans-activating VP16Ets2 constructs was due to dosage effects. The very high level of expression of E2TAD seen in the reverted E2TAD#52 line (Fig. 6C) was also seen in several other reverted E2TAD lines, and RNase protection analysis revealed that the Ets2TAD mRNA was approximately 100-fold more abundant than the endogenous Ets2 mRNA (data not shown).

Ets2 Constructs Act Downstream of Ras Expression or MAP Kinase Activation. To determine that the Ets2 constructs were not reverting the transformed phenotype of DT cells by simply extinguishing the expression of oncogenic Ras, we confirmed that the high level of Ras normally seen in DT cells was still present in the reverted clones, as had been previously shown for EtsDBD constructs (23). Western blot analysis using pan-Ras antibody (Fig. 7A) shows that indeed, a DT line containing empty pCIN vector expressed much higher levels of Ras than NIH 3T3 cells, and (even on a shorter exposure) that reverted cell lines expressing either full-length Ets2 or the Ets2TAD still expressed high levels of Ras similar to that of DT cells. Therefore, the effects on the transformed phenotype by the Ets2 constructs were not due to reduced Ras expression.

The expressed Ets2 proteins are likely to act well downstream of Ras, and we postulated that they would also act downstream of the MAP kinases ERK1 and ERK2. These ERKs are targets of Ras/Raf/MEK signaling, but are still upstream regulators of Ets2 transcriptional activity (12). We therefore analyzed MAP kinase activity in several cell lines, using an immune complex assay. For this assay, the ERK1 and ERK2 were immune precipitated from whole cell extracts, and their activity was assayed using myelin basic protein as a substrate. Quantitation of the signals shown in Fig. 7B by phosphorimager analysis revealed that ERK activity was induced 10-fold by serum in NIH3T3 cells and 4-fold in DT cells. As has been previously observed (33), the basal ERK activity in the stably Ras-transformed cells was only modestly higher than in non-transformed cells. Fig. 7B shows there was not a significant difference between the basal levels of ERK activity found in the parental DT cells and in representative Ets2 construct-rever ted cell lines, indicating that the Ets2 constructs were not altering ERK activity in the reverted cell lines.

**Ets2 Expression Reduces Tumorigenicity of DT Cells in Nude Mice**—To determine whether the Ets2-mediated morphological reversion or increased anchorage-dependence seen in vitro reflects a reduced ability to form tumors, we performed tumorigenicity assays in nude mice. The growth of 6–8 tumors for each cell line in nude mice was followed by external measurement every 2 days, as described in Methods. The volume of tumors derived from the DT cell lines expressing various Ets2 constructs (cell lines shown above to exhibit reversion in vitro) were compared with that of a DT cell line containing an empty pCIN expression vector (Table I). The rapid tumor growth of the pCIN#5 cell line was not significantly different than another empty vector DT cell line pCIN#6 (n = 8), or to the parental DT line (n = 4), (data not shown). However, the tumor volumes were significantly smaller than pCIN#5 for both full-length Ets2 cell lines and both VP16-Ets2 cell lines on days 6, 8, and 10 (Table I). As described above (Fig. 3B and data not shown), these cell lines had the same attached growth rate as the pCIN#5 line in culture, which suggests that the expressed Ets2 specifically inhibited tumorigenicity. Reductions in tumor growth were also seen in the Ets2TAD#53 and Ets2DBD#10 cell lines, but the smaller tumor volumes were statistically significant only on days 6 and 8 for the Ets2TAD line and only on day 8 for the Ets2DBD line. Due to the very high initial growth rate of the control cell lines, by day 10 the tumors from pCIN or parental DT cells were quite large, and started to
Ets2 Reverts Ras-mediated Transformation

It has been previously observed that stable expression of just the DNA binding domain (DBD) of the Ets factors Ets1, Ets2, or PU.1 reverses multiple features of the transformed phenotype in Ras-transformed NIH3T3 cells (23). In transient assays, these Ets DBD constructs exhibit a dominant inhibitory effect on Ets-dependent transcription, and on Neu/ErbB-2- and Ras-mediated activation of Ets-dependent gene expression (11, 22, 23). We show here that elevated expression of full-length Ets2, an activator of Ets-dependent transcription, can also specifically reverse the transformed phenotype of Ras-transformed NIH3T3 cells. The reverted phenotype in multiple stable Ets2-expressing cell lines included a reduction in anchorage-dependent growth, tumorigenicity, and saturation density. In addition, these Ets2 reverted cell lines exhibited more normal cell morphology, and reappearance of actin stress fibers which are lost in transformed fibroblasts. The inhibition of anchorage-independent growth by highly expressed Ets2 was specific, as anchorage-dependent growth was not inhibited in these cell lines. Reversion of the DT-derived Ets2-expressing cell lines was likely a direct consequence of Ets2 expression and not from selection of a secondary mutation, as 7/11 randomly picked stable DT cell lines generated with the Ets2 expression plasmid exhibited a strong (≥75%) reduction in anchorage-independent growth.

To test a potentially more specific inhibitory construct for Ets-dependent transcription, we also assayed the reversion activity of expressing the portion of Ets2 that does not bind DNA, the Ets2TAD. We previously found that high expression of this Ets2TAD, which presumably interacts with some limiting protein, could block Ras or Neu-mediated activation of an Ets-dependent reporter gene (11). In contrast to the ability of the Ets2DBD or full-length Ets2 to more completely reverse transformation, we show here that expression of Ets2TAD partially reversed the transformed phenotype of DT cells. Ets2TAD expression strongly inhibited anchorage-independent cell growth, but did not reverse transformed cell morphology. These findings suggest that the functions of Ets proteins in anchorage-independence and transformed morphology are separable, and that Ets factors impact on multiple pathways required in cellular transformation. In light of the differences in morphological reversion activity of Ets2 and the Ets2TAD, potential targets in reversion for Ets2 may include Rho family members or integrin-linked kinases.

Because phosphorylation of the Thr-72 residue of Ets2 has been found to be essential for the Ras-mediated increase in Ets2 transactivation activity (10, 11), we tested whether a Thr to Ala mutation would disrupt the reversion activity of highly expressed Ets2, both in the context of the Ets2 TAD, and in full-length Ets2. Although the Ets2(A72) mutation may have slightly reduced Ets2 reversion activity, the data showed that the Thr-72 residue was not essential for reversion activity in either construct. Although Thr-72 phosphorylation is not essential for reversion activity, we can not exclude that these Ala-72 constructs could still act by competing for a kinase, as the kinase recognition sequence may be distinct from the phosphorylation site.

Inhibition of Ras transformation in fibroblasts has previously been observed only with Ets constructs that inhibit expression of Ets-dependent transcription, and thus it was somewhat unexpected that expression of full-length Ets2 had strong and broad reversion activity. It was previously hypothesized that the Ets DBD acts as a dominant negative Ets protein which blocks the activation of genes whose persistent activation by Ras signaling is required for maintaining the transformed phenotype (34). To assess whether the transactivation activity of the overexpressed Ets2 contributes to its reversion activity, we synthetically increased Ets2 transactivation activity by N-terminal addition of the VP16 transactivation domain. This VP16-Ets2 construct showed strongly enhanced transactivation activity, and had the strongest apparent reversion activity of any of the Ets2 constructs we tested, both in the in vitro assays and the tumorigenicity assays. These results further indicated that activation of Ets-dependent transcription can also lead to reversion. A related finding was recently reported in the colon cancer cell line DLD-1, where ectopic expression of full-length Ets1 caused a partial reversion of the transformed phenotype of these cells, and an Ets1 mutant that had lost its transactivation activity also lost its reversion activity (35). These findings with DLD-1 cells, in which activated K-Ras has been shown to play a key role in their transformation (36), are consistent with our present data indicating that increasing Ets-dependent transcription can reverse Ras-mediated cellular transformation. This ability of highly expressed Ets1 and Ets2 to reverse cellular transformation is in contrast to the initial characterization of Ets1 and Ets2 as proto-oncogenes, whose overexpression could transform NIH3T3 cells (20, 21). However, the rare appearance of transformed cells in these experiments suggested that selection for a secondary event was required for transformation (20, 21). In several experiments, we have failed to observe direct transforming activity of highly expressed Ets2 in NIH3T3 cells.3

Although it is difficult to compare the reversion strength of different expression constructs in stable cell lines, in our parallel analysis of the reversion activity of full-length Ets2 and the Ets2DBD, it appeared as though the full-length Ets2-expressing cell lines tested were more reverted than the Ets2DBD lines in all of the transformation assays. The fact that the Ets2DBD-expressing lines were less well reverted than similar previously described lines (23) is likely due to the fact that in the previous study, strong selection for morphological reversion (flat, highly adherent cells) was applied prior to scoring or assaying the reverted phenotype. Given the dose response for reversion seen with the randomly picked Ets2DBD (Fig. 6B), it is likely one could find more reverted Ets2DBD cell lines. We postulate that lack of sufficient Ets2 construct expression was also responsible for the eventual tumor growth in the tumorigenicity assays (Table I). It has been reported that, in the absence of selection, the neomycin resistance gene can silence a nearby CMV promoter (37). When we assayed the level of introduced full-length Ets2 mRNA in a tumor that had grown up after significant delay, we found that the overall Ets2 expression level had dropped to below what was required to observe reversion in vitro (Fig. 6A). Thus, although this study showed a clear in vivo effect of full-length Ets2 expression inhibiting tumor growth, a more stable expression system will

3 C. K. Galang and C. A. Hauser, unpublished data.
Ets2 Reverts Ras-mediated Transformation

be required to assay the long term efficacy of reversing tumorigenicity by altering Ets-dependent transcription.

There are several distinct mechanisms that could explain how Ets2 constructs with seemingly opposite activities (inhibition or activation of Ets-dependent transcription) could revert Ras-mediated transformation. If these Ets2 constructs act on common targets, it is possible they do so by displacing an Ets family negative regulator which becomes more active in Ras-transformed cells. In such a model, the activity of an Ets-family repressor would be required to maintain the transformed state. The inhibitory Ets2 constructs would displace this repressor, and the activating constructs would displace it and additionally activate expression of the genes whose expression causes reversal. Such a repressor must act in a promoter context distinct from E18 or Py2, as we have found that Ets2DBD expression inhibits the transcription of these reporter genes in both NIH3T3 and DT cells (Ref. 11 and data not shown). Another possible common mechanism of reversion involves interaction of the Ets constructs with AP-1 family proteins, as both the Ets domain and the Ets2 TAD have been reported to physically interact with AP-1 family members involved in cellular transformation (38–40). However, we found that expression of the Ets2 TAD or DBD does not influence Ras or Her2Neu activation of an AP-1-dependent reporter gene (11). Alternatively, the inhibitory and activating Ets2 constructs may have distinct targets. One possible target for reversal by activating Ets2 constructs is p21Cip1/Waf1, as high level stimulation of Raf, an upstream activator of Ets activity, activates p21Cip1 expression (41–43). Expression of p21Cip1 is also induced by an Ets transcription factor (44), and overexpression of p21Cip1 can reverse anchor-age-independent growth of transformed cells without interfering with their attached growth (45). Other possible targets for reversion through activation of Ets-dependent transcription include the tumor suppressors p53 and Rb. The p53 promoter has tandem Ets sites arrayed very similarly to the Rb promoter has tandem Ets sites arrayed very similarly to the Ras promoter has tandem Ets sites arrayed very similarly to the Rb promoter has tandem Ets sites arrayed very similarly to the Ras promoter has tandem Ets sites arrayed very similarly to the Rb promoter has tandem Ets sites arrayed very similarly to the Rb promoter has tandem Ets sites arrayed very similarly to the Rb promoter has tandem Ets sites arrayed very similarly to the Rb promoter has tandem Ets sites arrayed very similarly to the Rb promoter has tandem Ets sites arrayed very similarly to the Rb promoter has tandem Ets sites arrayed very similarly to the Rb promoter has tandem Ets sites arrayed very similarly to the Rb promoter has tandem Ets sites arrayed very similarly to the Rb promoter has tandem Ets sites arrayed very similarly to the Rb promoter has tandem Ets sites arrayed very similarly to the Rb promoter has tandem Ets sites arrayed very similarly to the Rb promoter has tandem Ets sites arrayed very similarly to the Rb promoter has tandem Ets sites arrayed very similarly to the Rb promoter has tandem Ets sites arrayed very similarly to the Rb promoter has tandem Ets sites arrayed very similarly to the Rb promoter has tandem Ets sites arrayed very similarly to the Rb promoter has tandem Ets sites arrayed very similarly to the Rb promoter has tandem Ets sites arrayed very similarly to the Rb promoter has tandem Ets sites arrayed very similarly to the Rb promoter has tandem Ets sites arrayed very similar
Ets2 Reverts Ras-mediated Transformation

J. H. (1996) Oncogene 12, 775–784
45. Funaoka, K., Shindoh, M., Yoshida, K., Hanzawa, M., Hida, K., Nishikata, S., and Fujinaga, K. (1997) Biochem. Biophys. Res. Commun. 236, 79–82
46. Venanzoni, M. C., Robinson, L. R., Hodge, D. R., Kola, I., and Seth, A. (1996) Oncogene 12, 1199–1204
47. Savoysky, E., Mizuno, T., Sowa, Y., Watanabe, H., Sawada, J., Nomura, H., Ohsugi, Y., Handa, H., and Sakai, T. (1994) Oncogene 9, 1839–1846
48. Eliyahu, D., Michalovitz, D., Eliyahu, S., Pinhasi-Kimhi, O., and Oren, M. (1999) Proc. Natl. Acad. Sci. U. S. A. 86, 8763–8767
49. Kvinin, L., Pitkanen, K., and Laiho, M. (1995) Oncogene 8, 2703–2711
50. Albanese, C., Johnson, J., Watanabe, G., Eklund, N., Yu, D., Arnold, A., and Pestell, R. G. (1995) J. Biol. Chem. 270, 23589–23597
51. O’Hagan, R. C., Tozer, R. G., Symons, M., McCormick, F., and Hassell, J. A. (1996) Oncogene 13, 1323–1333
52. Janknecht, R., Monte, D., Baert, J. L., and de Launoit, Y. (1996) Oncogene 13, 1745–1754
53. Janknecht, R. (1996) Mol. Cell. Biol. 16, 1550–1556
54. Gille, H., Sharrocks, A. D., and Shaw, P. E. (1992) Nature 358, 414–417
55. Li, C., Lusis, A. J., Sparkes, R., Tran, S. M., and Gaynor, R. (1992) Genomics 13, 658–664
56. Hauser, C. A., Der, C. J., and Cox, A. D. (1994) Methods Enzymol. 238, 271–276
57. de Wet, J. R., Wooli, K. V., DeLasa, M., Helinski, D. R., and Subramani, S. (1987) Mol. Cell. Biol. 7, 725–737
58. Hauser, C. A., Westwick, J. K., and Quilliam, L. A. (1995) Methods Enzymol. 235, 412–426
59. Reuter, C. W., Catling, A. D., and Weber, M. J. (1995) Methods Enzymol. 255, 245–256
60. Neznanov, N. S., and Oshima, R. G. (1993) Mol. Cell. Biol. 13, 1815–1823
61. Neznanov, N. S., Umezawa, A., and Oshima, R. G. (1997) J. Biol. Chem. 272, 27549–27557