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c-*ets-2* protooncogene has mitogenic and oncogenic activity

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ABSTRACT **An expression vector containing the murine c-*ets-2* protooncogene cDNA was introduced into NIH 3T3 cells by DNA transfection. The cells transfected with this construct showed foci of densely growing, morphologically altered cells, when grown either in low-serum (0.05%) or in serum-free medium. The c-*ets-2*-derived foci contained additional copies of the c-*ets-2* gene, Northern blot analysis demonstrated overexpression of a c-*ets-2*-specific 2.5-kilobase RNA, and *ets*-specific antiserum recognized a 56-kDa protein. Overexpression of the c-*ets-2*-encoded protein stimulated cell proliferation and abolished their serum requirement. The c-*ets-2* transfected cells formed colonies in semisolid medium and induced tumors in nude mice, indicating that c-*ets-2* can be a transforming gene when overexpressed in these cells. This work demonstrates that a member of the c-*ets* gene family has transforming and mitogenic activity. In addition, the role of c-*ets-2* in cell proliferation and its location in the minimal Down syndrome region on chromosome 21 implicates its involvement in the phenotypic changes associated with Down syndrome.**

Protooncogenes were originally discovered as the cellular progenitors of viral oncogenes transduced by the acute transforming retroviruses (1). These genes can be activated by several mechanisms to become efficient transforming genes (2). The protooncogenes are highly conserved throughout evolution, suggesting that they probably play an important role in cell proliferation, cell differentiation, and/or development.

The human *ETS2* gene, first identified in our laboratory, is a member of a family of genes related to the v-*ets* of the avian leukemia virus E26. This gene has been localized to the long arm of chromosome 21 (3) and encodes a 56-kDa nuclear protein (4). c-*ets-2* genes have been cloned and sequenced from a variety of organisms including *Xenopus* (Z. Q. Chen, L. Burdett, J. Lautenberger, and T.S.P., unpublished data) chicken (5), mouse, and human (6) and were found to be highly conserved. The pattern of mRNA expression of c-*ets-2* is very similar to that of c-*myc* and c-*fos*; it is induced in regenerating liver and is superinduced in the presence of protein synthesis inhibitors (7). It would appear that c-*ets-2*, as with other protooncogenes, may play an important part in regulating cellular growth and differentiation. The *ETS2* gene is translocated from chromosome 21 to chromosome 8 in the specific translocation t(8;21) known to be associated with acute myelogenous leukemia (8, 9). The location of the *ETS2* gene at 21q22 has suggested that it may be a contributing genetic element in the cluster of genes whose amplification is associated with Down syndrome (DS) (9, 10), and this concept is supported by the observation that the *ETS2* gene is found in three copies in partial trisomies 21 associated with the DS phenotype (11, 12).

Comparative human and murine genetic maps show that *ETS2* belongs to a group of genes on chromosome 21 that are also present syntenically on mouse chromosome 16 (13). Not surprisingly, in mice with trisomy 16, a few DS phenotypic features common with DS patients are also present (10, 14). Such animal models should be valuable in providing information on the relationship between overproduction of gene products and the phenotypic manifestations of DS. An alternative approach that we explored was to introduce the *ETS2* protooncogene into cells by transfection, using an expression vector we have constructed. Unlike the mouse system, this *in vitro* system will enable us to investigate how an increase in one gene, the *ets-2* gene, may affect that delicate balance of regulatory processes that maintain essential function(s) of the cell. Such alterations could perturb other mitogenic signals and thus an increase in expression of the proto-*ets-2* gene might have drastic ramifications on the steady-state equilibrium of the cell relevant to the manifestation of DS. In fact, we shall demonstrate that, in our model transfection system, the overexpression of the *ets-2* gene does markedly alter the recipient cells, mitogenically stimulating them and abolishing their serum requirements. Furthermore, these *ets-2* transfected cells form colonies in semisolid medium and are tumorigenic in nude mice.

MATERIALS AND METHODS

Construction of the c-*ets-2* Expression Vector pMME-18. The parent vector (pMVBneo) used to construct the c-*ets-2* expression vector contains the mouse metallothionein promoter, the neomycin gene to confer resistance against G418, and the bidirectional poly(A) signals from simian virus 40 (SV40) (see Fig. 1) (15). This vector also contains a unique *Bam*HI site downstream of the metallothionein promoter. To construct the c-*ets-2* expression vector, the plasmid pMVBneo was digested with *Bam*HI and treated with calf intestine phosphatase. The c-*ets-2* gene was obtained by digesting pA3 (mouse *ets-2* cDNA clone) with *Eco*RI and *Ava* I (6) and isolating the 1.6-kilobase (kb) fragment. The 1.6-kb fragment was repaired with dXTPs and Klenow fragment to make the ends flush and was then ligated to *Bam*HI linkers (16). This *Bam*HI-flanked c-*ets-2* gene was then ligated to *Bam*HI-digested and calf intestine phosphatase-treated pMVBneo. The ligation mixture was transformed into competent DH5α cells (Bethesda Research Laboratories). The vectors with c-*ets-2* in correct orientation (pMME-18) and reverse orientation (pMME-11) were isolated and used for transfection.

Cell Culture and Transfection. NIH 3T3 fibroblasts (17) were maintained in Dulbecco's modified minimum essential medium (DMEM) supplemented with 8% calf serum. The transfection of cells with calcium phosphate DNA precipitate and selection in medium containing G418 have been described (17). Briefly, cells were seeded at a density of 2×10^5 cells per dish and were transfected with 1 μg of plasmid DNA.

Focus Formation Assay. The G418-resistant colonies obtained after transfection with c-*ets-2* expression vectors were pooled and grown in a defined medium containing low serum (0.05% calf serum) or no serum. This defined medium (QBSF-

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Abbreviations: DS, Down syndrome; SV40, simian virus 40.

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51) was obtained from Quality Biologicals (Gaithersburg, MD) and it allows the growth of NIH 3T3 cells when supplemented with epidermal growth factor or fibroblast growth factor (18). Medium was changed every 2–3 days and foci of densely growing cells appeared after 3–4 weeks. Some of the foci were cloned and grown to mass culture for DNA, RNA, and protein analysis.

Southern and Northern Blot Analysis. High molecular weight DNA was prepared as described (16) and digested with restriction enzymes. The digested DNA was run on a 0.8% agarose gel followed by transfer onto nitrocellulose. The nitrocellulose blot was hybridized with a mouse c-*ets-2* probe containing only the last two exons (D.K.W., unpublished results). Total RNA was extracted from transfected cells by a guanidine isothiocyanate/CsCl centrifugation method (16). RNA samples (10 μg) were run on formaldehyde-containing agarose gels and transferred to nitrocellulose paper. The nitrocellulose blots were probed with a mouse c-*ets-2* cDNA probe. Detailed conditions for hybridization and washing have been described (16).

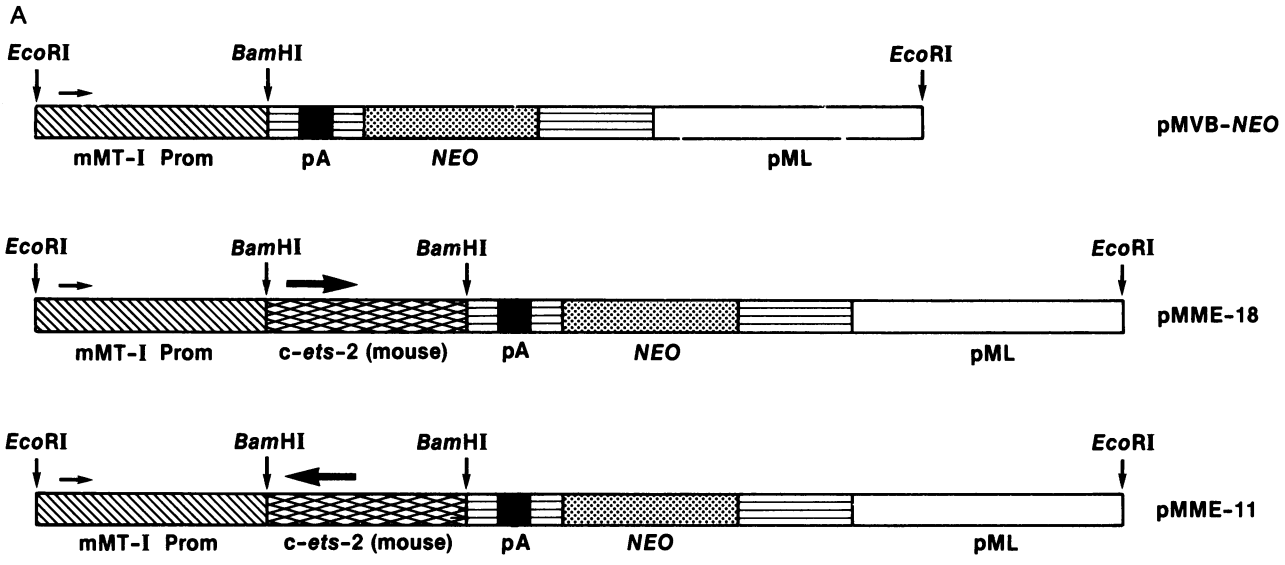
Protein Extraction and Immunoprecipitation. Cells were starved in methionine-free medium for 0.5 hr before labeling with 1 mCi of [^{35}S]methionine for 1 hr (1 Ci = 37 GBq). After labeling, the cells were washed with phosphate-buffered saline and lysed with RIPA buffer containing protease inhibitors (4, 19). The cell extracts were centrifuged at 100,000 × *g* for 30 min and the supernatant was used for immunoprecipitation with *ets*-specific antibody (ZC-50) (4, 19). Details of the immunoprecipitation and SDS/PAGE have been described (4, 19).

Colony-Forming Assay in Semisolid Medium. Colony-forming assays were done in 0.3% agarose and have been described by Moscovici *et al.* (20). Cells were seeded at concentrations of 10^4 and 10^5 cells per dish in soft agar in 60-mm plates and were fed once a week with an agar overlay containing DMEM and 8% calf serum. Colonies were scored after 2 weeks.

Tumorigenicity in Nude Mice. Athymic nude mice were subcutaneously inoculated with c-*ets-2* transfected cell lines grown in either serum-free or low-serum medium (21). The cell suspensions were adjusted to 10^7 cells per ml and 0.1 ml was injected into three animals per experiment. The mice were examined for tumor development every week. In most cases, the tumors appeared in approximately 3 weeks. Cell lines derived from tumors were examined for DNA and RNA by Southern and Northern blot analyses.

RESULTS

Construction and Transfection of the c-*ets-2* Expression Vector into Murine Fibroblasts. The mammalian expression vector (pMVBneo), which was used to construct the c-*ets-2* expression vector, contains the mouse metallothionein promoter upstream of a unique *Bam*HI site (Fig. 1*A*) (15). This



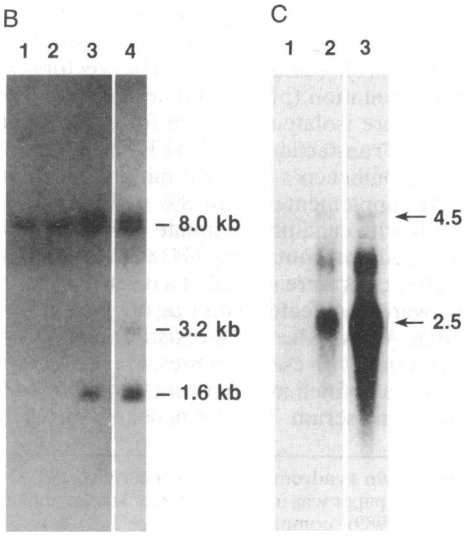


FIG. 1. (*A*) Schematic representation of the *ets-2* expression vectors. pMVBneo is the parent vector, which contains mouse metallothionein promoter (mMT-I Prom) and a unique *Bam*HI site downstream for cloning of DNA fragments. □, Plasmid ML; ▤, SV40 sequences; pA, a bidirectional polyadenylylation signal of the SV40 virus; NEO, the SV40 virus early promoter and neomycin-resistance gene. The c-*ets-2* fragment containing the entire coding sequences was obtained by cleaving the pA3 cDNA clone (6) with *Eco*RI and *Ava* I. This 1.6-kb *Eco*RI/*Ava* I fragment was then repaired with four dXTPs and the Klenow fragment and was ligated to *Bam*HI linkers. The c-*ets-2* cDNA fragment flanked by *Bam*HI was then inserted at the unique *Bam*HI site of pMVBneo. The resultant two vectors with c-*ets-2* sequences in correct orientation (pMME-18) and opposite orientation (pMME-11) were isolated and used for transfection. (*B*) Integration of pMME-18 into the NIH 3T3 genome. High molecular weight DNA was extracted (16) from cell lines transfected with pMME-18 or pMVBneo and digested with *Bam*HI. The DNA was transferred onto nitrocellulose paper (16) and hybridized with ^{32}P-labeled c-*ets-2* probe. The DNA was prepared from NIH 3T3 cells (lane 1), cells transfected with pMVBneo (lane 2), and cells transfected with pMME-18 (lanes 3 and 4). The 8.0-kb band represents the endogenous c-*ets-2* fragment and the 1.6-kb band represents the c-*ets-2* cDNA fragment. The c-*ets-2* probe contained the last two exons and intron of the mouse gene. (*C*) Expression of the c-*ets-2* gene in NIH 3T3 cells. Total cellular RNA was extracted from cells transfected with the c-*ets-2* expression vector and examined by Northern blot analysis. Lanes: 1, NIH 3T3 cells; 2, cells transfected with the c-*ets-2* expression vector (pMME-18); 3, agar clone of the c-*ets-2* (pMME-18) transfected cells.

vector also contains a functional neomycin gene under SV40 promoter control to confer resistance to the drug G418 as well as poly(A) signals derived from the SV40 virus that are located downstream from the *Bam*HI site (Fig. 1*A*). Two c-*ets-2*-containing expression vectors, pMME-18 (correct orientation) and pMME-11 (opposite orientation), were constructed by ligation of a 1.6-kb *Bam*HI fragment containing the entire coding sequences of murine c-*ets-2* into the pMVBneo vector (Fig. 1*A*) (6, 16).

Biological Activities of the c-*ets-2* Expression Vector (pMME-18). The c-*ets-2* expression vectors (pMME-18 and pMME-11) were transfected into the NIH 3T3 cells by the calcium phosphate-DNA precipitate technique (17). After 2 weeks of drug selection, resistant colonies were pooled and tested for their growth in a defined culture medium containing either 0.05% serum or no serum (18). The cells transfected with the pMME-18 vector were able to grow and expand over an extended period of time in either serum-free or low-serum medium; in contrast, control cells or cells transfected with pMME-11 grew relatively slowly in the medium containing 0.05% serum and did not grow in serum-free medium. After 30 days, pMME-18 transfected cells showed foci of densely growing and morphologically altered cells (Fig. 2). No such foci were observed in control cells transfected with the vector alone (data not shown) or the vector containing the *ets-2* insert in reverse orientation (Fig. 2). To determine whether the growth alterations observed are mediated by the transfected *ets-2* gene, we expanded several of the densely growing foci. DNA extracted from these cell lines was digested with *Bam*HI and Southern blots were analyzed by hybridization (16) using an *ets-2*-specific probe. All the cell lines tested showed an 8.0-kb endogenous c-*ets-2* band and also the characteristic 1.6-kb internal *Bam*HI fragment released from the vector (Fig. 1*B*). One of the pMME-18 transfected cell lines showed, in addition to the 1.6-kb band, a 3.2-kb *ets*-specific band (Fig. 1*B*, lane 4). This band could have originated either by loss of one *Bam*HI site or by rearrangement of the gene during transfection. Southern blot analysis with enzymes that do not cut within the c-*ets-2* insert indicated that these cells contained multiple copies of the transfected sequences integrated at multiple sites (22).

Expression of c-*ets-2*-Specific mRNA. To establish whether the transfected c-*ets-2* gene is expressed, total cellular RNA was prepared from cell lines 3AQ-1, 3AQ-2, and 3AQ-3, derived from different foci, and analyzed for the presence of *ets-2*-specific mRNA. Control cells express low levels of a unique 4.5-kb RNA transcribed from the endogenous c-*ets-2* gene. However, in the transfected cell line 3AQ-2 (Fig. 1*C*), as well as the cell lines 3AQ-1 and 3AQ-3 (data not shown), we detected an abundant level of 2.5-kb RNA in addition to the low level of the endogenous 4.5-kb mRNA. The data are consistent with the 2.5-kb RNA being transcribed from the transfected *ets-2* sequences under the control of the mouse metallothionein promoter. That the 2.5-kb mRNA is directed from the metallothionein promoter was further confirmed by the observation that the 2.5-kb RNA, and not the 4.5-kb RNA, was induced when the cells were grown in the presence of 2 μM Cd^{2+} (data not shown).

c-*ets-2* Transfected Cells Produce High Levels of $p56^{c\text{-}ets\text{-}2}$ Product. We have also compared the expression of $p56^{c\text{-}ets\text{-}2}$

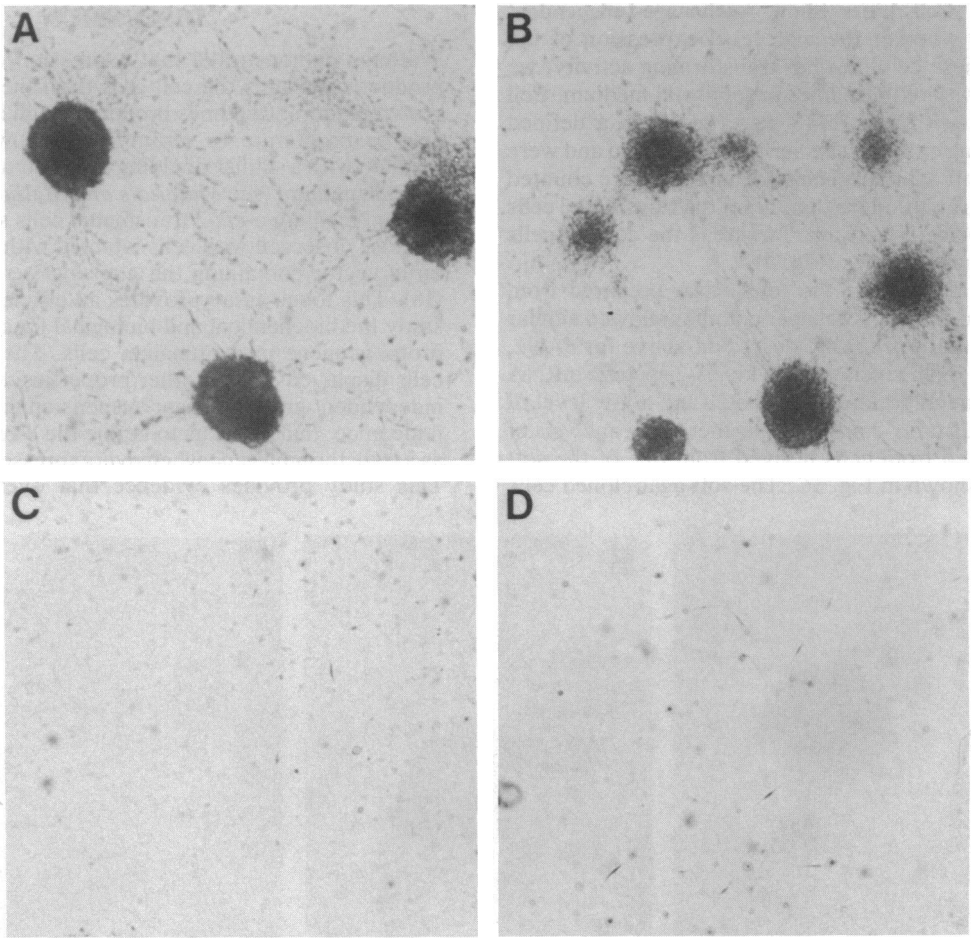


FIG. 2. Transfection of NIH 3T3 cells with c-*ets-2* expression vector. Cells were transfected with pMME-18 (*A* and *B*) and pMME-11 (*C* and *D*). The G418-resistant colonies were pooled and grown in either low serum (*A* and *C*) or serum-free (*B* and *D*) medium. The foci appeared in approximately 4 weeks.

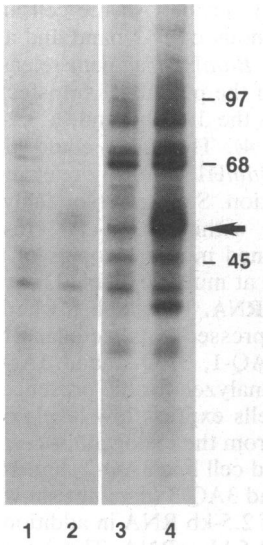


FIG. 3. Expression of 56-kDa c-*ets-2*-encoded protein. Cells transfected with the c-*ets-2* expression vector (pMME-18) were labeled with [^{35}S]methionine for 1 hr. The ^{35}S-labeled proteins were extracted and immunoprecipitated with *ets-2*-specific antiserum (19) in the presence (lanes 1 and 3) or absence (lanes 2 and 4) of excess peptide. Lanes: 1 and 2, NIH 3T3 cells; 3 and 4, cells transfected with the c-*ets-2* expression vector (pMME-18). p56$^{c\text{-}ets\text{-}2}$ is indicated by an arrow. Numbers on right are kDa.

from these cell lines by metabolically labeling the cells with [^{35}S]methionine and analyzing the proteins after immunoprecipitation with *ets*-specific antiserum (4, 19). The results are shown in Fig. 3. Cells transfected with the c-*ets-2* vector (pMME-18) produced high levels of a 56-kDa protein. Competition of the 56-kDa protein by the addition of excess peptide (Fig. 3) indicated that the 56-kDa protein is an *ets*-specific protein. Moreover, this protein was not detected in the nontransfected cells (Fig. 3).

c-*ets-2*-Derived Cell Lines Show Anchorage-Independent Growth. To test whether the high level expression of the c-*ets-2* gene in these cell lines has transforming activity, we analyzed the growth of these lines in semisolid medium. Cell lines transfected with pMME-18 were grown in a defined medium containing either 0.05% serum or no serum and were then seeded in soft agar suspension. Colonies were counted after 2 weeks of growth. More than 1% of the transfected cells gave rise to colonies in soft agar, whereas the control cells failed to show such colonies (Fig. 4).

Northern blot analysis of the total RNA prepared from cells lines derived from several agar colonies showed similar RNA expression pattern to that described above for 3AQ1, 3AQ2, and 3AQ3; high levels of a 2.5 kb *ets*-2 specific mRNA directed by the metallothionein promoter and a low level of 4.5 kb endogenous c-*ets*-2 mRNA. Northern blot analysis of the RNA extracted from cells derived from one of the soft agar colonies is shown in Fig. 1*C*. The soft agar cloned cells express 10- to 20-fold more *ets-2*-specific RNA (Fig. 1*C*, lane 3) than the uncloned cell line 3AQ-2 (Fig. 1*C*, lane 2).

Table 1. Tumorigenicity of the c-*ets-2* transfected cells

Cell line	Tumor incidence in 3 weeks or less
NIH 3T3	0/3
3AQ-1	3/3
3AQ-2	3/3
3AQ-3	3/3
pTS-1 transfectants	3/3

Three mice were injected subcutaneously at a concentration of 10^6 cells per mouse. Tumor formation was checked every week. The pTS-1-derived cell line was generated by transfection of a c-*mos* (mouse) gene linked to the 5′ long terminal repeat of Moloney murine sarcoma virus (23).

c-*ets-2*-Expressing Cell Lines Are Tumorigenic in Nude Mice. To determine whether the pMME-18 transfectants are tumorigenic in nude mice, trypsin-treated cell suspensions (10^6 cells per animal) were inoculated into athymic *nu/nu* mice and examined every week for tumor development. Except for the control cells, all the cell lines tested (Table 1) induced tumors in approximately 3 weeks; the tumorigenicity observed was in fact comparable to the c-*mos* transformed cells (21, 23). Histological analysis of the tumors revealed that they are all fibrosarcomas. Molecular analysis of the cell lines derived from these tumors showed that they contain c-*ets-2*-specific DNA and express the 2.5-kb c-*ets-2*-specific mRNA (data not shown).

DISCUSSION

We have demonstrated that expression of the c-*ets-2* gene product stimulates the cell growth in the absence of serum growth factors. Cell lines producing high levels of c-*ets-2* are able to proliferate in serum-free or low-serum-containing medium with a similar doubling time to that observed with the normal medium (see *Materials and Methods*; ref. 22). In this defined medium c-*ets-2* transfected cells showed foci similar to those observed with cells infected with a murine leukemia virus vector containing the *gag myb ets* gene of E26 virus (18). This focus assay provides an efficient assay system to study the biochemical and biological functions of the c-*ets-2* protooncogene in mammalian cells. The *ets-2* transformed cells displayed several other properties, such as anchorage-independent growth in agar suspension and tumorigenesis in nude mice, that allow us to define the c-*ets-2* protooncogene as a transforming gene when overexpressed in NIH 3T3 cells. This study provides evidence that overexpression of the

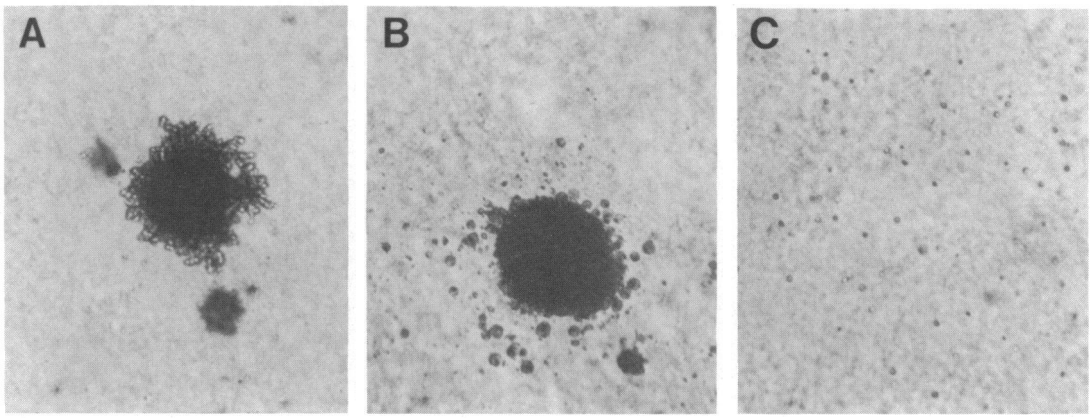


FIG. 4. Anchorage-independent growth of NIH 3T3 cells transfected with the c-*ets-2* expression vector (pMME-18). pMME-18 transfectants were seeded in soft agar suspension at a density of 10^5 cells per plate; before seeding into the soft agar suspension, the cells were grown in medium containing either low serum (*A*) or no serum (*B*). (*C*) Control cells. The soft agar colonies appeared in approximately 2 weeks.

c-*ets-2* gene is necessary to exhibit transforming and mitogenic activity. Previously, it was reported that the *gag myb ets* fusion product had mitogenic effects in avian and murine systems (18, 24). Together, these results suggest that the *ets* domain is in fact essential for the growth-promoting activity.

Since focus formation occurs after prolonged cell culture in a defined medium containing no serum or low serum, it is possible that a secondary event may also be required for such a phenotype. Clearly, *ets-2* expression is necessary to induce foci because cell lines derived from these foci produced high levels of *ets-2* mRNA. Moreover, the soft agar cloned cells expressed much higher levels of *ets-2* mRNA than the uncloned cell lines. In addition, the growth rates of all the *ets-2* cell lines have a positive correlation with the expression of *ets-2* mRNA.

In spite of the fact that DS is associated with a trisomy for the region of chromosome 21q22, only a limited number of expressed genes may be actually responsible for the manifestation of the DS pleiotropic phenotype. The chromosomal location and putative proliferative role of c-*ets-2* as shown here makes it a likely candidate for investigation. The *in vitro* cellular transfection assay presented in this paper is consistent with a role for c-*ets-2* in the process. In addition, the assay we have described will be useful to determine what regions within *ETS2* are required for mitogenesis or tumorigenesis and therefore may enable one to define the *ETS2* domain(s) essential for any chromosome 21-associated diseases.

The authors wish to dedicate this paper to the memory of Dr. Max Tishler, a scientific colleague who was an inspiration to all who knew him. We thank George Pavlakis and Barbara Felber for the gift of pMVBneo, Dee Thompson for technical assistance, Greg Beal for preparing the high molecular weight DNA, Richard Ascione and Steve Kottaridis for critical reading of the manuscript, and Cheryl M. Nolan for preparing the manuscript.

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