Enhanced Expression of c-myc and Decreased Expression of c-fos Protooncogenes in Chemically and Radiation-transformed C3H/10T1/2 CI 8 Mouse Embryo Cell Lines

Taro Shuin, Paul C. Billings, Johan R. Lillehaug, Steven R. Patierno, Pradip Roy-Burman, and Joseph R. Landolph

ABSTRACT

c-abl, c-fos, c-Ha-ras, c-myc, and c-mos were expressed whereas c-sis, c-fms, c-rel, c-src, and c-myb expression was not detectable in C3H/10T1/2 CI 8 (10T1/2) cells and in eight chemically and radiation-transformed 10T1/2 cell lines. The expression of c-abl, c-fos, c-Ha-ras, and c-myc was growth-related in nontransformed 10T1/2 cells. c-abl and c-fos expression increased at confluence by 5- and 9-fold, respectively, compared to that in log phase cells. c-Ha-ras and c-myc transcripts were most abundant in log phase cells and decreased by 70 and 50%, respectively, in confluent cells. There were no significant growth-related changes in the expression of c-Ha-ras, c-myc, or c-abl in methylcholanthrene-transformed CI 15 cells. The c-fos transcript was not detected in CI 15 cell cultures. c-ABL, c-FOS, c-Ha-Ras, and c-Myc were expressed in whole C3H mouse embryo tissue, mouse liver, and 10T1/2 cell lines. Sizes of these protooncogene transcripts in 10T1/2 cells were the same as those in whole embryo tissue, except that 10T1/2 cells did not express the 8.2-kilobase abl transcript.

At subconfluence, equivalent low levels of c-mos expression were observed in nontransformed and in the eight transformed 10T1/2 cell lines. The level of c-abl expression was similar in the nontransformed and in the eight transformed cell lines, but there was a new 8.2-kilobase transcript in the transformed MCA CI 15 cell line. c-fos was expressed in 10T1/2 cells but was not detectable or greatly reduced in eight transformed cell lines. c-Ha-Ras was expressed to a similar extent in eight transformed cell lines and in nontransformed 10T1/2 cells. In the UVC-4 transformed cell line, extra 3.3-kilobase Ha-ras and 7.5-kilobase Ki-ras transcripts were observed. c-myc was expressed at 4- to 7-fold higher levels in six transformed cell lines compared to 10T1/2 cells. There were no major rearrangements in or amplification of the c-myc gene in three transformed cells overexpressing this gene 5-fold.

These studies show that enhanced expression of c-myc and decreased expression of c-fos correlate with the chemically and radiation-transformed states of 10T1/2 cells. Changes in c-fos and c-myc oncogene expression may be causally linked to late stages of neoplastic transformation in these chemically and radiation-transformed 10T1/2 cell lines.

INTRODUCTION

Protooncogenes are evolutionarily conserved genes that are expressed and code for proteins believed to play essential but poorly understood roles in the growth and differentiation of normal mammalian cells (1, 2; reviewed in Ref. 3). Protooncogenes may become mutated and/or rearranged and subsequently transduced by retroviruses, leading to the formation of oncogenes (reviewed in Refs. 3 and 4). Oncogenes have been isolated from acutely transforming retroviruses and by transfection of DNA from transformed cell lines and tumors into NIH 3T3 cells (5, 6) and have been shown to be activated forms of normal cellular protooncogenes (7-9; reviewed in Ref. 4). Mutated Ha-ras or Ki-ras protooncogenes have been identified in human tumors and tumor cell lines (10-12) and in chemically induced murine tumors (8, 13, 14). Increased expression of c-myc, c-Ki-ras, or c-abl has also been observed in different human and murine malignancies (15-17). There is therefore substantial evidence that oncogenes play a role in the overall process of carcinogenesis (reviewed in Refs. 3 and 4). Oncogene-mediated acquisition of immortality has been shown to be a necessary step in neoplastic transformation, and cooperation of oncogenes from different complementation groups can induce neoplastic transformation of primary cultures of mammalian cells (4, 18, 19); hence, alterations in protooncogenes play important roles in the various steps mediating transformation of normal cells into tumor cells.

There is also evidence that chemically induced neoplastic transformation of cultured cells is a multistep process (20, 21). The 10T1/2<sup>2</sup> cell line (22), which exhibits postconfluence inhibition of cell division, anchorage-dependent growth, and a low frequency of spontaneous transformation and has undergone immortalization (22) is frequently used as an in vitro model system to study mechanisms of chemical carcinogenesis (23; reviewed in Refs. 24 and 25). Chemical carcinogens (23, 26) or radiations (27, 28) induce morphological transformation of 10T1/2 cells, and a high proportion of cell lines derived from these foci are tumorigenic in X-irradiated syngeneic mice (23). We developed an assay in 10T1/2 cells to detect carcinogen induced ouabain-resistant (Ouar) mutants (26) which have stable (29), specific (30), base substitution mutations (31) in the potassium transport system (29) that map to mouse chromosome 3 (32). We then showed that mutagenic organic chemical carcinogens induce transformation and mutation to ouabain resistance over the same concentration ranges in 10T1/2 cells (26), suggesting that mutation(s), likely in cellular protooncogenes, are a part of the transformation process induced by mutagenic organic carcinogens (reviewed in Ref. 24).

We next asked whether there are alterations in protooncogenes in chemically transformed 10T1/2 cells. In this study, we therefore compared expression of c-ABL, c-FOS, c-HA-RAS, c-MYC, c-MOS, c-SIS, c-FMS, c-REL, c-SRC, and c-MYB in exponentially growing and confluent 10T1/2 cells, compared expression of c-ABL, c-FOS, c-HA-RAS, and c-MYC between 10T1/2 cells and C3H mouse embryo tissue, and compared expression of c-ABL, c-FOS, c-HA-RAS, c-MYC, and c-MOS in nontransformed 10T1/2 cells and in eight chemically and radiation-transformed 10T1/2 cell lines.

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The abbreviations used are: 10T1/2, C3H/10T1/2 CI 8 mouse embryo cells; SSC, standard saline citrate (0.015 M sodium citrate-0.15 M sodium chloride); poly(A), polyadenylic acid; poly(A)*, polyadenylated; MCA, methylcholanthrene.
Materials and Methods

Cells and Cell Culture. 10T1/2 cells (22) and eight chemically and radiation-transformed 10T1/2 cell lines were used in this study. These transformed cell lines include two methylcholanthrene-transformed cell lines (MCA CI 15 and MCA CI 16; see Ref. 23), one X-ray-transformed cell line (XE-1, previously designated DR-AL-258 and generously donated by Dr. M. M. Elkind, Colorado State University), one neutron-transformed cell line (NES52, previously designated DR-AL-525/JW and also obtained from Dr. Elkind), two UV-transformed cell lines (UVOC and UV4C, previously designated TU2 and TU3 and obtained from Dr. G. Chan, Harvard University), and two bleomycin-transformed cell lines (Bleo CI 1 and Bleo CI 2, previously designated J2BB and J2BC1B1 and obtained from Dr. W. F. Benedict, University of Southern California; see Ref. 33). All these transformed cell lines except Bleo CI 1 were tumorigenic in nude mice, and all transformed cell lines exhibit anchorage-independent growth (Table 1).

Cells were cultured in Eagle's basal medium containing 10% fetal calf serum (Gibco, Grand Island, NY) without antibiotics at 37°C in a 5% CO2 incubator in Corning tissue culture flasks or in roller bottles (Corn ing Glass Co., Corning, NY) as described (25, 26). All cell lines were routinely checked for Mycoplasma by growth on Mycoplasma agar and by Hoechst fluorescent staining (34), and only those found to be negative for Mycoplasma were used in these studies. To obtain cells for DNA and RNA extractions, one million cells were seeded/850-cm2 roller bottle in 150 ml of medium. Medium was changed on day 7 postseeding only for cells to be harvested on day nine. Cells were harvested on the appropriate day by rinsing cells in each roller bottle with 100 ml of ice-cold isotonic phosphate-buffered saline, scraping cells from bottle into 100 ml of phosphate-buffered saline with a rubber policeman, and centrifuging cells. The growing state of cells at each time point was assessed by propidium iodide staining and cytofluorography using a Becton-Dickinson fluorescence-activated cell sorter according to the method of Hawkes and Bartholomew (35). On day 5 postseeding, nontransformed 10T1/2 cells were in log phase of growth, on day 7 cells were subconfluent, and by day 8 cells were confluent. The chemically and radiation-transformed cell lines used here did not reach a discrete confluent state as did nontransformed C3H/10T1/2 cells but continued to grow and pile up on each other (23); so the analogous state in the transformed cell lines is referred to as a state of high cell density.

Source and Description of Cloned Oncogenes Used as Hybridization Probes. pBR322 containing a 0.6-kilobase v-bas insert or a 1-kilobase v-sis insert was supplied by Dr. Stuart A. Aaronson, NIH, Bethesda, MD. A 0.6-kilobase HindIII-BamHI v-bas fragment was used for studying c-bas expression. Since v-bas is a variant form of c-Ha-ras, we refer in this manuscript to c-Ha-ras expression. A pBR322 containing v-mos plasmid, pMsV-S1, a pBR322 containing v-fos plasmid, pF0s1, and a pBR322 containing v-fms plasmid, pFms, were obtained from Dr. Inder M. Verma, Salk Institute, San Diego, CA. The 0.3- and 0.45-kilobase PstI v-mos fragments were used for the c-mos expression study, a 1.0-kilobase PstI v-fos fragment was used for the c-fos expression study, and the 1.5-kilobase PstI-v-fms fragment was used for the c-fms expression study. A pBR322 containing v-abl plasmid, pAB1-sub9, was supplied by Dr. David Baltimore, and a 3.2-kilobase HindIII-BamHI fragment of this was used for studying expression of c-abl. pBR322 containing a PvuII fragment of v-src was supplied by Dr. Peter K. Vogt, Department of Microbiology, University of Southern California, Los Angeles, CA, and the 0.8-kilobase PvuII fragment was used for the c-src expression study. pBR322 containing the second and third exon of c-myc, pM104BH (17), was supplied by Dr. Michael D. Cole, and a 2.5-kilobase Xbal-HindIII fragment containing the second and third exons of c-myc (17) was used to study expression of c-myc. pBR322 containing v-rel was supplied by Dr. Howard Temin, and the 0.8-kilobase EcoRI fragment was used for studying expression of c-rel. The 0.8-kilobase BamHI fragment of v-myc was supplied by Dr. Pradip Roy-Burman, Dept. of Pathology, University of Southern California. N-ras and Ki-ras probes were obtained through the courtesy of Dr. Yuen K. Fung, Children's Hospital of Los Angeles.

Extraction of Nucleic Acids and Blot Hybridization Procedures. High molecular weight DNAs were extracted with phenol and chloroform-isooamyl alcohol (1:1, v/v) by standard methods as previously described.7 For Southern blotting analysis, 10 to 20 /g of DNA were digested with a 5-fold excess of restriction enzymes for 5 h, electrophoresed in 1% agarose gels in Tris-borate buffer (pH 8.3), and transferred to nitrocellulose filters as described by Southern (36). After baking at 80°C for 2 h in a vacuum oven, filters were prehybridized and then hybridized with 32P-labeled c-onc specific fragments (2 x 106 cpm/ml) as described by Thomas (37). After hybridization, filters were washed three times with 2x SSC, 0.1% sodium pyrophosphate, and 0.05% sodium dodecyl sulfate at 50°C and then three times with 0.2x SSC, 0.1% sodium pyrophosphate, and 0.05% sodium dodecyl sulfate at 60°C.

Cytoplasmic RNAs were extracted from cultured cells according to the method of Favaloro et al. (38). Poly(A)-containing RNA was isolated on an oligodeoxythymidylic acid cellulose column as described by Aviv and Leder (39). For extracting RNA from tissue, tissue was disrupted by a Polytron mixer in a solution containing proteinase K (200 /g/ml), 0.14 M NaCl, 50 mM Tris-HCl, pH 8.4, 1% sodium dodecyl sulfate, and 10 mM EDTA. The concentration of the proteinase K was then increased to 400 /g/ml, the homogenized tissue was incubated for 10 min at 25°C and extracted with phenol-chloroform, and the DNA and RNA were precipitated with 100% ethanol. The DNA was spooled out, and the solution was kept at -20°C overnight.

To obtain pure RNA, the precipitate was dissolved in buffer (10 mM Tris, pH 7.5, 1 mM EDTA) and precipitated with 2 mM lithium chloride at 2°C overnight. Then, poly(A)-containing RNA was selected by chromatography on an oligodeoxythymidylic acid cellulose column and electrophoresed in 1% formaldehyde agarose gels in a circulating gel-running buffer (20 mM 3-(N-morpholino)propanesulfonic acid pH 7.0, 5 mM sodium acetate, and 1 mM EDTA). RNAs were transferred from gels to nitrocellulose filters by using 20x SSC according to the method of Southern (36). The nitrocellulose filters were baked at 80°C for 2 h in a vacuum oven, prehybridized, and finally hybridized with 32P-labeled v-one specific probe (2 x 106 cpm/ml) according to the method of Lee et al. (40). Ribosomal markers and a commercially available RNA ladder (Bethesda Research Laboratories, Gaithersburg, MD) were used to determine the molecular sizes of the c-one-specific transcripts.

* Numbers in parentheses, reference wherein the indicated cell line was derived.
RESULTS

Expression of c-abl, c-fos, c-ras, and c-myc in Different Growth States of Nontransformed 10T1/2 Cells. We first determined which of the 10 protooncogenes that we studied were expressed in 10T1/2 cells by RNA dot-blot analysis. Two, 1, and 0.5 μg of poly A-containing RNA were spotted onto nitrocellulose and hybridized with abl, fos, ras, mos, sis, fms, src, rel, myb, and c-myc probes. c-sis, c-fms, c-rel, c-src, and c-myb protooncogenes were not expressed at detectable levels, while we found expression of c-abl, c-fos, c-Ha-ras, c-myc, and c-mos protooncogenes (summarized in Table 3).

We next studied the expression of c-abl, c-fos, c-ras, and c-myc as a function of the growth state of 10T1/2 cells to determine optimal growth states at which to quantitate protooncogene expression. Figure 1a shows growth curves for both nontransformed and transformed 10T1/2 cell lines. The transformed cells grow to 1.3- to 4.4-fold higher densities than nontransformed 10T1/2 cells (Fig. 1a). Cytofluorographic analysis indicated that the percentage of cells in S phase on day 5 postseeding was similar in both nontransformed and transformed cells (18 to 20%) (Fig. 1b). In the nontransformed 10T1/2 cells, the percentage of cells in S phase decreased from 18% on day 5 to 12% on day 7 to 7% on day 9. In two transformed cell lines, MCA Cl 15 and MCA Cl 16, the percentage of cells in S phase remained constant at approximately 20% on days 5, 7, and 9 (Fig. 1b). Protooncogene expression was then compared in nontransformed and transformed cell lines at the same times postseeding. Poly(A)-containing RNAs were extracted from log phase (5 days after seeding), subconfluent phase (7 days after seeding), or confluent phase (9 days after seeding) cells, electrophoresed, and hybridized to v-abl, v-fos, v-ras, and c-myc probes.

Major c-abl transcripts of 5.6 and 7.2 kilobases were detected in logarithmically growing, subconfluent, and confluent nontransformed 10T1/2 cells (Fig. 2a). Compared to the amount in log phase cells, the amount of c-abl transcript increased 4.2-fold in confluent nontransformed 10T1/2 cells (average of two experiments; Fig. 2a, lanes 1 and 3, is a representative experiment). A major 2.2-kilobase c-fos transcript was detected in growing and confluent 10T1/2 cells (Fig. 2b), consistent with the 2.2-kilobase c-fos transcript detected in mouse embryos by Muller et al. (1, 2). The amount of c-fos transcript was very low in log phase cells (Fig. 2b, lane 1) but increased by 6.5-fold in subconfluent cells and 9-fold in confluent cells (average of two experiments; Fig. 2b, lanes 2 and 3, respectively, is a representative experiment). In these experiments, medium was changed on day 7 only for cells to be used on day 9. When we changed medium on 10T1/2 cells 2 days before each RNA analysis, there was no change in the fraction of cells in S phase or in steady state levels of poly(A)+ c-fos mRNA compared to cells that did not undergo a change of medium, indicating that the increase of c-fos mRNA levels as the cells approached confluence was not due to either medium changing or to enhanced inducibility of c-fos expression (data not shown).

There was a 1.4-kilobase c-Ha-ras transcript in 10T1/2 cells (Fig. 2c), consistent with the 1.4-kilobase transcript found by others in mouse embryos (1). The 1.4-kilobase c-Ha-ras transcript was most abundant in log phase cells (Fig. 2c, lane 1) and decreased by 35% in subconfluent cells and by 55% in confluent cells (average of two experiments; Fig. 2c, lanes 2 and 3, respectively, is one representative experiment). A 2.3-kilobase c-myc transcript was detected in growing and confluent 10T1/2 cells (Fig. 2d), which is approximately the same c-myc
ENHANCED EXPRESSION OF c-myc AND DECREASED EXPRESSION OF c-fos

Fig. 2. Expression of c-abl, c-fos, c-ras, and c-myc in 10T1/2 cells at log phase, subconfluent phase, and confluent phase. Five µg of poly(A)-containing RNAs were electrophoresed in 1% agarose gels and hybridized to 32P-labeled v-abl, v-fos, v-ras, or c-myc specific fragments. RNAs were extracted from 10T1/2 cells at lane 1, log phase, lane 2, subconfluent phase, and lane 3, confluent phase. a, expression of c-abl; b, expression of c-fos; c, expression of c-Ha-ras; d, expression of c-myc. Relative densitometric readings are at the bottom of each figs, with the reading at log phase for each probe arbitrarily being set to 1.0. kb, kilobase.

The amount of c-myc transcript was greatest in log phase 10T1/2 cells (Fig. 2d, lane 1) and decreased by 2-fold (average of two experiments) in subconfluent and confluent cells (Fig. 2d, lanes 2 and 3, respectively, shows one representative experiment). The growth-related changes in the expression of poly(A)* protooncogene transcripts in nontransformed 10T1/2 cells is summarized in Fig. 3. Expression of c-fos and c-abl increases as the cells approach the confluent state, whereas the steady state levels of c-myc and c-ras mRNA decrease as the cells become confluent.

Next, we determined the expression of the c-abl, c-fos, c-Ha-ras, and c-myc protooncogenes in a methylcholanthrene-transformed 10T1/2 clone, CI 15, as a function of the growth state (Fig. 4). c-abl transcripts of 7.2 and 5.6 kilobases were observed as in 10T1/2 cells. An additional minor 8.2-kilobase transcript was also observed. The amounts of all three transcripts did not change appreciably as the cells reached high density (the trans-
ENHANCED EXPRESSION OF c-myc AND DECREASED EXPRESSION OF c-fos

formed cells do not become confluent) compared to the values in log phase cells (Fig. 4a). c-fos was not expressed at detectable levels either in log phase or in high density CI 15 cells (Fig. 5). The 1.4-kilobase c-Ha-ras transcript was expressed at approximately the same levels in log phase, medium density, and high density cells (Fig. 4b). Finally, we found that the major 2.3-kilobase c-myc transcript was present at approximately the same levels in log phase and high density CI 15 cells (Fig. 4c). Protooncogene expression in MCA-transformed C1 15 cells is summarized in Fig. 5. It is obvious that c-fos and c-abl expression is not regulated in C1 15 cells in the same manner that it is in 10T1/2 cells as a function of the growth state of cells.

Comparison of Expression of c-abl, c-fos, c-Ha-ras, and c-myc in Mouse Embryo Tissue and 10T1/2 Cells. 10T1/2 cells were originally derived from embryos of C3H mice (22). To determine whether there were changes in the gross level of expression of protooncogenes or in transcript sizes after establishment of the permanent cell line, 10T1/2, we therefore next compared the expression of four protooncogenes in 10T1/2 cells and in whole C3H mouse embryos. Poly(A)+ RNAs from subconfluent (day 7 postseeding) 10T1/2 cells and from 14-day-old embryos were examined for protooncogene expression, and poly(A)-containing RNAs from the liver of adult C3H female mice were also included for comparison.

c-abl transcripts of 7.2 and 5.6 kilobases were observed in adult liver, 10T1/2 cells, and fetal tissue by Northern gel analysis (Fig. 6a; summarized in Table 2). There was a minor 8.2-kilobase transcript in MCA CI 15 cells and in fetal tissue but no detectable levels of this transcript in 10T1/2 cells (shown clearly in Fig. 6b, lanes 2 and 3). Adult liver had approximately equal amounts of 7.2 and 5.6-kilobase transcripts. 10T1/2 cells and whole embryo tissue had more 5.6 than 7.2-kilobase transcript. A 2.2-kilobase c-fos transcript was observed in 10T1/2 cells and whole embryo tissue but not in liver. A higher molecular weight hybridizing transcript of 14-kilobase was faintly observed in adult liver tissue (summarized in Table 2). A 1.4-kilobase c-Ha-ras transcript was observed in all three preparations. Adult liver tissue, 10T1/2 cells, and fetal tissue all expressed a 2.3-kilobase c-myc transcript. An additional 14-kilobase transcript was also detected in adult liver. These data are summarized in Table 2. The significant finding is that 10T1/2 cells expressed the same four protooncogenes as whole mouse embryos did and that the transcript sizes were the same.

Expression of Protooncogenes in Nontransformed 10T1/2 Cells and in Eight Chemically and Radiation-transformed 10T1/2 Cell Lines. The expression of a number of protooncogenes in subconfluent 10T1/2 cells and in eight subconfluent chemically and radiation-transformed cell lines was next compared at day...
Table 2: Summary of protooncogene expression in IOTI/2 cells, whole mouse embryo tissue, and adult mouse liver

<table>
<thead>
<tr>
<th>Transcript (kilobases)</th>
<th>IOTI/2 cells</th>
<th>Whole mouse embryo tissue</th>
<th>Adult mouse liver</th>
</tr>
</thead>
<tbody>
<tr>
<td>c-abl</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5.6</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>7.2</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>8.2</td>
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<td>-</td>
<td>-</td>
</tr>
<tr>
<td>c-fos</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.2</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>14</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>c-Ha-ras</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.4</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>c-myc</td>
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</tr>
<tr>
<td>2.3</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>14.0</td>
<td>-</td>
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</tr>
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</table>

Fig. 6. a, expression of c-abl in adult liver, in IOTI/2 cells, and in fetal tissue. Five µg of poly(A)-containing RNA were electrophoresed and hybridized to the 32P-labeled v-abl specific probe. RNAs were extracted from lane 1, adult liver tissue from female C3H mice, lane 2, subconfluent phase 10T1/2 Cl 8 cells, and lane 3, 14-day-old fetus of C3H mice. Autoradiograms were quantitatively evaluated by scanning with a densitometer. Relative densitometric readings are at bottom with the reading for 10T1/2 Cl 8 cells arbitrarily set to 1.0. b, expression of c-abl in 10T1/2 cells, Cl 15, and fetal tissue. Ten µg of poly(A)-containing RNAs were electrophoresed in 0.8% agarose gels, blotted, and hybridized to a 32P-labeled v-abl specific fragment. kb, kilobase.

7 postseeding. Table 1 describes the properties of the transformed cell lines used in this study. Two, 1, and 0.5 µg of poly(A)-containing RNA were spotted onto nitrocellulose filters and hybridized with abl, fos, ras, mos, sis, fms, src, rel, myb, and myc probes. No expression of c-sis, c-fms, c-rel, c-src, and c-myc protooncogenes was detected, whereas expression of c-abl, c-fos, c-Ha-ras, c-mos, and c-myc protooncogenes was found by dot-blot hybridization in all these nine cell lines (Table 3). We found by Southern analysis that c-sis, c-fms, c-src, and c-myc genes were present in the DNA of 10T1/2, Cl 15, Cl 16, Bleo Cl 1, Bleo Cl 2, NE525, and XE-1 cells, and there were no differences in EcoRI restriction fragments in these DNAs between 10T1/2 cells and the eight transformed cell lines (data not shown); therefore, the oncogene probes used in this study do hybridize to corresponding protooncogenes in these cells, and the oncogene probes used would detect transcription of the protooncogenes if transcripts were present. C-rel did not bind to the DNA of 10T1/2 cells or the eight transformed cell lines (not shown).

We then performed Northern analysis on poly(A)-selected RNA to determine sizes of transcripts from c-abl, c-fos, c-Ha-ras, c-myc, and c-mos protooncogenes. A Northern analysis of the expression of c-abl in 10T1/2 cells and eight transformed cell lines is shown in Fig. 7. 10T1/2 cells and all eight transformed cell lines expressed two c-abl specific transcripts of 7.2 and 5.6 kilobases. The 5.6-kilobase transcript was more abundant than the 7.2-kilobase transcript in all the cell lines. All cell lines expressed the same relative amounts of the two c-abl transcripts as determined in two separate experiments. An extra, but minor, higher molecular weight transcript of 8.2 kilobases was observed in MCA-transformed Cl 15 cells (Fig. 7) and in fetal cells (Fig. 6b) but not in 10T1/2 cells (Fig. 6a, lane 2). This is more clearly shown in Fig. 6b, lane 2.

The expression of c-fos, c-Ha-ras, and c-myc in 10T1/2 cells and in eight transformed 10T1/2 cell lines is shown in Figs. 8 to 10. c-fos was expressed to a significant degree in subconfluent 10T1/2 cells, and the size of the c-fos specific transcript was 2.2 kilobases (Fig. 8). Interestingly, the 2.2-kilobase c-fos transcript was present but at a 4.4-fold lesser extent in XE-1 than in 10T1/2 cells, and was not detected in the other seven transformed cell lines (average of data from two separate experiments). Aliquots of the same RNA preparations were used for the experiments in Figs. 7 and 8 for determination of c-abl and c-fos expression. Since c-abl was expressed to the same extent in nontransformed and transformed cell lines, the decreased expression of c-fos is a real phenomenon and is not due to altered efficiencies of transfer of RNA between nontrans-
Table 3  Summary of protooncogene expression in nontransformed and in chemically and radiation-transformed C3H/10T1/2 Cl 8 cell lines

All measurements were made in cells 7 days postseeding.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>sis</th>
<th>fins</th>
<th>src</th>
<th>myb</th>
<th>mos</th>
<th>abl</th>
<th>fos</th>
<th>ras</th>
<th>myc</th>
</tr>
</thead>
<tbody>
<tr>
<td>C3H/10T1/2</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>MCA CI 16</td>
<td>-</td>
<td>-</td>
<td>-</td>
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* Levels of c-mos transcripts were at the limits of detection.
* Four- to 7-fold increases in c-myc expression were observed in these cell lines.
* An extra 8.2-kilobase transcript was observed in this cell line.
* An extra 7.5-kilobase Ki-ras and an extra 3.3-kilobase Ha-ras transcript were observed in this cell line.

Fig. 7. Expression of c-abl in 10T1/2 cells and eight transformed 10T1/2 cell lines. Ten μg of poly(A)-containing RNAs were electrophoresed and hybridized to a 32P-labeled v-abl specific fragment. Lanes 9 and 10 were hybridized on different days. kb, kilobase.

Fig. 8. Expression of c-fos in 10T1/2 cells and eight transformed cell lines. Ten μg poly(A)-containing RNAs were electrophoresed in 1% agarose gels, blotted, and hybridized to a 32P-labeled v-fos specific fragment. kb, kilobase.

Fig. 9. Expression of c-Ha-ras in 10T1/2 and in eight transformed cell lines. Ten μg poly(A)-containing RNAs were electrophoresed in 1% agarose gels, blotted, and hybridized to the 32P-labeled v-ras probe. Lanes 9 and 10 are results from a separate experiment. kb, kilobase.

Enhanced expression of c-myc and decreased expression of c-fos

We examined the cell lines showing the highest (5- to 7-fold) levels of increased c-myc expression for evidence of rearrangements or amplification of c-myc in the DNA of these cells, in order to understand the reasons for increased c-myc expression. DNAs of 10T1/2 CI 8, Bleo CI 1, CI 16, and UVC3 were digested with EcoRI, HindIII, BamHI, and SstI and hybridized with the c-myc probe in Southern analysis (36). EcoRI sites are located in only the flanking region of the c-myc gene, whereas there are internal sites in the c-myc gene for HindIII (exon I, exon III), BamHI (exon I, intron I), and SstI (exon I, introns I and II). No gross gene rearrangements within the DNA fragment of the 21-kb c-myc locus nor amplifications of this gene were observed in these three transformed cell lines expressing high levels of c-myc used in this study (data not shown).

Transformed and transformed cell lines in the blotting procedure.

There was a 1.4-kilobase c-Ha-ras specific transcript in all cell lines (Fig. 9). All transformed cell lines expressed similar amounts of the 1.4-kilobase c-Ha-ras transcript, and the amount in the eight transformed cell lines varied from 1.1- to 1.5-fold that in 10T1/2 cells as determined by densitometry (average of data from two separate experiments). In the UV-transformed cell line, UVC-4, two extra higher molecular weight transcripts of 7.5 and 3.3 kilobases were observed in addition to the more abundant 1.4-kilobase transcript. The 7.5-kilobase transcript was a Ki-ras specific transcript and the 3.3-kilobase transcript was a Ha-ras specific transcript (data not shown).

A 2.3-kilobase myc specific transcript was observed in 10T1/2 cells and all eight transformed cell lines (Fig. 10). Two- to 7-fold increases in the amount of c-myc transcript were observed in the transformed cell lines compared to that in 10T1/2 cells as determined by densitometric scanning. While the 2.2- and 3.0-fold increases in c-myc were marginal increases, the 4.2- to 7.0-fold increases in the other six transformed cell lines were significant (average of results from two separate experiments; Fig. 10 is a representative experiment). Cell lines Bleo CI 1, CI 16, and UVC3 showed the highest increases, 7-, 6-, and 5-fold, respectively, in the amount of 2.3-kilobase c-myc transcript. Since aliquots of the same RNA preparations were used to determine c-Ha-ras and c-myc expression and since c-Ha-ras expression was similar in non-transformed and transformed cell lines, the 5-, 6-, and 7-fold increases in c-myc in transformed cell lines are considered to be significant.
expression of the various protooncogenes as a function of the growth of nontransformed 10T1/2 cells. Significant differences in expression of protooncogenes were observed in different growth phases of nontransformed 10T1/2 Cl 8 cells. The expressions of c-abl and c-fos were more pronounced at confluence; the level of poly(A)^+ c-fos RNA was increased 9-fold at confluence, and the level of poly(A)^+ c-abl RNA was 5-fold greater at confluence than in log phase; conversely, the expression of c-Ha-ras and c-myc decreased as cells progressed from log phase to confluence. The amount of c-myc transcript was 2-fold more in asynchronous log phase cells than in confluent cells, and the amount of c-ras transcript was 3-fold greater in log phase cells than in confluent cells. Our results are consistent with those of Campisi et al. (42), who found that c-myc expression decreased during the quiescent phase of nontransformed of BALB 3T3 A31 cells. In contrast to the growth-related changes in expression of c-fos, c-Ha-ras, c-myc, and c-abl in nontransformed 10T1/2 cells, methylenetetrafluoroethane-transformed Cl 15 cells showed no significant growth-related changes in expression of these protooncogenes. The 2.2-kilobase c-fos poly(A)^+ RNA transcript was not detectable in Cl 15 cells, indicating that the expression of c-fos, which is maximal in confluent 10T1/2 cells, is aberrant and regulated differently in MCA Cl 15 cells compared to 10T1/2 cells.

Subconfluent 10T1/2 cells expressed a 2.2-kilobase c-fos specific transcript, whereas the poly(A)^+ c-fos transcript was not observed in the eight transformed cell lines studied except for Cl-1, which showed a small amount of c-fos transcript. Unexpectedly, the amount of c-fos transcript in log phase 10T1/2 cells was low and similar to that in the eight transformed cell lines.

Peptide growth factors stimulate transcription of c-fos (43, 44), and c-fos exhibits cell cycle dependent changes in transcription (43). It has therefore been proposed that in fibroblasts, c-fos expression is stringently controlled and may have a role in control of cell proliferation, whereas there are other cell types which express c-fos constitutively and in which expression of c-fos may be important in differentiation (44). It has also been proposed that transformation by c-fos may be due to a deregulation of temporal control of c-fos (43). Interestingly, 12-O-tetradecanoylphorbol-13-acetate-induced differentiation of HL-60 cells to macrophage-like cells is accompanied by induction of c-fos expression (45). Colony-stimulating factor also stimulates c-fos expression in macrophages (46), and platelet-derived growth factor stimulates expression of the c-fos gene in BALB/c 3T3 (46) and NIH 3T3 cells (47). Others have found that c-fos mRNA is barely detectable in quiescent cultures of serum-deprived (0.1% serum) NIH 3T3 or BALB/c 3T3 cells (47).

In this study we found expression of c-fos poly(A)^+ mRNA to be maximal in confluent C3H/10T1/2 cells grown in high (10%) serum levels. It is important to note that in previous studies, cells were rendered quiescent by culturing in medium containing only 1% of the required amount of methionine and only 0.5% serum (46). In contrast, our cells were grown in complete medium containing 10% fetal calf serum, which is known to contain high levels of mitogens such as platelet-derived growth factor. The observation here that logarithmically growing 10T1/2 cells exposed to the highest amount of fresh platelet-observed growth factor-containing serum express the lowest levels of c-fos RNA suggests that some other cellular events, for example cell contact, must occur in order to obtain maximal c-fos RNA expression in the 10T1/2 Cl 8 cells. It is also significant that confluent 10T1/2 cells express high levels of c-fos mRNA but are not transformed. This is consistent with
other reports that the c-fos transcript may be significantly different from the transforming v-fos transcript due to alterations at the 3' end of the gene (reviewed in Ref. 48).

As high as 4- to 7-fold increases in c-myc expression were observed in six transformed cell lines examined in our study. In methylcholanthrene-transformed Cl 15, c-myc expression did not decrease to the same extent in high density cells as it did in nontransformed 10T1/2 cells. This effect indicates a loss of regulation of c-myc expression in high density Cl 15 cells, similar to the results of Campisi et al. (42). A more striking observation was the increase in c-myc expression among different transformed cell lines. UVC3, Cl 16, and Bleo Cl 1 showed 5- to 7-fold increased levels of the c-myc transcript. Since Bleo Cl 1, which showed the highest level of c-myc expression, forms foci and grows in soft agar but is not appreciably tumorigenic, enhanced expression of myc correlates with focus-forming ability and anchorage independence in chemically and radiation-transformed C3H/10T1/2 cell lines. In other cell and tumor systems, gene rearrangement or amplification and increased expression of c-myc have been frequently observed (16, 17). Rearrangement or amplification of the c-myc gene was not found in the UVC3, CI 16, or Bleo cell lines used in this study. This suggests that mechanisms other than gene amplification or rearrangement are responsible for increased c-myc expression in these three cell lines. It will be of interest to determine whether in DNA sequence at the c-myc locus have occurred in these chemically and radiation-transformed cells and to cotransfect DNA from these three cell lines along with a mutated ras oncogene into primary rat embryo cells (18) to determine the biological significance of enhanced expression of myc in these cell lines in relation to transformation. The role of the c-myc gene product in normal cell physiology is not yet understood, although it is known that lymphocyte mitogens and platelet-derived growth factor activate expression of the myc gene (49, 50) and that elevated levels of c-myc RNA weakly promote cell growth (49).

10T1/2 cells and eight different transformed 10T1/2 cell lines studied expressed similar levels of 5.6- and 7.2-kilobase c-abl specific transcripts; however, an extra 8.2-kilobase c-abl transcript was observed in the MCA-transformed Cl 15 cell line. It is not clear whether appearance of this 8.2-kilobase c-abl transcript is transformation related or can result from clonal variation, since another MCA-transformed cell line, Cl 16, did not have this transcript. Further work will be required to investigate this question. The amount of 1.4-kilobase c-Ha-ras transcript was similar in the transformed cell lines we examined and in nontransformed 10T1/2 cells. UVC4 showed extra 3.3-kilobase C-Ha-ras and 7.5-kilobase c-Ki-ras transcripts in addition to the expected 1.4-kilobase transcript. The 7.5 and 3.3-kilobase transcripts were not observed in 10T1/2 cells and the other seven transformed cell lines.

c-mos has been reported not to be expressed in rodent 10T1/2 (51) or human cells except in some plasmacytoma cell lines (52). We found very little c-mos expression in 10T1/2 cells and other transformed cell lines. In addition, no significant difference in c-mos expression was seen between 10T1/2 cells and the transformed cell lines we examined. The significance of the low c-mos expression in nontransformed and transformed 10T1/2 cell lines found here needs to be further clarified. This level is so low that differences in the fetal calf serum, cell stocks, or sensitivity of the assays used by us and by Gattoni et al. (51) may explain the very low level of c-mos expression found by us versus the lack of expression found by Gattoni et al. (51).

In conclusion, three major points emerge from this study: (a) the expression of protooncogenes studied in 10T1/2 cells was similar to that in whole embryo tissue. The transcript sizes were in general the same except that an extra 8.2-kilobase c-abl transcript was detected in whole embryo tissue but not in 10T1/2 cells; (b) the expression of c-myc at 5- to 7-fold higher levels in three transformed cell lines was significant. As previously observed in other types of tumors (16, 17), increased expression of c-myc may be related to late stages of chemical and radiation transformation in these three cell lines; and (c) the low expression of poly(A)+ c-fos mRNA in growing 10T1/2 cells and its high expression in confluent 10T1/2 cells show that increasing amounts of the c-fos transcript correlate with the approach of C3H/10T1/2 cells to the confluent state. We found little or no expression of poly(A)+ c-fos mRNA in eight chemically and radiation-transformed 10T1/2 cells, and we therefore conclude that expression of c-fos is deregulated in transformed 10T1/2 cells.

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Enhanced Expression of c-myc and Decreased Expression of c-fos Protooncogenes in Chemically and Radiation-transformed C3H/10T1/2 Cl 8 Mouse Embryo Cell Lines

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