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Cellular Transformation and Malignancy Induced by ras Require c-jun

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***ras* is an important oncogene in experimental animals and humans. In addition, activated ras proteins are potent inducers of the transcription factor AP-1, which is composed of heterodimeric complexes of Fos and Jun proteins. Together with the fact that deregulated expression of some AP-1 proteins can cause neoplastic transformation, this finding suggests that AP-1 may function as a critical ras effector. We have tested this hypothesis directly by analyzing the response to activated ras in cells that harbor a null mutation in the c-*jun* gene. The transcriptional response of AP-1-responsive genes to activated ras is severely impaired in c-*jun* null fibroblasts. Compared with wild-type cells, the c-*jun* null cells lack many characteristics of ras transformation, including loss of contact inhibition, anchorage independence, and tumorigenicity in nude mice; these properties are restored by forced expression of c-jun. Rare tumorigenic variants of ras-expressing c-*jun* null fibroblasts do arise. Analysis of these variants reveals a consistent restoration of AP-1 activity. The results provide genetic evidence that c-jun is a crucial effector for transformation by activated ras proteins.**

Members of the ras family of GTPases, initially identified by virtue of their oncogenic potential, are involved in a number of biological processes. Signaling by ras proteins is critically dependent on the state of their guanine nucleotide, such that ras-GTP is active and ras-GDP is inactive. Regulation of the ras guanine nucleotide is complex, with both nucleotide exchange factors and GTPase-activating proteins playing important roles. These activities, in turn, are controlled by a variety of environmental stimuli, including peptide growth factors (for reviews, see references 8 and 10). The nature of the downstream targets of ras has only recently begun to be elucidated. The best-characterized target of ras signaling is the raf protein kinase, which participates in a mitogen-activated protein kinase (MAPK) cascade leading to activation of the ERK1 and ERK2 kinases (53, 54, 55, 60). ras is believed to have other possible effectors, including phosphatidylinositol 3′-OH kinase and the ral guanine nucleotide exchange factor (23, 30, 46, 51). In addition, ras appears to indirectly regulate a number of other candidate effectors, including the stress-activated protein kinase/Jun N-terminal kinase (SAPK/JNK), which phosphorylates and activates the c-jun N-terminal activation domain, and the rho family of GTPases, which regulate actin stress fiber assembly (16, 39, 44). Even less clear than the identities of the putative effectors are their relative roles in eliciting cellular responses. Genetic data strongly support a model in which activation of the raf protein kinase is critical for neoplastic transformation by ras. Mutant ras proteins that cannot interact with raf but can interact with other ras targets are incompetent for transformation (56). Activated raf kinase phosphorylates and activates MEK, which then activates MAPK. Constitutively active forms of raf and MEK also show transforming activity, suggesting that activated MAPK is capable of eliciting biologic responses (13, 36).

Prominent among the nuclear targets of MAPK is the transcription factor AP-1. AP-1 is a sequence-specific DNA-binding transcription factor composed of a mixture of proteins encoded by the Fos and Jun family genes. AP-1 activity is induced by stimuli that activate MAPKs, including the ras GTPases and phorbol esters (3, 17). The mechanisms of AP-1 activation are only partially understood. Synthesis of c-*fos* mRNA is subject to control by the ternary complex proteins SAP1 and Elk1, both of which are believed to be direct targets for activation by MAPK (21, 37). Regulation of Jun proteins by MAPKs is less well understood, although agents that activate MAPKs, including growth factors and phorbol esters, also regulate the expression of c-*jun* mRNA and protein (32, 47). In addition, c-jun transcriptional activity is controlled by the phosphorylation of serines 63 and 73 by the SAPK/JNKs (43, 50). The extent to which this regulation of c-jun activity is under the control of ras is not yet clear.

The mixture of proteins that constitute AP-1 activity is both complex and dynamic, with the overall level of AP-1 activity and the abundance of specific family members changing under different conditions. Three observations have focused attention on the role of c-jun as a possible downstream effector for ras proteins. First, c-*jun* is oncogenic under certain conditions; second, c-*jun* activity is induced in response to ras activation; and third, overexpression of dominant negative c-*jun* alleles can inhibit transformation by ras proteins (1, 11, 18, 34, 39, 50). However, none of these observations directly address the issue of whether c-jun functions as a ras effector. In particular, the interpretation of experiments utilizing dominant negative Jun mutants is complicated by the fact these mutants are expected to interfere with the function of all Jun family members. Therefore, to directly test the hypothesis that c-jun is required for transformation by ras, we have analyzed the transforming response to ras in cells carrying a homozygous null mutation of

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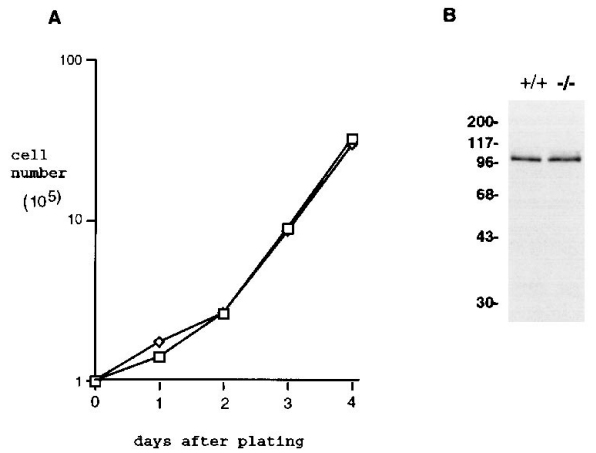

FIG. 1. Wild-type and c-*jun* null cells immortalized by SV40 T Ag show similar growth rates. (A) c-$jun^{+/+}$ (squares) and c-$jun^{-/-}$ (diamonds) cells were immortalized by expression of SV40 T Ag. The resulting cell lines were plated in DMEM with 10% FCS at 10^5 cells per 6-cm-diameter dish, and cell numbers were counted every 24 h. (B) The characteristics of expression of T Ag in the two cell lines were compared by Western blotting using a monoclonal antibody specific for T Ag. Sizes are indicated in kilodaltons.

the c-*jun* gene. We find that these cells are markedly impaired both in the AP-1 transcriptional response and in the ability to be transformed by activated ras proteins. Rare variants of c-*jun* null cells that are transformed in a ras-dependent manner do arise; analysis of these variants demonstrates that they all show increased AP-1 DNA binding activity compared with the nontransformed parent. Taken together, the results provide clear genetic evidence that c-jun and AP-1 are essential for transformation by ras proteins.

MATERIALS AND METHODS

Cells and viruses. Primary embryo fibroblasts (28) were isolated from 11.5-day-old mouse embryos by previously published methods. Wild-type and c-*jun* null cells were transfected with plasmid pOT, which directs expression of the simian virus 40 (SV40) large T antigen (T Ag), and immortalized cell lines were derived. cDNAs encoding H-ras 61L, c-jun, c-jun ΔLZ, JunB, or JunD were cloned into the pBabe series of retroviral vectors (40). c-jun ΔLZ contains a deletion of three amino acids (284 to 286) in the leucine zipper and is defective for both dimerization and DNA binding. H-ras 61L (a gift from C. Der) was cloned into pBabe-puro, which allows for selection in puromycin. All of the Jun family proteins were cloned into pBabe-Hygro, which allows for selection in hygromycin. Proviral DNAs were transfected into ψ-2 cells, and stably transfected cells were isolated. Supernatants from these cells were then used to infect the T Ag-derived cell lines. Selection used 3 μg of puromycin per ml and 200 μg of hygromycin per ml. Cell genotypes were confirmed by Southern blotting. Cell photomicrographs were taken on a Zeiss Axiovert microscope.

Gel shift analysis. Nuclear extracts were prepared by previously described methods (2). Gel shift experiments typically used 5 μg of nuclear extract in buffer C (20 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid] [ph 7.9], 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM dithiothreitol, 25% glycerol), 3 μg of poly(dI-dC), and a ^{32}P-labeled double-stranded oligonucleotide corresponding to the human collagenase AP-1 site (top strand, 5′ AGCTT GTGAGTCAGCCGGAT 3′). Samples were incubated at room temperature for 20 min and then run on 4% polyacrylamide gels in 0.5% Tris-borate-EDTA. Control experiments used an Oct-1 probe to verify that equal amounts of Oct-1 DNA binding activity were present.

Protein and RNA analysis. Jun proteins were detected by Western blotting (immunoblotting) of nuclear extracts. Extracts were prepared as described above, and 30 μg of nuclear extract per sample was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Samples were blotted onto nylon membranes and probed with an antiserum against c-jun, JunB, or JunD (JunB and JunD antibodies were a generous gift of Rodrigo Bravo). SV40 T Ag was detected by immunoblotting of nuclear extracts with the monoclonal antibody KT3. Detection was by chemiluminescence. ras proteins were immunoprecipitated from cells metabolically labeled with ^{35}S-amino acids by using the ras-specific monoclonal antibody Y259. The immunoprecipitates were then separated by SDS-PAGE and visualized by fluorography.

RNA isolation and Northern (RNA) blotting were performed as previously described (5, 58). Stromelysin mRNA was detected by using a ^{32}P-radiolabeled cDNA fragment from the mouse stromelysin cDNA (a generous gift from Lynn Matrisian).

Transformation assays. Saturation density was measured by plating 4×10^5 cells in 6-cm-diameter dishes in Dulbecco's minimal essential medium (DMEM) with 10% fetal calf serum (FCS). Twelve hours after plating, the medium was replaced with DMEM supplemented with 0.5, 2, or 5% FCS. After an additional 4 days, the cell number was determined by counting. For each cell type, the saturation density was determined in triplicate at least twice.

Soft-agar colony assays were carried out by seeding 2×10^4 cells in soft agar (0.4% agar in DMEM with 5% FCS). Dishes were fed twice per week, and macroscopic colonies were counted after 14 days. For ras-expressing c-$jun^{+/+}$ cells, which have a high cloning efficiency in soft agar, cells were also plated at 2×10^3 and 500 cells per plate.

For tumorigenicity assays, nude mice were injected with 10^7 cells in phosphate-buffered saline subcutaneously. The mice were monitored twice weekly for the appearance of tumors.

RESULTS

The availability of mice harboring targeted disruptions of the c-*jun* gene provided an opportunity to examine the role of c-jun in transformation by ras. Mutation of the c-*jun* gene results in embryonic lethality; in addition, fibroblasts isolated from c-$jun^{-/-}$ embryos grow poorly in culture, and despite many attempts, we have not succeeded in generating 3T3-like cell lines from c-*jun* null cells (19, 28, 57). To study the biochemical and biologic responses to ras, immortalized cell lines were generated by expression of SV40 T Ag (see Materials and Methods). Matched fibroblast cell lines derived from wild-type and c-*jun* null cells were characterized. These cell lines do not senesce; they express equivalent levels of T Ag, grow at the same rate (Fig. 1), and are similar in appearance morphologically (see Fig. 3a). In addition, they are not transformed, as measured by several different criteria (see below), consistent with previously published data showing that expression of T Ag in primary embryo fibroblasts results in efficient immortalization but not cellular transformation (4, 27, 41). These cell lines are therefore suitable host cells in which to assay the cellular response to ras. All of the experiments described below were carried out in these T Ag-immortalized cell lines.

Transformation by ras requires c-jun. Wild-type and c-*jun* null fibroblasts were infected with a recombinant retrovirus directing expression of an activated *ras* allele (H-ras 61L). Pools of infected cells were isolated and analyzed for expression of activated ras protein by immunoprecipitation from metabolically labeled cells. Activated ras proteins were expressed at equivalent levels in c-$jun^{+/+}$ and c-$jun^{-/-}$ cells (Fig. 2A).

ras-expressing c-$jun^{+/+}$ and c-$jun^{-/-}$ cells were analyzed for biochemical evidence of AP-1 activation. Expression of activated ras proteins resulted in approximately a fivefold induction of c-jun protein in wild-type cells, as measured by Western blotting, while no c-jun protein was detected in c-$jun^{-/-}$ cells (Fig. 2B); this result is similar to the induction of c-jun protein that is observed in 3T3 fibroblasts following transformation by ras (42). The induction of c-jun protein was accompanied by an increased level of AP-1 DNA binding activity in ras-expressing wild-type cells, while AP-1 DNA binding activity was only slightly induced in ras-expressing c-$jun^{-/-}$ cells (Fig. 2C). This increase in AP-1 DNA binding activity was translated into an increase in expression of the stromelysin gene, a known AP-1-responsive gene (Fig. 2D) (38). Taken together, these data indicate that c-jun expression is required for activation of the AP-1 response by ras in fibroblasts. Additionally, they suggest that c-jun is the Jun family protein responsible for the increased AP-1 DNA binding activity that is observed in response to activated ras.

Having demonstrated that the AP-1 response to activated ras was markedly attenuated in c-$jun^{-/-}$ cells, we analyzed

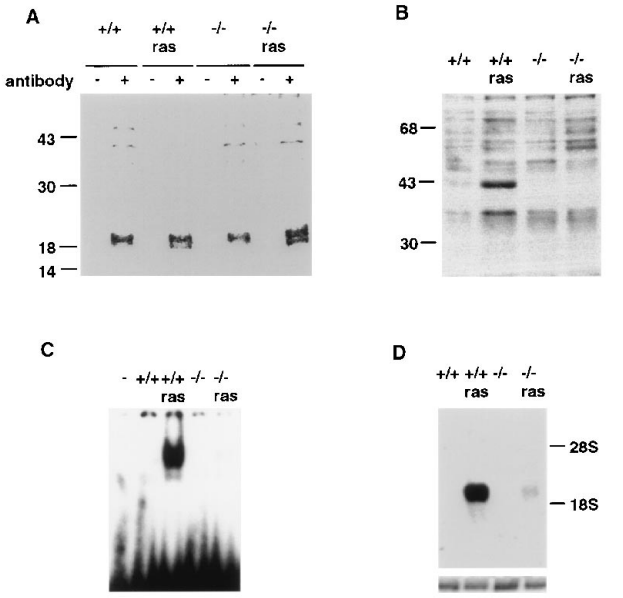

FIG. 2. c-jun expression is required for AP-1 activation by ras. (A) c-*jun*$^{+/+}$ and c-*jun*$^{-/-}$ cells were infected with a recombinant retrovirus directing expression of H-ras 61L. Cells of the indicated genotypes, either expressing or not expressing activated ras, were metabolically labeled with ^{35}S-labeled amino acids, and ras proteins were immunoprecipitated with a monoclonal antibody to ras proteins. The top band represents the endogenous wild-type cellular ras proteins, while the more rapidly migrating species (arrowhead) represents activated ras protein. Sizes are indicated in kilodaltons. (B) Nuclear extracts from cells of the indicated genotypes were analyzed for the expression of c-jun protein by Western blotting. Sizes are indicated in kilodaltons. (C) The same cell types were analyzed for total AP-1 DNA binding activity by gel shift analysis. Nuclear extracts were prepared from each cell type and incubated with a ^{32}P-labeled probe corresponding to the human collagenase AP-1 site. The samples were separated by electrophoresis on 4% polyacrylamide gels in 0.5% Tris-borate-EDTA. (D) The same cell types were analyzed by Northern blotting for expression of stromelysin mRNA (top panel) or glyceraldehyde-3-phosphate dehydrogenase as a loading control (lower panel).

several parameters related to the transformed phenotype. ras-expressing c-*jun*$^{+/+}$ cells, in contrast to ras-expressing c-*jun*$^{-/-}$ cells or cells not expressing activated ras, showed a typical transformed phenotype, with a refractile and rounded appearance (Fig. 3a). The ras-expressing wild-type cells had undergone a loss of contact inhibition, as measured by cell density at saturation of the culture (Fig. 3b and Table 1); this loss of contact inhibition required both activated ras expression and c-jun expression, as it was not observed in cells with other genotypes. The same cells were tested for loss of anchorage dependence by growth in soft agar. Once again, ras-expressing c-*jun*$^{+/+}$ cells, but not other cells, formed colonies in soft agar (Table 1). Finally, we tested the ability of these cells to form tumors after subcutaneous injection into nude mice. All animals injected with ras-expressing c-*jun*$^{+/+}$ cells developed tumors by 10 days after injection, while tumors were not evident in animals injected with other cell types (Fig. 3c and Table 1). At later time points (14 to 16 weeks), animals injected with c-*jun*$^{-/-}$ cells developed tumors in a ras-dependent manner (see below). Thus, by four different criteria, i.e., morphology, loss of contact inhibition, anchorage-independent growth, and tumorigenesis, neoplastic transformation by ras requires c-jun expression.

Rescue of transformation in c-*jun*$^{-/-}$ cells by c-jun. If c-*jun*$^{-/-}$ cells are not transformed in response to expression of activated ras solely as a result of loss of c-jun expression, then it should be possible to transform these cells by expression of c-jun. To test this hypothesis, ras-expressing c-*jun* null cells were infected with a retrovirus directing expression of c-jun. Western blotting confirmed that c-jun protein was expressed at levels approximating those of ras-expressing c-*jun*$^{+/+}$ cells (data not shown). The expression of c-jun in ras-expressing c-*jun*$^{-/-}$ cells resulted in an induction in stromelysin expression to levels similar to those seen in ras-expressing c-*jun*$^{+/+}$ cells (data not shown), demonstrating that the diminution in stromelysin expression in ras-expressing c-*jun* null cells was due to the absence of c-jun. These cells showed morphologic changes similar to those of ras-expressing c-*jun*$^{+/+}$ cells and displayed an increased saturation density, demonstrating loss of contact inhibition (Fig. 3a and Table 1). c-*jun*$^{-/-}$ ras-Jun-expressing cells formed soft-agar colonies much more efficiently than the c-*jun*$^{-/-}$ ras-expressing parent cells (Table 1). When injected into nude mice, the c-*jun*$^{-/-}$ cells expressing ras and ectopic c-jun formed progressively growing tumors at 2 weeks, while the c-*jun*$^{-/-}$ ras-expressing parent cells did not (Table 1). Thus, all of the phenotypes produced by expression of activated ras protein in c-*jun*$^{+/+}$, but not c-*jun*$^{-/-}$, cells were efficiently rescued by expression of recombinant c-jun in c-*jun* null cells. This rescue required active c-jun protein; expression of a c-jun mutant protein incapable of dimerization or DNA binding did not rescue any of the phenotypes described above (Fig. 3a and Table 1). In addition, transformation required the expression of activated ras, as expression of c-jun in c-*jun*$^{+/+}$ or c-*jun*$^{-/-}$ cells did not result in transformation (Table 1). These data demonstrate that loss of c-jun protein is in fact the cause of the defective ras response in c-*jun* null cells. Furthermore, forced expression of c-jun in the absence of activated ras did not induce transformation, suggesting that induction of c-jun is not the sole mechanism by which ras proteins function.

The ability of exogenously expressed c-jun protein to rescue ras responsiveness provides a novel assay for the analysis of Jun function. The data in Fig. 2 suggest that c-jun is the sole Jun family member that is induced in response to expression of activated ras in these cells. Analysis of the expression of *junB* and *junD* mRNAs and proteins confirmed that they are expressed at the same levels in the presence or absence of activated ras (data not shown). This finding suggested the possibility that ectopic expression of either JunB or JunD might rescue the ras response in c-*jun*$^{-/-}$ cells. To test this idea directly, ras-expressing c-*jun*$^{-/-}$ cells were infected with recombinant retroviruses directing expression of either JunB or JunD and analyzed as described above. Both JunB and JunD rescue transformation by ras in c-*jun*$^{-/-}$ cells, although the efficiency of rescue is reduced compared with that of c-jun (Table 1). In addition, we observed that there is not complete correlation between all of the assays used to measure transformation. For example, JunD inefficiently restores soft-agar colony formation but efficiently rescues tumorigenicity (Table 1). The basis of this incomplete rescue is not clear.

c-*jun*$^{-/-}$ cells generate tumorigenic variants. In the experiments described above, c-*jun*$^{-/-}$ cells expressing activated ras failed to generate tumors at 2 weeks, while ras-expressing c-*jun*$^{+/+}$ cells generated rapidly growing tumors at this time (Fig. 3). At later time points (14 to 16 weeks), however, animals injected with ras-expressing c-*jun*$^{-/-}$ cells developed progressively enlarging tumors. These tumors arose in a ras-dependent manner, as they were not observed in animals injected with cells not expressing ras or in c-*jun*$^{-/-}$ cells expressing exogenous c-jun. The tumors were dissected, and cells were isolated and propagated in culture; upon reinjection into animals, these cells were rapidly tumorigenic. Molecular analysis confirmed that the tumorigenic cells were composed of c-*jun*$^{-/-}$ cells. Southern blotting demonstrated that each of the

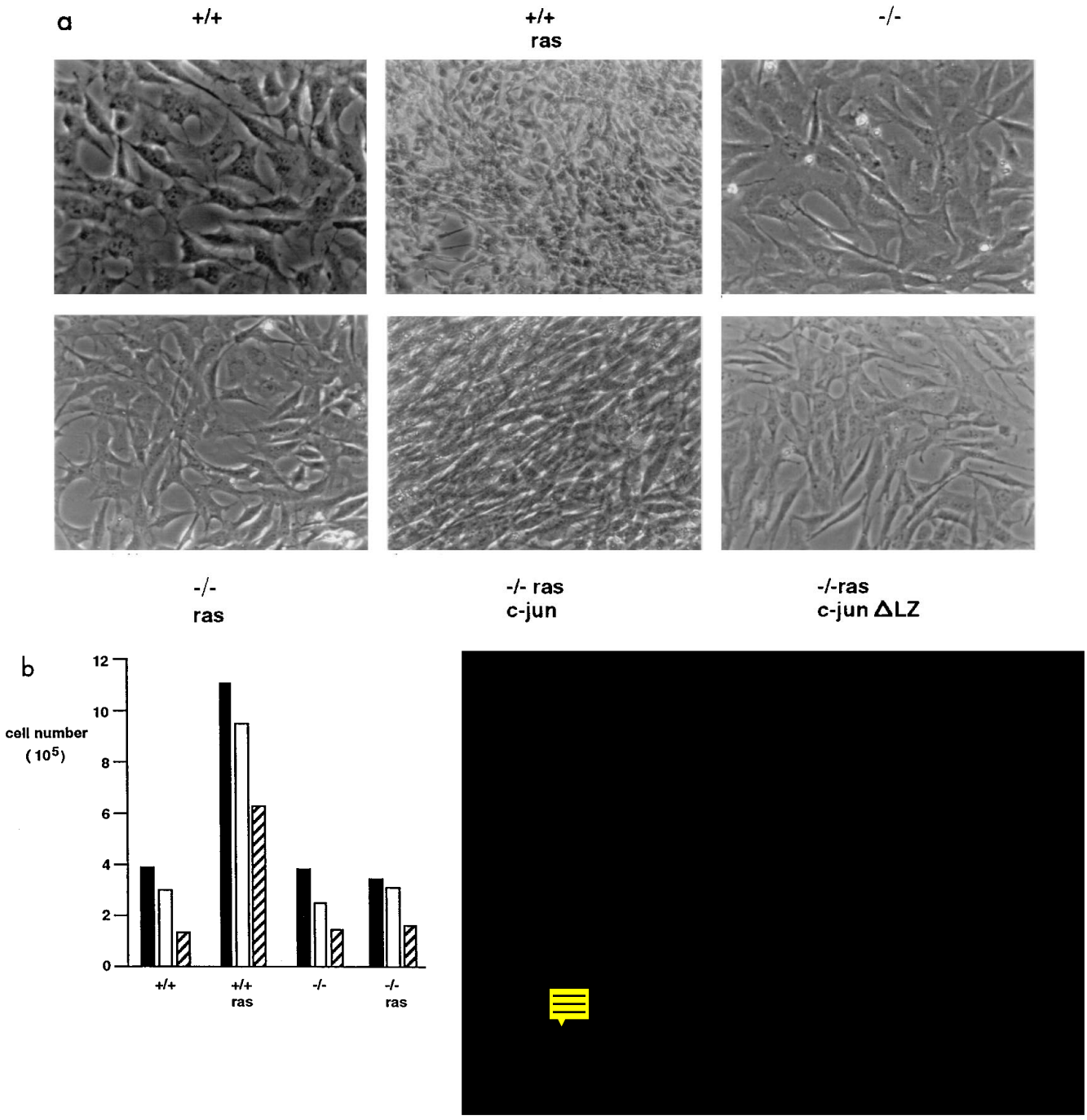

FIG. 3. Transformation by ras requires c-jun expression. (A) Phase-contrast photomicrographs of cells expressing the indicated genes. (B) Fibroblasts expressing the indicated genes were seeded at 4×10^5 cells per 6-cm-diameter dish. After 12 h, the medium was changed to DMEM with 5% FCS (black bars), 2% FCS (white bars), or 0.5% FCS (hatched bars). Cells were counted after growth for an additional 4 days. Each experiment was carried out in triplicate at least twice. (C) Cells of the indicated genotypes were injected subcutaneously into nude mice (10^7 cells per animal). The photographs were taken 2 weeks after injection.

tumors was composed of cells containing a single ras provirus; however, the site of integration was different in each of the tumors (data not shown). Therefore, these tumors represent four independent events.

The tumorigenic variants of ras-expressing c-*jun*$^{-/-}$ cells were analyzed for other aspects of the transformed phenotype. All of these cell lines showed altered morphology (Fig. 4). In addition, they showed loss of contact inhibition, and three of the four grew efficiently in soft agar (Table 2). Therefore, these cells are phenotypically similar to ras-expressing c-*jun*$^{+/+}$ cells.

Tumorigenic variants of ras-expressing c-*jun* null cells could arise by two possible mechanisms. One mechanism would involve the activation of AP-1 activity in a c-jun-independent manner. Alternatively, these cells could have arisen by a bypass mechanism, in which AP-1 activity does not contribute to the transformed state of the cells. It should be possible to distin-

TABLE 1. Characteristics of c-*jun* cells

c-*jun* genotype	Exogenous genes	Saturation density[a] (10^6)	Soft-agar colonies[b]	No. of nude mice with tumors[c]
+/+		2.6	0	0
+/+	*ras*	9.4	1,400[d]	4
−/−		2.4	0	0
−/−	*ras*	3.3	1	0
+/+	c-*jun*	3.1	0	0
−/−	c-*jun*	2.7	0	0
−/−	*ras* + c-*jun*	8.2	458	4
−/−	*ras* + c-*jun* ΔLZ	3.7	0	0
−/−	*ras* + *junB*	6.8	126	4
−/−	*ras* + *junD*	5.1	7	4

[a] Cells were plated at 4×10^5/6-cm-diameter dish in DMEM with 10% FCS. Twelve hours after plating, the medium was changed to DMEM with 2% FCS, and cells were counted after an additional 4 days.

[b] Cells (2×10^4) were plated in soft agar as described in Materials and Methods. Macroscopic colonies were counted after 14 days.

[c] Cells (10^7) suspended in phosphate-buffered saline were injected subcutaneously into nude mice, which were then monitored for the appearance of tumors. Each group contained four mice.

[d] The high soft-agar colony formation rate of these cells made it difficult to count colonies after plating of 2×10^4 cells. Therefore, these cells were also plated at 2×10^3 and 500 cells per dish to determine soft-agar colony-forming efficiency.

guish between these different mechanisms by analyzing the AP-1 status of these cells. We therefore analyzed the AP-1 DNA binding activity present in nuclear extracts of these cells. The results showed that in all cases, the level of AP-1 DNA binding activity was higher than that in the nontumorigenic parental cells, although in all cases it was less than that observed in ras-expressing c-$jun^{+/+}$ cells (Fig. 5a). Western blotting demonstrated that in each case, the amounts of JunB protein present in the nuclear extract were similar in the nontumorigenic c-$jun^{-/-}$ ras-expressing cells and in the tumorigenic variants. In contrast, each of these variants showed an increase in JunD protein compared with the nontumorigenic parent (Fig. 5b). These results suggest that in the absence of c-jun, increased expression of JunD protein may contribute to ras-dependent transformation. The mechanism by which JunD is induced in the tumorigenic variants is not clear. The nontumorigenic parental cells and the tumorigenic variants express equivalent levels of *junD* mRNA (data not shown), and therefore the induction of JunD protein that is observed does not occur at the transcriptional level. Whether induction occurs by translational control or by a posttranslational mechanism involving altered rates of protein turnover remains to be determined. These experiments demonstrate that even in the absence of c-jun protein, induction of AP-1 activity is correlated with transformation by ras.

DISCUSSION

We have used fibroblasts immortalized by SV40 T Ag to assess the role of c-jun in the transforming response to ras. T Ag-immortalized c-$jun^{-/-}$ cells showed a defective ras response compared with their c-$jun^{+/+}$ counterparts. That this defect in oncogenic signaling is due to selective loss of c-jun is demonstrated by the rescue by ectopic expression of c-jun. Our ability to isolate rare transformed variants from c-$jun^{-/-}$ cells shows that in every case, bypass involves an increase in AP-1 activity. Thus, several different lines of evidence converge to demonstrate an important role for AP-1 in transformation by ras proteins.

c-jun and the cell cycle. Fibroblasts isolated from c-*jun* null embryos fail to grow in culture. When c-$jun^{-/-}$ cells are treated with growth factor, the cells fail to divide and are blocked in the G_1 phase of the cell cycle (28). As induction of AP-1 activity is implicated in the mitogenic response to growth factors, this growth defect presumably represents an inability to synthesize a component critical for cell cycle progression. The ability of SV40 T Ag to bypass the growth requirement for c-jun expression may provide some insight into the mechanism of the cell cycle arrest in c-*jun* null cells and the role of AP-1 proteins in cell cycle progression in response to growth factors. T Ag possesses a number of biochemical activities, including the ability to bind to and functionally inactivate pRb and p53 (15, 33), both of which are capable of mediating G_1 arrest. Further experiments may help to clarify the mechanism of the cell cycle arrest in c-$jun^{-/-}$ cells.

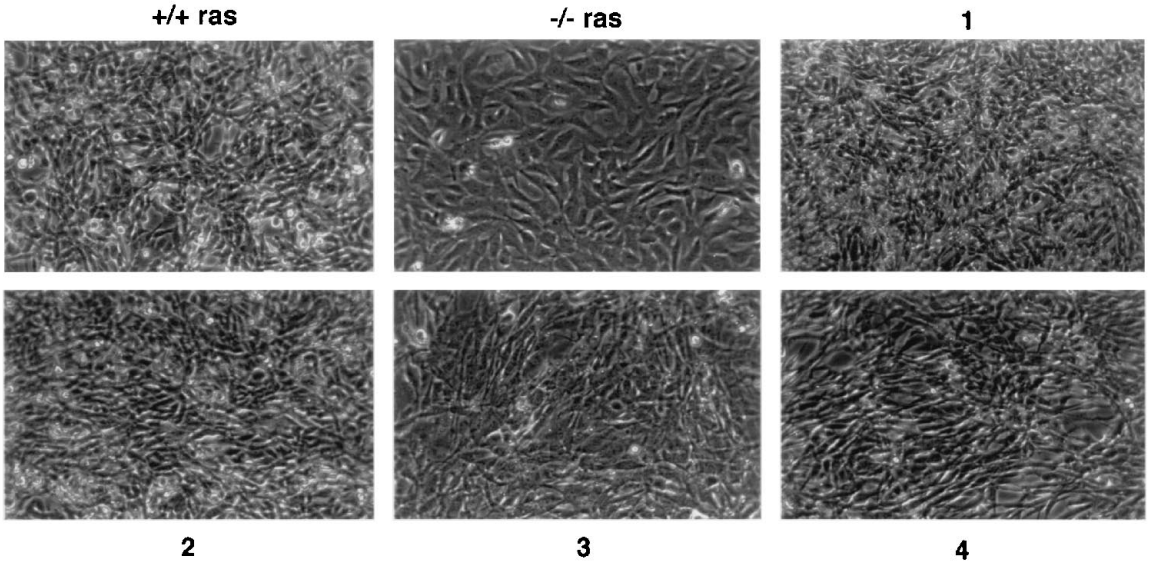

FIG. 4. Morphologies of tumorigenic variants of ras-expressing c-$jun^{-/-}$ cells. Animals injected with ras-expressing c-$jun^{-/-}$ cells developed tumors after 14 to 16 weeks. The tumors were dissected, and cells were plated in culture. Phase-contrast photomicrographs of the tumorigenic cells are shown.

TABLE 2. Characteristics of variants of ras-expressing c-*jun* cells

c-*jun* genotype	Saturation density[a] (10^6)	Soft-agar colonies[b]	No. of nude mice with tumors[c]
+/+ ras	10.3	31	4/4
−/− ras	3.2	0	0/4
Variants			
1	11.7	22	1/1
2	12.4	29	1/1
3	6.8	1	1/1
4	9.5	37	1/1

[a] Cells were plated at 4×10^5/6-cm-diameter dish in DMEM with 10% FCS. Twelve hours after plating, the medium was changed to DMEM with 2% FCS, and cells were counted after an additional 4 days.

[b] Cells (500) were plated in soft agar as described in Materials and Methods. Macroscopic colonies were counted after 14 days. For variant 3, plating 2×10^4 cells resulted in 46 colonies, a result that can be compared with those shown in Table 1.

[c] Cells (10^7) suspended in phosphate-buffered saline were injected subcutaneously into nude mice, which were then monitored for the appearance of tumors.

c-jun as an oncogenic effector. Members of the ras family of GTPases are implicated in formation of many different tumor types. The precise mechanisms of transformation by ras proteins remain unclear, but several pieces of evidence suggest that AP-1 is an important downstream ras effector. These include the observations that AP-1 activity is induced by ras, that activating mutations in some AP-1 family members are oncogenic, and that expression of dominant negative c-*jun* alleles inhibits transformation by ras (1, 11, 14, 16, 18, 34). We have provided direct genetic evidence that efficient transformation by activated ras proteins requires c-jun. In addition, we have shown that the rare transformed variants that do arise in c-*jun* null cells show increased AP-1 activity. Taken together, these data strongly suggest that AP-1 activity is crucial for transformation by the ras GTPases.

Transcriptional control and oncogenic potential for c-jun are most clearly demonstrated in the context of AP-1 activity. However, c-jun has also been shown to interact with a number of other transcription factors, including the glucocorticoid receptor, the myogenic proteins, and the CREB family member ATF-2 (6, 17, 26, 29, 35, 49, 59). It is possible that transformation by c-jun is a result of modulation of the activity of one or more of these transcription factors. Whatever the precise mechanism, the results provide genetic evidence that c-jun is a critical ras effector.

Our data can be compared with those of studies evaluating the role of c-fos in neoplastic transformation by ras proteins. Recent data demonstrate a crucial role for c-fos in the progression of skin cancer in animals induced by a combination of an activated *ras* transgene and topical application of phorbol esters (48). However, experiments similar to those described here show that c-fos expression is not required for ras transformation of fibroblasts (24). This finding suggests that there may be some cell type variability in the requirements for specific AP-1 proteins, perhaps reflecting cell type differences in expression of other AP-1 family members. It will therefore be important to evaluate the role of c-jun in oncogenic transformation by ras proteins in a different cellular context.

In addition to ras, several other oncogenic signaling molecules, including the growth factor receptor tyrosine kinases, the src family of cytoplasmic tyrosine kinases, and the raf serine/threonine kinase, activate AP-1 activity. Whether transformation by these molecules requires c-jun remains to be demonstrated. c-$jun^{-/-}$ embryonic stem cells grow poorly as teratocarcinomas, suggesting that c-jun is an important oncogenic effector for the growth of these tumors (20). Likewise, a role for AP-1 proteins as effectors in normal physiologic signaling by ras proteins remains to be demonstrated, although expression of dominant negative Jun mutants in *Drosophila melanogaster* suggests that Jun does play a role in signaling by the sevenless tyrosine kinase and ras proteins (9).

AP-1 regulation by ras proteins. AP-1 apparently receives multiple ras-dependent signals. Activation of the MAPK/ERK pathway by ras most clearly influences AP-1 activity by increasing the expression of the Fos family proteins (21, 37). Essentially all of the AP-1 DNA binding activity present in our ras-transformed cells is sensitive to treatment with an antibody that recognizes the Fos family proteins (data not shown), demonstrating that the AP-1 activity in these cells is composed exclusively of Fos-Jun heterodimers and not Jun-Jun homodimers. As Fos proteins contribute to AP-1 DNA binding activity only as part of a Fos-Jun complex, ras signaling through Fos proteins is expected to be dependent on the expression of Jun proteins. It is not clear if MAPK/ERK signals directly regulate the expression or activity of Jun family proteins, although many activators of the MAPK/ERKs do activate c-jun (32, 47).

The Jun family of proteins are also regulated by phosphorylation of serine residues in their activation domains by the SAPK/JNKs (43, 50). In some, but not all, cell types, these kinases can be activated by ras signaling (7, 31, 39, 50). Analysis of the ability of a c-jun mutant protein containing alanine substitutions at residues 63 and 73 shows that it efficiently rescues soft-agar colony formation and high saturation density but not tumorigenicity (data not shown). We have not yet determined the phosphorylation state of serines 63 and 73 or the activity of SAPK/JNK in this system, which may provide insight into the ability of ras proteins to regulate this signaling pathway. In any event, our results provide clear genetic evidence that c-jun is a crucial component of ras signaling through AP-1. Also unclear is the potential regulation of AP-1 by other potential ras effectors, including phosphatidylinositol 3′-OH kinase, fos-regulating kinase (FRK), and the ral GTPase.

JunD as a possible oncogenic effector. The Jun family of proteins consists of c-jun, JunB, and JunD. Previous functional studies have suggested that JunD may function as a negative regulator of AP-1 activity and impair transformation by ras proteins (42). Our data suggest that JunD efficiently rescues tumorigenicity, but not soft-agar colony formation, by ras-expressing c-$jun^{-/-}$ cells. In addition, the analysis of tumorigenic c-$jun^{-/-}$ variants shows a consistent increase in JunD protein levels. These data suggest that JunD can contribute to positive regulation of AP-1 activity. The reasons for this discrepancy

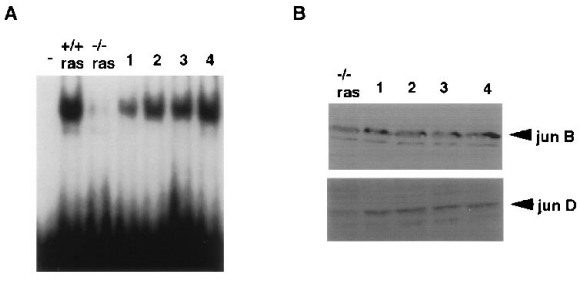

FIG. 5. Tumorigenic variants of ras-expressing c-$jun^{-/-}$ cells show increased AP-1 DNA binding activity. (A) Nuclear extracts were prepared from cells of each of the indicated genotypes and analyzed by gel shift for AP-1 DNA binding activity, using a probe corresponding to the collagenase AP-1 site. (B) The same extracts were analyzed for the expression of JunB and JunD proteins by Western blotting.

are not immediately apparent, but data from several other cell types support the idea that JunD can function either as a positive regulator or as a negative regulator of AP-1 signaling (12, 22, 52). These data suggest that the role of JunD may be dependent on the cell context, with the levels of other AP-1 family proteins perhaps contributing to some of the differences observed.

The mechanism by which JunD expression is induced in c-*jun* null tumorigenic cells is unclear. Northern blotting demonstrates that there is no change in mRNA levels, and so the increase in JunD protein presumably occurs at either a translational or a posttranslational level. Further experiments may provide some insight into this potentially novel mechanism of control of AP-1 activity.

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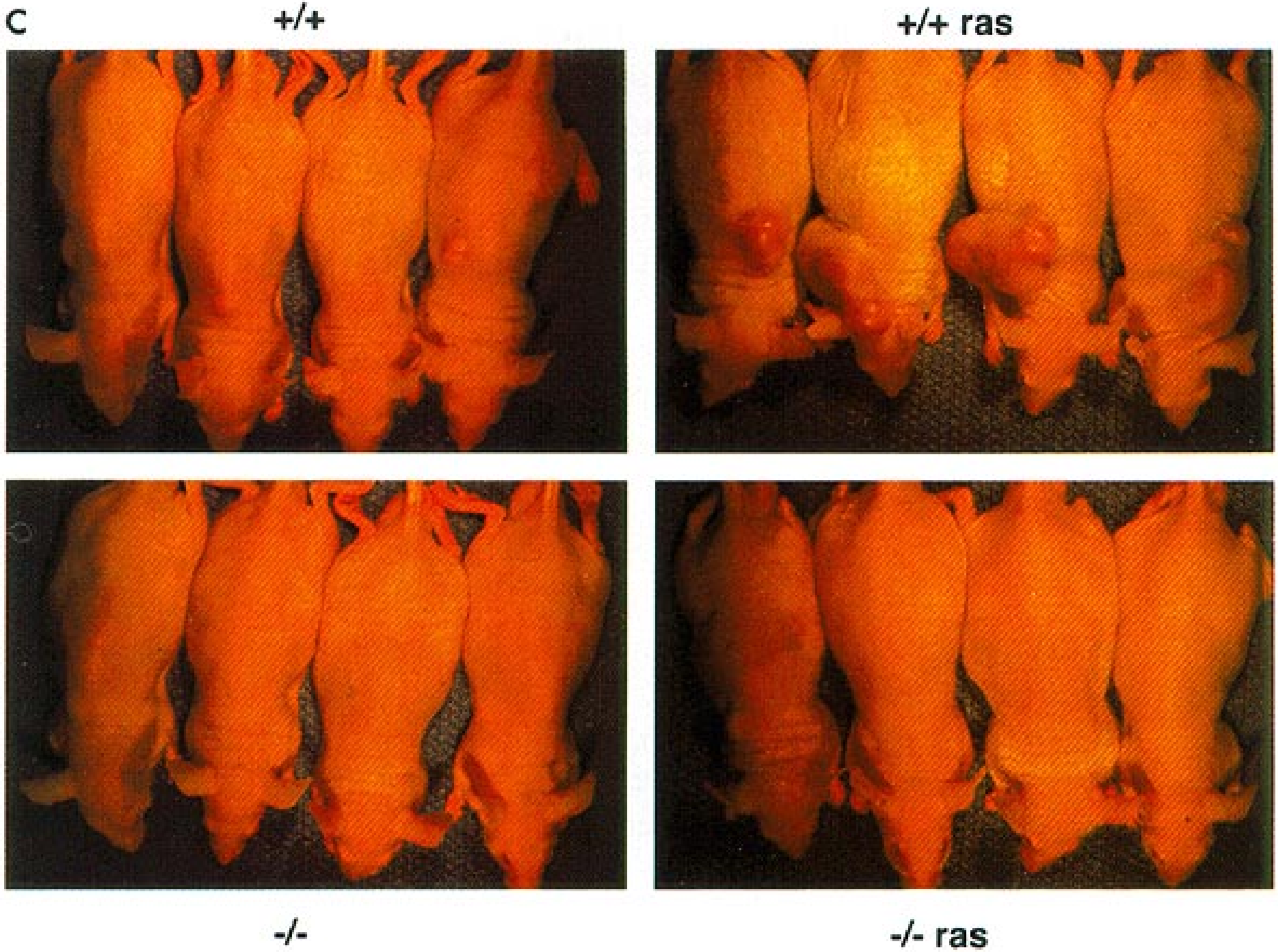
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