Oncogene-mediated Multistep Transformation of C3H10T1/2 Cells

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ABSTRACT

We have examined the response of the mouse embryonic cell line C3H10T1/2 to transfection with the activated human c-H-ras oncogene and the gag-myc oncogene from avian myelocytomatosis virus 29. C3H10T1/2 cells are not morphologically transformed following transfection with the gag-myc oncogene. A low level of focus formation is observed following transfection of the c-H-ras oncogene. When C3H10T1/2 cells are cotransfected with the ras and myc oncogenes, focus formation is increased by an average of 13 fold. In addition, C3H10T1/2 ras/myc foci have a distinct, transformed morphology which correlates with an increased potential for anchorage-independent growth. Although morphological transformation in this system is largely a function of ras oncogene expression, our studies demonstrate that it is potentiated by the presence of a functional gag-myc protein. Oncogene-mediated multistep transformation, which was first described in primary embryo cultures, is not a general property of established cell lines. The C3H10T1/2 cell line is an exception and provides a model system in which partially transformed phenotypes, in a progression toward malignant transformation, can be isolated and studied.

INTRODUCTION

It is now believed that most cancers arise from the mutational alteration of genetic sequences within somatic cells. Perhaps the strongest evidence for this hypothesis has come from the discovery that the oncogenes of retroviral genomes are derived from sequences found in normal cellular DNA (reviewed in Ref. 1). Additional evidence comes from the identification of point mutations which activate the transforming potential of cellular protooncogenes (reviewed in Ref. 2) and from studies which link certain cancers to chromosomal translocations (3-5). More than two dozen protooncogenes have been identified in mammalian genomes and although the molecular basis for the activation of many of these protooncogenes and their retroviral counterparts is known, the actual mechanism by which the proteins of these activated genes transform cells has not been described (reviewed in Ref. 6). Understanding how individual protooncogene products transform cells is complicated further by the observation that cellular transformation is often a multistep process involving the sequential activation of more than one protooncogene product (7-11).

Multistep transformation can be studied using established cell lines. Ruley et al. (12) have described a rat fibroblast cell line, REFS2, which is refractory to transformation by the activated ras protooncogene, but can be transformed when cotransfected with ras and a cooperating oncogene such as adenovirus E1A or myc. We have begun studies utilizing the mouse embryonic fibroblast cell line C3H10T1/2. C3H10T1/2 fibroblasts display strong postconfluent growth inhibition, a very low frequency of spontaneous transformation (13) and have been shown to be good recipients in DNA transfection studies (14). In this paper, we demonstrate that oncogenic transformation of C3H10T1/2 cells is a multistep process requiring oncogene cooperativity. Transfection of C3H10T1/2 cells with an activated myc oncogene does not result in morphological transformation. Transfection of C3H10T1/2 cells with an activated ras protooncogene results in a low incidence of focus formation, while a fully transformed phenotype, which we define morphologically and as well as by anchorage-independent growth in soft agarose, is produced only when C3H10T1/2 cells are cotransfected with both the ras and the myc oncogenes. We demonstrate further that full transformation of C3H10T1/2 cells depends on the expression of the myc oncogene since cotransfections using myc mutants defective for avian cell transformation (15) do not show cooperativity with ras. In contrast to the REF52 cell line where a close examination of ras transfectants and myc transfectants is hampered by stability problems (12, 16), the C3H10T1/2 cell line offers the advantage of examining phenotypes resulting from ras, myc, or ras and myc expression in the same cell line. We propose that the cooperative transformation of the C3H10T1/2 cell line will be an excellent model system in which the molecular events associated with multistep transformation can be dissected.

MATERIALS AND METHODS

Plasmid DNAs. Plasmid pT24 contains the activated human c-H-ras gene cloned into the BamHI site of pBR322 (17). Plasmid pMC29 contains the complete gag-myc coding sequences of avian myelocytomatosis virus 29 in a pSV2 vector (15). Deletion plasmids CH204, CH205, and CH301 were derived by restricting and religating appropriate restriction sites in pMC29 (15). Plasmid pKOneo containing the bacterial neomycin gene (18) was used to obtain individual C3H10T1/2 neo and C3H10T1/2 myc/neo clones. All plasmids used in transfections were purified using CsCl/EtBr density gradient ultracentrifugation (19).

Transfections. C3H10T1/2 cells were obtained from the American Type Culture Collection. Passage 8-18 cells were used exclusively in all transfections. Cells used at later passages showed an increase in spontaneous transformation and a decrease in ras/myc cooperativity. C3H10T1/2 cells were maintained in DMEM5 supplemented with 10% FCS (GIBCO, Grand Island, NY) and 1% penicillin-streptomycin (GIBCO). Transfections were carried out using a modification of the procedure of Wigler et al. (20). On the day prior to transfection, cells were seeded at 3.0-5.0 x 10^5 cells/100 mm culture dish. Six h prior to transfection, each plate was refed DMEM + 10% PCS and after 4 h a 1:3 in DMEM + 5% PCS for focus assays or 1:6 in DMEM + 10% PCS. Twenty-four h following transfection the plates were split 1:3 in DMEM + 10% FCS and after 4 h a solution (10 mg/ml) of chloroquine (Sigma, St. Louis, MO) was added to a final concentration of 10 mg/ml. Calcium phosphate precipitates containing test DNAs and 30 µg C3H10T1/2 carrier DNA per dish were prepared. A 3:1 molar ratio of myc to ras plasmid DNAs (total 200 ng/plate) was used for all cotransfections. pKOneo was added at 50 ng/plate. Chloroquine was removed by refeeding with DMEM + 10% FCS immediately before precipitates were added. Cells were incubated with precipitates for 5 to 6 h and then refeed with DMEM + 10% FCS. Twenty-four h following transfection the plates were split 1:3 in DMEM + 5% FCS for focus assays or 1:6 in DMEM + 10% FCS + 400 µg/ml G418 (GIBCO) for neomycin selection. Ten to 14 days after splitting, C418 colonies were cloned or plates were fixed

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2. To whom requests for reprints should be addressed, at Department of Biological Sciences, Purdue University, West Lafayette, IN 47906.
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5. The abbreviations used are: DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum.
ras AND myc COOPERATE TO TRANSFORM C3H10T1/2 CELLS

with methanol, stained with Giemsa, and scored for foci.

Anchor-independent Growth. Foci from ras or ras/myc transfected plates were cloned using glass cloning cylinders (Bellico Glass, Vineland, NJ). After culture expansion, 1 x 10^6 cells from each clone were mixed with 2 ml of 0.5% low gelling temperature agarose (Bio-Rad, Richmond, CA) in DMEM + 10% FCS and layered onto a 60-mm culture dish containing a 5-ml prehardened feeder layer of 1.0% low gelling temperature agarose (FMC SeaPlaque, Rockland, ME) in DMEM + 10% FCS. Plates were fed 1 ml DMEM + 10% FCS every 5–7 days. After 14 days the plates were stained with iodonitrotetrazolium violet (1 mg/ml; Sigma, St. Louis, MO) (21) and were scored macroscopically for colony formation. A clone was scored positive if ≥1 macroscopic colony (approximately 5 x 10^2 cells) was observed.

RESULTS

The C3H10T1/2 cell line exhibits a high degree of contact-inhibited growth in culture (13). However, this stable property can be affected if C3H10T1/2 cells are exposed to any one of a variety of cancer causing agents (reviewed in Ref. 22). In order to investigate what effect activated oncogenes have on the growth characteristics of C3H10T1/2 cells, we examined the response of C3H10T1/2 cells following transfection with either the activated c-H-ras oncogene, the viral myc oncogene or both oncogenes.

Focus Assays. In our previous studies, we determined that transfection of NIH3T3 fibroblasts with the activated human c-H-ras gene on plasmid pT24 yields approximately 3.0–5.0 x 10^5 foci/µg plasmid DNA (17). The results of similar focus assays with plasmid pT24 and C3H10T1/2 fibroblasts are presented in Table 1. Eight independent transfection experiments were performed and an average of 1.1 x 10^3 foci/µg ras DNA was observed. Focus formation was correlated strictly with transfection of the c-H-ras gene since parallel experimental groups without ras never produced a transformed phenotype. The low frequency of ras focus formation with C3H10T1/2 cells is due to the inability of the cells to incorporate and express transfected genes. Control transfections using the selectable marker neomycin (18) and C3H10T1/2 or NIH3T3 cells resulted in 3.0 x 10^3 G418-resistant colonies/µg neomycin DNA, regardless of the recipient cell line (data not shown). We conclude that the difference in ras focus formation obtained with NIH3T3 and C3H10T1/2 cells represents a difference in the transformation potential, not the transfection competence, of the two cell types.

The frequency of ras focus formation in C3H10T1/2 cells can be increased by pretreatment of the cells with chloroquine (23) (Table 1, experiments 5–8). Chloroquine is a drug that inhibits cellular lysosome activity. Thus, when cells are treated with chloroquine prior to transfection, the intracellular transport of calcium phosphate DNA precipitates is not impaired. The increased number of ras foci in the experimental groups using chloroquine suggests that ras focus formation in C3H10T1/2 may be dependent, in part, on the number of ras genes that become incorporated and expressed in a C3H10T1/2 genome. Spontaneous foci did not appear on control transfected C3H10T1/2 cells pretreated with chloroquine (Table 1).

The morphology of the foci resulting from transfection of C3H10T1/2 cells with the c-H-ras gene is shown in Fig. 1, E and F. Previously, Reznikoff et al. (23) described three distinct focus morphologies produced after treatment of C3H10T1/2 cells with various doses of chemical carcinogens. Using the morphological guidelines set forth by that study, our ras foci are of the Type I variety, consisting of tightly packed, spindle-shaped cells with diffuse focus boundaries. Since the response of C3H10T1/2 cells to transfection with an activated ras oncogene was lower than expected, we next examined the possibility that focus formation in C3H10T1/2 cells may require the cooperative action of a second oncogene. Ruley et al. (12) and Franza et al. (16) have demonstrated that
ras AND myc COOPERATE TO TRANSFORM C3H10T1/2 CELLS

The morphology of the foci resulting from cotransfection of C3H10T1/2 cells with ras and myc oncogenes is dramatically different from the morphology of foci produced from the transfection of the ras gene alone (Fig. 1, G and H). ras/myc foci have characteristics which fit both the Type II and Type III foci classification proposed by Reznikoff et al. (24). ras/myc foci are multilayered, consist of rounded, highly refractile cells, and show distinct focus boundaries. Macroscopically, ras/myc foci are detected easily by eye and stain darkly with Giemsa.

On the basis of our transfection data and the morphological comparison of ras and ras/myc foci, we conclude that the C3H10T1/2 cell line shows oncogene cooperativity. Cotransfection of the ras and myc oncogenes results in a significant increase in the number of transformed foci and the production of a new and distinct focus morphology.

Soft Agar Assays. Studies by Reznikoff et al. (24) using chemically mutagenized C3H10T1/2 cells demonstrated that cells having a Type I focus morphology did not give rise to tumors when injected into syngeneic animals, while those foci having a Type II or Type III phenotype were highly tumorigenic. These results suggest that the Type I transformed phenotype represents one step in a progression of events leading to malignant transformation. Since C3H10T1/2 ras foci have a Type I morphology and C3H10T1/2 ras/myc foci more closely resemble foci of the Type II and III morphology, we investigated the relative tumorigenicity of ras and ras/myc transformed cells.

To define a level of cellular tumorigenicity for C3H10T1/2 ras and C3H10T1/2 ras/myc foci, we assayed the anchorage independent growth of cloned foci in soft agarose. The results of these assays are presented in Table 2. Approximately 1.0 × 10⁶ cells from each of 14 cloned ras foci and 13 cloned ras/myc foci were plated in soft agarose and examined macroscopically for colonies after 14 days in culture. Only two of the 14 (14%) ras clones were competent for anchorage independent growth by this assay, while nine of the 13 (69%) ras/myc clones were capable of such growth. Control cultures of myc/neo C3H10T1/2 cells and neo C3H10T1/2 cells did not form colonies in soft agarose. With few exceptions, anchorage independent growth is a reliable indicator of cellular tumorigenicity. Therefore, we conclude that C3H10T1/2 cells transformed by the cooperative action of the c-H-ras and gag-myc oncogenes exhibit an increased tumorigenic potential over C3H10T1/2 cells transformed by the activated ras gene alone.

Expression of myc Is Required for Cooperative Transformation. The increase in focus formation following cotransfection of C3H10T1/2 cells with pt24 and pMC29 could be due to an increase in c-H-ras transcription caused by the recombination of the pt24 ras coding sequences with the pMC29 retroviral promoter (LTR) during transfection. Chang et al. (25) have shown that the c-H-ras protooncogene under the control of a retroviral promoter is active in NIH3T3 focus assays. To examine if the expression of the pMC29 gag-myc gene is critical to the cooperative event, several deletion mutants of pMC29, two of which are defective for avian fibroblast transformation, were used in C3H10T1/2 focus assays. The structures of the pMC29 deletion mutants are diagrammed in Fig. 2. The biological properties of the mutant gag-myc proteins with respect to avian embryo cell transformation and intracellular location also are indicated in Fig. 2 and have been described in detail elsewhere (15). The results of the cotransfections with pt24 are presented in Table 3. CH205 and CH301, two mutants which are unable to transform avian cells, did not cooperate with ras in C3H10T1/2 focus assays. In fact, the cotransfections with CH205 and CH301 produced fewer foci than transfection with ras alone, suggesting that the expression of these defective gag-myc proteins may have a negative effect on C3H10T1/2 cell growth. The 20 foci appearing with each of these transformation defective gag-myc mutants possessed the characteristic ras Type I focus morphology. In contrast, mutant CH204, which is competent for avian embryo cell transformation, showed wild-type cooperativity with ras and, as expected, produced colonies with ras/myc.

Table 2 Anchorage-independent growth of cloned C3H10T1/2 ras and C3H10T1/2 ras/myc foci in soft agarose

<table>
<thead>
<tr>
<th>No. clones tested</th>
<th>No. clones positive</th>
<th>%a</th>
</tr>
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<tbody>
<tr>
<td>ras foci</td>
<td>14</td>
<td>2</td>
</tr>
<tr>
<td>ras/myc foci</td>
<td>13</td>
<td>9</td>
</tr>
<tr>
<td>C3H10T1/2 neo clones</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>C3H10T1/2 myc/neo clones</td>
<td>4</td>
<td>0</td>
</tr>
</tbody>
</table>

a Clones were scored as positive if ≥1 colony was macroscopically visible on the culture dish.

% , number of clones showing growth/total number of clones tested and represents the relative efficiency of anchorage independent growth for each experimental group of clones.

C3H10T1/2 neo clones were selected as described in "Materials and Methods." For the myc/neo clones, the presence of gag-myc mRNA was assayed by RNA slot-blot analysis (data not shown).

Fig. 2. Structure and biological properties of the pMC29 gag-myc mutant proteins. The 2628 base pair open reading frame of the gag-myc oncogene codes for the p110 gag-myc fusion protein. Deletion mutants were constructed using available restriction sites (H, Hinc II, B, Ball). The number of amino acids (a.a.) deleted in each mutant is indicated by Δ. Mutant CH301 contains a 54 amino acid deletion plus an additional nine amino acids from the 3′ nontranslated (NT) portion of the gag-myc gene transcript. Results of avian embryo cell transformation assays and intracellular location studies with the wild-type and mutant gag-myc proteins are indicated at right. Details of these experiments have been published previously (15).
activated c-H-ras oncogene, a small number of foci appear on gag-myc oncogene is critical to the enhanced quantitative and qualitative transformation typical of C3H10T1/2 ras/myc cotransfections.

DISCUSSION

The C3H10T1/2 cell line has been used to study the sensitivity of cells to various cancer-causing agents and has served as a standard for these types of studies because of its stable, nontransformed phenotype (reviewed in Ref. 22). In this paper we demonstrate that C3H10T1/2 is also a model system that can be used to study multistep transformation mediated by activated oncogenes. An examination of multistep transformation requires a cell line where partially transformed phenotypes can be isolated and studied. In contrast to other available culture systems, this can be accomplished using the C3H10T1/2 cell line.

C3H10T1/2 cells are not morphologically transformed when transfected with an activated myc oncogene. Using neomycin cotransfection, we have obtained cell lines which by RNA slot-blot analysis stably express gag-myc mRNA (data not shown). These lines are not morphologically transformed and none of the lines so far tested show anchorage independent growth in soft agarose. When C3H10T1/2 cells are transfected with the activated c-H-ras oncogene, a small number of foci appear on the culture dishes. C3H10T1/2 ras foci have a transformed morphology, but grow poorly in soft agarose, suggesting that they are partially transformed at best. Cotransfection of C3H10T1/2 cells with the myc and ras oncogenes results in a large number of foci having a morphology distinct from C3H10T1/2 ras foci and showing anchorage independent growth in soft agarose.

The ability of the myc oncogene product to potentiate transformation by the ras oncogene has been demonstrated in primary embryo fibroblast cultures (7, 8), in the REF52 cell line (12, 16) and, as a result of this study, in the C3H10T1/2 cell line. In contrast to the aforementioned systems, the C3H10T1/2 cell line shows morphological transformation (and some growth in soft agarose) when transfected with the ras oncogene alone. This low level response to ras may depend on the amount of ras expressed by a C3H10T1/2 cell following transfection. Our experiments with chloroquine pretreatment suggest that an increase in transfection efficiency and the corresponding increase in the number of ras genes a cell receives as a result has a positive effect on C3H10T1/2 ras focus formation. We are investigating this observation further by quantitating the levels of ras gene expression in ras foci and comparing that to levels expressed in ras/myc foci and in ras/neo C3H10T1/2 cells not displaying a transformed morphology.

The low incidence of ras focus formation in C3H10T1/2 cells may be a consequence of other factors, such as a heterogeneous response of the C3H10T1/2 cell population to the transforming effects of activated ras gene expression. Cell cycle effects at the time of transfection, or differences in the position of a transfected cell with regard to untransfected neighbor cells, may play an important role in the final decision of a cell expressing the ras oncogene to become transformed. There is experimental evidence for both of these hypotheses. Hsiao et al. (14) have shown that stimulation of C3H10T1/2 cells with tumor promoters at the time they are transfected with ras increases the incidence of focus formation up to 40 fold. Herschmann and Brankow (26) have shown that the morphology of UV transformed C3H10T1/2 cells is influenced greatly by cell-to-cell contact with nontransformed C3H10T1/2 cells. In this regard, it will be interesting to assess the behavior of cloned C3H10T1/2 ras foci when cotransfected with untransformed C3H10T1/2 cells or with C3H10T1/2 cells expressing the viral gag-myc oncogene.

Our experiments with the gag-myc deletion mutants clearly demonstrate that myc activity is required to potentiate the transforming effects initiated by ras. The role of the myc gene product in cellular metabolism is not known. The myc protein is nuclear (27–29) and experimental evidence suggests that the protein may act to mediate cellular growth (30, 31), possibly by influencing DNA synthesis (32). Originally, it was proposed that myc activity was required for the immortalization or establishment of cells in culture and that myc induced immortalization was a prerequisite for ras transformation (7, 8). Our studies demonstrate that all immortalization functions are not equivalent and that immortalization alone is not sufficient for a cell to respond to the transforming effects of the ras oncogene. The cooperative effect of myc may include an immortalization function (which can be assayed in primary embryo cells), but also additional functions that contribute to transformation in previously established cell lines. The multistep transformation system we have described in this paper provides an opportunity to assay for additional myc functions. We intend to use this system to define regions of the myc protein that are essential to ras/myc cooperativity and to isolate new genes whose products mimic ras or myc function and which may be involved in the metabolic pathway of ras/myc mediated cellular transformation.

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