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Endogenous AP-1 Levels Necessary for Oncogenic Activity Are Higher Than Those Sufficient to Support Normal Growth

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We investigated the role of endogenous AP-1 in human tumor cell lines by introducing SupJunD-1, a dominant-negative mutant of AP-1, using vesicular stomatitis virus G protein (VSV-G)-pseudotyped retrovirus vectors. Single inoculation of six human tumor cell lines, originating from osteosarcomas, non-small cell lung carcinomas or cervical carcinomas, with recombinant SupJunD-1 virus at a high multiplicity of infection readily inhibited colony formation in soft agar. We detected no significant changes in expression levels of AP-1 components c-Jun or Fra-1, adhesion molecules CD44 or E-cadherin, or cell cycle regulator p53, which are encoded by genes previously reported to be under the control of AP-1 in some mouse or human cell lines. By varying the dosage of VSV-G-pseudotyped retrovirus, we were able to change the proviral copy number of *supjun*D-1 from 1 to approximately 10 and monitor suppression of endogenous AP-1 function as assessed by growth characteristics of the tumor cell lines, we found a SupJunD-1 dosage which significantly suppressed anchorage-independent growth without affecting the cellular growth in monolayer cultures at all. We conclude that endogenous AP-1 levels necessary for oncogenic activity are much higher than those sufficient to support normal growth.

Key Words: **AP-1; human tumor cell lines; osteosarcomas; non-small cell lung carcinomas; cervical carcinomas; dominant-negative mutants; VSV-G pseudotyped retrovirus; p53; anchorage-independent growth.**

Abbreviations used: CEF, chicken embryo fibroblasts; VSV-G, vesicular stomatitis virus G protein; MOI, multiplicity of infection; IRES, internal ribosomal entry site; EMCV, encephalomyocarditis virus; MLV, murine leukemia virus; DMEM, Dulbecco's modified Eagle medium; TRE, TPA response element; SDS, sodium dodecyl sulfate; i.u., infectious units; LTR, long terminal repeat; HPV, human papillomavirus; LCR, long control region.

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Tumor formation is causally related to the acquisition of a series of genetic lesions. Most of the currently known lesions cause either a gain of transforming function of dominant oncogenes or a loss of tumor suppressor function of tumor suppressor genes. This raises the possibility that correction of a genetic abnormality in the cancer cell by the transduction of either a tumor suppressor gene or a dominant-negative mutant of oncogene could mediate a sufficient therapeutic effect. The functions of AP-1 have been investigated largely in CEF or established rodent fibroblasts and endogenous AP-1 was shown to play important roles not only in normal cell growth but also in several transformed cells induced by oncogenes (1, 2). This transcription factor is composed of Fos family proteins (c-Fos, Fra-1, Fra-2 and FosB) and Jun family proteins (c-Jun, JunB and JunD). All Fos family members can form stable heterodimers with any of the Jun family members, but the Jun family members can also form unstable dimers among themselves (3–5). Although Fos and Jun hetero- and homodimers possess basic domains that bind to similar DNA-binding sites (TGAC/GTCA, AP-1-binding sites), each dimer has a distinct regulatory function that either positively or negatively modulates transcription (6, 7). High-level expression of most members of the *fos* or *jun* gene families cause cellular transformation of CEF (3, 8, 9) or established rodent fibroblasts. Wild-type JunD has no transforming activity, but some JunD mutations, which arise spontaneously during propagation of the *jun*D gene in a retrovirus vector can acquire transforming potential (10). Thus, uncontrolled expression and qualitative changes of any component of AP-1 can induce cellular transformation.

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We previously constructed dominant-negative mutants of v-Fos (SupFos-1), c-Jun (SupJun-1) and JunD (SupJunD-1) (2, 11, 12). SupJun-1 and SupJunD-1 are identical to wild-type c-Jun and JunD, except for a large N-terminal deletion spanning the entire transactivation domain. Both the basic region and the leucine zipper motif remain intact in these mutants. Like wild type, SupJun-1 or SupJunD-1 forms heterodimers with Fos family proteins and Jun family proteins. These complexes bind to DNA *in vitro* (2) but are unable to transactivate gene expression. In addition to all components of AP-1, these mutants efficiently suppress transformation by oncogenes v-*src*, v-*yes*, v-*fps*, c-Ha-*ras* and activated *raf* in CEF (2) and rodent cell lines (13–15) by inhibiting activation of endogenous AP-1.

Here, we examined whether inhibition of endogenous AP-1 suppresses oncogenic potential of human tumor cell lines originating from osteosarcomas, non-small cell lung carcinomas and cervical carcinomas. Unlike c-Jun, JunD is not oncogenic and the dominant-negative mutants SupJunD-1 and SupJun-1 are equally effective in suppressing anchorage-independent growth of v-*src*-transformed CEF (12). Therefore, we have chosen SupJunD-1 as the dominant-negative mutant in this study.

MATERIALS AND METHODS

Plasmid construction. pBabe-IRES was generated by ligating the 4.2-kb *Eco*RI–*Eco*RV fragment of pBabe (16) and the 0.6-kb *Eco*RI–*Pml*I fragment of pIR-1 (17) that carries the entire IRES from EMCV. A 0.7-kb DNA fragment of the puro gene was amplified by PCR using a pair of primers (5′-AGACGACCTTCCATGGCCGAGTACAAGC-3′ and 5′-TCGGACATATGGGGTCGTGCGCTCCTTT-3′) and pPUR (Gibco/BRL) as the template. The PCR product was digested with *Nco*I and *Nde*I and ligated into the *Nco*I–*Nde*I site of pBabe-IRES to generate pBabe-IRESpuro. *Bgl*II restriction endonuclease digestion of pMF-GnlslacZ (18) yield 3.1-kb fragment containing the nlslacZ gene and pDS-supJunD-1 (12) yield 0.3-kb fragment containing the entire *supjun*D-1 gene. Each DNA fragment was inserted into the unique *Bam*HI site of pBabe-IRESpuro to generate pBabe-lacZ-IRESpuro and pBabe-supjunD-1-IRESpuro. The *Bgl*II DNA fragment carrying the *supjun*D-1 gene was ligated into the unique *Bam*HI site of pBluescript SK^+ to generate pBS-supjunD-1. The 1.5-kb *Kpn*I fragment carrying U5, the packaging signal and the *gag* gene was excised from pBabe-IRESpuro and ligated into the unique *Kpn*I site of pBluescript SK^+ to generate pBS-gag.

Cell lines and DNA transfection. The prepackaging cell line, PtG-S2 (19) carries the *gag* and *pol* genes from MLV and a *VSV-G* gene that is silent before the introduction of Cre recombinase. Cell lines PtG-S2, rat fibroblast 3Y1, human osteosarcomas U2-OS and Saos-2, human non-small cell lung carcinomas A549 and H1299 and HPV induced-human cervical carcinomas HeLa and SiHa were maintained in high glucose DMEM (Gibco/BRL) supplemented with 10% fetal calf serum and incubated at 37°C. PtG-S2 and its derivatives were grown in the presence of 4 μg/ml of blasticidin S (Funakoshi, Tokyo, Japan) and 1 mg/ml of G418 (Gibco/BRL). Neo or Ras virus-infected NIH3T3 cells (20) were maintained in low glucose DMEM (Gibco/BRL) supplemented with 10% calf serum and 400 μg/ml of G418 and incubated at 37°C. Virus-infected NIH3T3 cells, U2-OS cells and A549 cells were selected with 2 μg/ml of puromycin (Sigma, St. Louis, MO) and virus-infected Saos-2 cells were selected with 1 μg/ml of puromycin. PtG-S2 cells were seeded at 2×10^6 cells/100 mm dish and after 1 day, transfected with pBabe-IRESpuro, pBabe-lacZ-IRESpuro, or pBabe-supjunD-1-IRESpuro (4 μg) by the use of Lipofectamine Plus Reagent (Gibco/BRL). Two days after the transfection, cells were seeded and incubated with 1 μg/ml puromycin for 3 or 4 days more to propagate mixed populations of puromycin-resistant colonies.

Retrovirus preparation. PtG-S2 and its derivatives were seeded at 1.0×10^6 cells/100-mm dish, incubated at 37°C kept for 1 day and infected with AxCANCre (21) at an MOI of 10. Blasticidin S, G418 and puromycin were removed from the culture medium immediately before AxCANCre infection. Two days after adenovirus infection, cultures were incubate at 32°C to obtain slightly more stable production of VSV-G-pseudotypes. Media a containing retrovirus were collected every day and replaced with fresh media. About 2×10^5 IU/ml of LacZ virus (pBabe-lacZ-IRESpuro), control virus (pBabe-IRESpuro) and SupJunD-1 virus (pBabe-supjunD-1-IRESpuro) were produced and further concentrated by ultracentrifugation to 1×10^8 IU/ml. For titration of VSV-G-pseudotyped retrovirus, indicator 3Y1 cells expressing vector RNA or *supjun*D-1 RNA was evaluated by *in situ* hybridization using digoxigenin-labeled antisense RNA. Digoxigenin-labeled probes were synthesized from linear plasmids using DIG RNA Labeling Kit (Boehringer-Mannheim) and *in situ* hybridization was performed as described previously (23).

Colony formation in soft agar and growth curve analysis. Ras-transformed NIH3T3 cells and human tumor cell lines were inoculated with either control virus or SupJunD-1 virus and seeded at 6×10^4 cells (U2-OS), 1×10^4 cells (NIH3T3/Ras and Saos-2), 1×10^3 cells (A549, HeLa and SiHa) or 3×10^2 cells (H1299) into 60-mm dishes in suspensions of 0.38% Noble agar (Difco) in DMEM (low glucose) supplemented with 10% fetal calf serum on top of a bed of 0.5% Bacto agar (Difco) in the same complete medium. The cultures were incubated at 37°C for 13 to 28 days and colonies were counted. To quantitate doubling times and saturation densities, cells were seeded into 60-mm dishes and cell numbers were counted every 24 h using two plates.

Gel shift analysis. Nuclear extracts for gel shift analysis were prepared as described previously (2, 11). Nuclear extracts (2.5 μg protein per lane) and poly(dI:dC) (0.5 μg) were incubated in 20 μl of binding buffer (10 mM Tris–Cl (pH 7.5), 1.2 mM EDTA, 10 mM dithiothreitol, 5% glycerol, 1 mM phenylmethylsulfonyl fluoride) at 4°C for 15 min. ^{32}P-labeled double-stranded probe DNA (0.2 μg) was added and incubated at 4°C for another 15 min. For competition analysis, a 30-fold molar excess of unlabeled oligonucleotide was added to the mixture 15 min before addition of the labeled probe. For supershift analysis, 4 μl of the appropriate antiserum was added to the mixture 15 min before addition of the labeled probe. The antisera were rabbit polyclonal anti-Fos-pep-1 (22) that is reactive with all Fos family proteins (designated hereafter anti-Fos antiserum), anti-c-Jun (#10) (9), which is reactive with all Jun family proteins (designated hereafter as anti-Jun antiserum) and anti-JunD (329; Biotechnology, Santa Cruz, CA). The DNA-protein complexes were separated by electrophoresis on a nondenaturing 5% acrylamide gel at 4°C and shifted bands were detected by autoradiography. The double-stranded DNA probe including collagenase TRE and its mutant probe were described previously (2, 11).

Protein analysis. For Western blotting, whole cell extracts (30 μg) were prepared under denaturing conditions, separated by electrophoresis on SDS–10% polyacrylamide gels and transferred to nitrocellulose and immunostained with rabbit polyclonal anti-c-Jun (H-79; Santa Cruz), anti-Fra-1 (N-17; Santa Cruz), anti-p53 (FL-393; Santa Cruz), murine monoclonal anti-CD44 (A3D8; Sigma) or anti-E-cadherin (clone 36; Transduction Laboratories, Lexington, KY) and visualized by ECL Western blotting detection system (Amersham, Buckinghamshire, England).

RESULTS

Strategy for the introduction of SupJunD-1, a dominant-negative mutant of AP-1. For introduction of the *supjun*D-1 gene into human tumor cell lines, we used a system that was developed by our group (19). VSV-G-pseudotyped retrovirus vector has advantage over conventional amphotropic retrovirus vectors in that it has a broad host range, very high transfection efficiency and can be readily concentrated ($>10^9$ i.u./ml) by ultracentrifugation (24). Furthermore, we recently demonstrated that VSV-G-pseudotyped retrovirus vector can transduce entire populations of several cell lines in an MOI-dependent manner. Using NIH3T3 or human tumor cell lines, the ratio of cells transfected with the VSV-G-pseudotyped vector corresponded closely with the equation $1 - e^{-\mathrm{MOI}}$, as predicted from Poisson distribution of transfection of an entire cell population (25). The copy number of integrated vector proviral DNA and the increase in expression of the exogenous gene was nearly linear with the dose of the VSV-G-pseudotyped vector (25). Therefore, we can regulate the copy number of integrated vector DNA by changing the MOI. We used an MLV-based vector with an IRES where a single transcript carrying both an exogenous gene and a *puro* gene are driven by the same LTR promoter. Translation of the *puro* gene is enabled by the IRES.

Biological effects of high level expression of SupJunD-1. For high level induction of SupJunD-1, all cell lines were transduced with SupJunD-1 virus at an MOI of 10. If transduction of the entire cellular population follows a Poisson distribution, only a single cell out of 2×10^4 cells would escape transduction. After a single inoculation at an MOI of 10, cells were grown for 2 days without selection and then examined for cellular morphology, anchorage-independent growth and cellular growth in monolayer cultures. In several cell lines tested, 6–20 copies of the proviral DNA per cell were detected (25 and data not shown) by Southern blot analysis.

The suppressive effect of SupJunD-1 expression on anchorage-independent growth was examined in the human solid tumors cell lines U2-OS, Saos-2, A549, H1299, HeLa and SiHa as well as Ras transformed NIH 3T3 (NIH3T3/Ras). Introduction of 10 copies per cell of the *supjun*D-1 transgene into all six human cell lines and NIH3T3/Ras efficiently suppressed anchorage-independent growth (Fig. 1, Table 1). The colony-forming activity of U2-OS and A549 was reduced to less than 10% and in Saos-2 and HeLa cells, reduction in colony size as well as colony number was observed (Table 1, data not shown). These results indicate that some minimum level of endogenous AP-1 activity is essential for oncogenic activity in human tumor cell lines.

Next we analyzed the growth curves of monolayer cultures of control virus- or SupJunD-1 virus-transduced

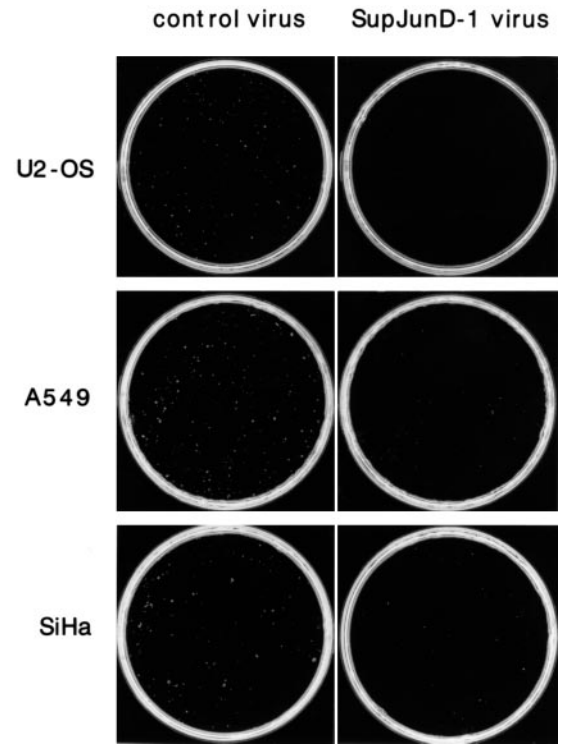


FIG. 1. Colony formation of SupJunD-1-transduced human tumor cell lines in soft agar. U2-OS (originated from osteosarcoma), A549 (originated from non-small cell lung carcinoma) and SiHa (originated from cervical carcinoma) were transduced with control virus or SupJunD-1 virus at an MOI of 10 and were seeded into soft agar and kept at 37° for 21–26 days.

cell lines using the parallel cultures used for the analysis of anchorage-independent growth. While transduction with control virus had no effect on cell growth at all, introduction of approximately 10 copies of *supjun*D-1 transgene significantly reduced growth rate in NIH3T3/Ras and U2-OS cells, slightly reduced growth rate in Saos-2 and A549 cells and significantly reduced saturation density of NIH3T3/Ras, Saos-2 and HeLa cells (Table 1). SupJunD-1 transgene, even at high copy numbers, affected neither growth rate nor saturation density in H1299 and SiHa cells (Table 1).

Dosage effect of SupJunD-1 transgene on anchorage-independent growth and growth in monolayer cultures. To determine the effect of low supjunD-1 transgene copy number on growth characteristics, the human tumor cell lines and NIH3T3/Ras cells were transduced with SupjunD-1 virus at an MOI of 0.1 and selected for 1 week with puromycin in order to yield cells having a single copy of the supjunD-1 transgene. The growth rate and saturation density did not differ significantly between monolayer cell cultures carrying a single copy of either the SupjunD-1 virus or control virus (Table 1). To determine the effects of SupJunD-1 expression levels on growth properties of human tumor cell lines more in detail, we scrutinized U2-OS and A549, by

TABLE 1

Suppression of Cell Growth by SupJunD-1 Virus

Cell lines	Copy number of provirus/cell	Ratio (SupJunD-1 virus-transduced cells/control virus-transduced cells)		
		Colony formation in soft agar[a]	Doubling time[b]	Saturation density[c]
NIH3T3/Neo	1	—[d]	0.98 ± 0.10	0.90 ± 0.07
	10	—	1.22 ± 0.06	0.49 ± 0.01
NIH3T3/Ras	1	0.39 ± 0.03	0.94 ± 0.05	0.98 ± 0.09
	10	0 ± 0	1.11 ± 0.03	0.58 ± 0.04
U2-OS	1	0.41 ± 0.02	0.99 ± 0.01	0.97 ± 0.02
	3	0.06 ± 0.01	0.93 ± 0.01	0.95 ± 0.03
	5	0.06 ± 0.01	1.80 ± 0.01	0.93 ± 0.06
	10	0.02 ± 0.01	1.93 ± 0.01	1.02 ± 0.01
Saos-2	1	0.39 ± 0.02	1.03 ± 0.02	0.92 ± 0.01
	10	0.17 ± 0.05	1.22 ± 0.01	0.62 ± 0.07
A549	1	0.32 ± 0.01	0.98 ± 0.01	1.02 ± 0.07
	3	0.08 ± 0.01	0.98 ± 0.01	1.05 ± 0.03
	5	0.07 ± 0.02	1.17 ± 0.01	0.80 ± 0.01
	10	0.08 ± 0.01	1.28 ± 0.01	0.82 ± 0.04
H1299	10	0.39 ± 0.01	0.96 ± 0.09	0.81 ± 0.03
HeLa	10	0.32 ± 0.03	1.01 ± 0.01	0.71 ± 0.05
SiHa	10	0.21 ± 0.09	0.93 ± 0.03	1.01 ± 0.01

[a] The number of colonies formed by SupJunD-1 virus-transduced cells was divided by that formed by control virus-transduced cells.
[b] The doubling time of SupJunD-1 virus-transduced cells was divided by that of control virus-transduced cells.
[c] The saturation density of SupJunD-1 virus-transduced cells was divided by that of control virus-transduced cells.
[d] No colony was formed in both of the transduced cells.

transducing with SupJunD-1 virus at MOIs of 3 and 5 and subsequently selecting with puromycin for a week. Introduction of three copies of the *supjun*D-1 transgene, the colony formation in anchorage-independent cells was suppressed more than 90%, but cell growth in monolayer cultures was not affected at all (Fig. 2, Table 1). These results indicate that regulation of monolayer growth and anchorage-independent growth is fully separable. Results (Figs. 2 and 3) further suggest that the endogenous AP-1 levels essential for oncogenic function are much higher than the AP-1 levels sufficient for normal growth in monolayer cultures.

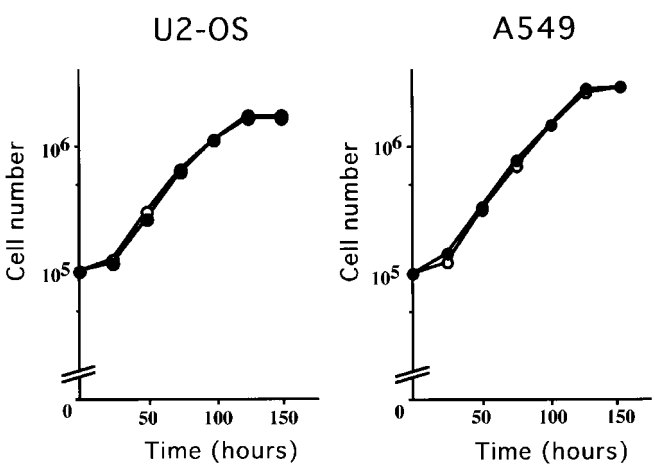


FIG. 2. Growth curves of control virus-transduced (open circles) or SupJunD-1 virus-transduced (closed circles) cell lines. Cells were seeded in 60-mm dishes and cell numbers were counted at the intervals indicated.

Changes in Fos/Jun heterodimer DNA binding activity by high level expression of SupJunD-1. To determine whether transduction of SupjunD-1 virus (at an MOI of 10) affects the specific DNA binding activity of endogenous AP-1 (Fig. 4), gel shift assays were performed. Incubation of DNA probe containing an AP-1 DNA binding site with nuclear extracts from cells transduced with control virus displayed a single shift (band A). However, incubation of the same DNA probe with nuclear extracts from cells transduced with SupjunD-1 virus displayed the band of the control extract (band A) as well as an additional, faster migrating complex (band B) (Fig. 4A). These bands were due to specific binding of endogenous AP-1 because the band was competed with the excess unlabeled probe but not with the excess unlabeled mutant AP-1 probe. Addition of anti-Fos or anti-Jun antiserum to mixtures containing DNA probe and nuclear extracts from control virus-infected or SupJunD-1 virus-infected cells resulted in the disappearance of band A and the appearance of slower migrating probe DNA bands (supershift) (Fig. 4B), while preimmune serum did not result in a supershift. These results indicate that band A is formed by AP-1 heterodimer composed of endogenous Fos and Jun family proteins.

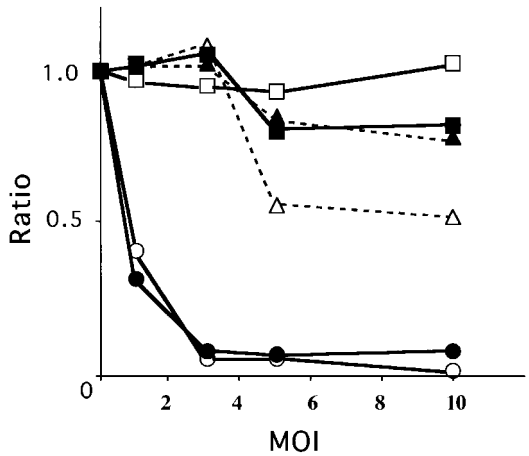


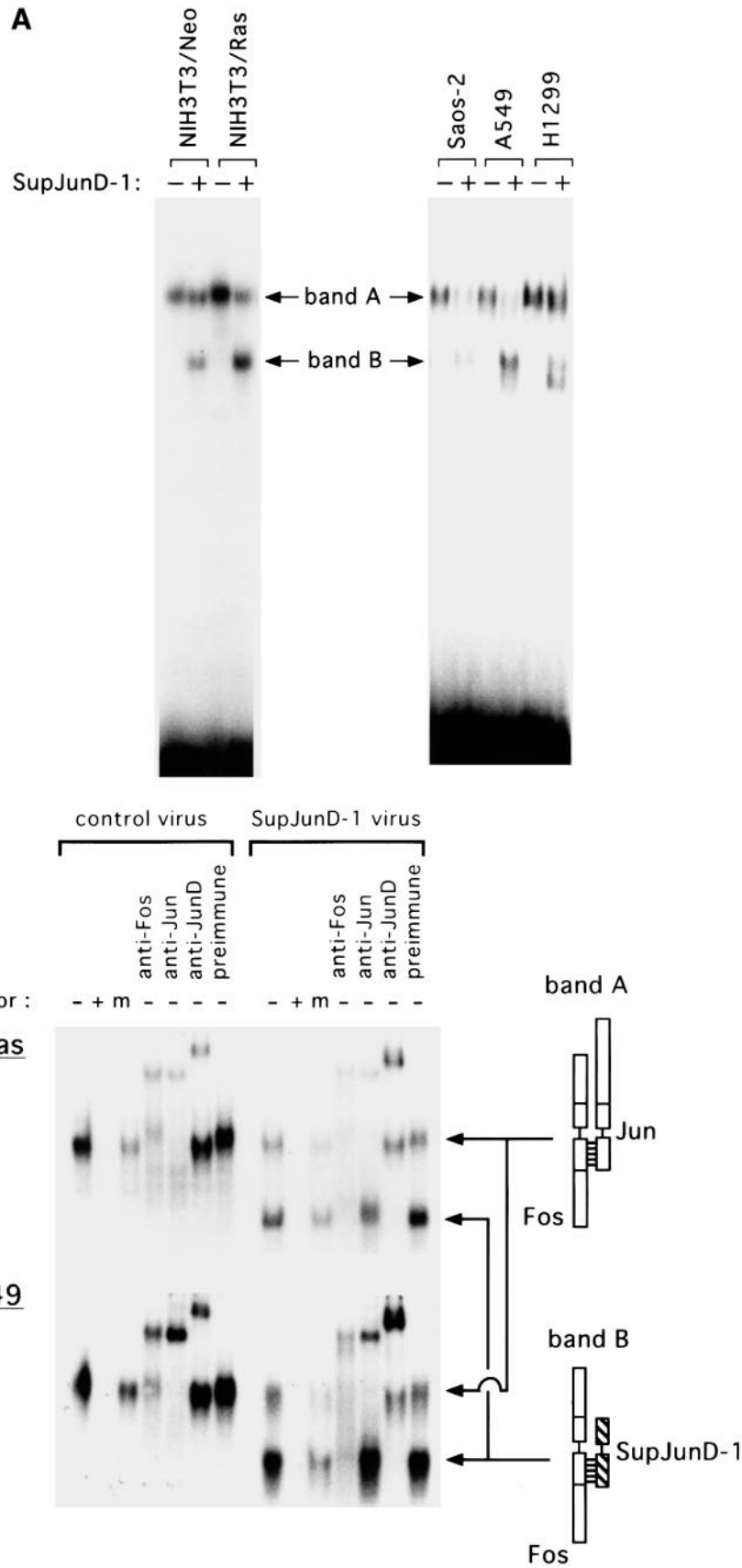
FIG. 3. Relation between suppression of cell growth and SupJunD-1 transgene dosage. U2-OS cells (open symbols) or A549 cells (closed symbols) were transduced with control virus or SupJunD-1 virus at the indicated MOIs. The ratios (SupJunD-1 virus-transduced cells/control virus-transduced cells) of colony numbers formed in soft agar (circles), growth rates (reciprocal of doubling times) (triangles), and saturation densities (squares) are shown.

The faster migrating band B was fully reactive with either the anti-Fos or the anti-JunD antiserum but completely non-reactive with either the anti-Jun or preimmune serum (Fig. 4B). Since this anti-Jun antiserum, which is reactive with all the full length Jun family proteins, is non-cross-reactive with SupJunD-1 protein that lacks N-terminal region of wild-type JunD, these results indicate that band B represents DNA probe bound to heterodimers formed between endogenous Fos family proteins and exogenous SupJunD-1. Since we did not detect any band that was insensitive to anti-Fos antiserum in this assay, dimers formed between endogenous Jun family proteins and SupJunD-1 were nondetectable.

When incubated with AP-1 DNA probe, a twofold greater intensity of band A was evident in reactions containing nuclear extracts from NIH3T3 cells transduced with Ras virus than from NIH3T3 cells transduced with control virus (Fig. 4A). This supports earlier observations that *ras* oncogene elevates endogenous AP-1 DNA binding activity (2). Incubation of DNA probe with nuclear extracts from NIH3T3/Ras, Saos-2 and A549 cells transduced with SupJunD-1 virus resulted in decreases in band A intensity and appearance of band B (Fig. 4A). Band A intensity was not affected when nuclear extracts from NIH/Neo and H1299 cells were assayed (Fig. 4A). This might reflect the difference in the composition of Fos and Jun family proteins in each cell line. In all the cell lines transduced with SupJunD-1 virus, the intensity of band B was much denser than that of band A. These results indicate that high level expression of SupJunD-1 caused formation of heterodimers between Fos family proteins and SupJunD-1, which are expected to have very low transactivation activity, leading to drastic changes in both amount and composition of endogenous DNA binding heterodimers as designed.

Expression of putative target genes of AP-1 in cells expressing SupJunD-1. We examined whether expression of SupJunD-1 modulates expression of genes previously suggested to be under control of AP-1 in certain conditions. These genes might encode members of AP-1, adhesion molecules and cell cycle regulators and modulation of expression of these molecules could potentially affect anchorage independent-growth. In mouse, AP-1 DNA binding sites are present in the promoter region of c-*jun* gene and in the first intron of *fra*-1 gene and positive feed-forward regulation of these members of AP-1 was suggested (26, 27). There is similar autoregulatory loop involving expression of Fra-2 and c-Jun in CEF cells (28, 29). Expression levels of c-Jun and Fra-1 in NIH3T3/Ras were higher than in NIH3T3/Neo (Fig. 5, left panel), suggesting that these two AP-1 components were induced by *ras* oncogene. This result is consistent with an earlier study suggesting that that c-Jun and Fra-1 mediate Ras transformation of murine fibroblasts (30). Following introduction of SupJunD-1, c-Jun and Fra-1 expression was lower in NIH3T3/Ras cells than in control virus-transduced NIH3T3/Neo cells (Fig. 5, left panel). Therefore, SupJunD-1 probably blocks positive autoregulation of c-Jun and Fra-1 gene transcription. Therefore SupJunD-1 could contribute to suppression of anchorage-independent growth not only by forming nonfunctional heterodimers with endogenous AP-1 but also by reducing the amount of some AP-1 members in this transformed mouse cell line. Expression of c-Jun and Fra-1 in human tumor cell lines, however, was not altered by the transduction with SupJunD-1 virus except for slight reductions of Fra-1 in Saos-2 and HeLa cells (Fig. 5, right panel) suggesting that AP-1 levels are maintained largely by mechanisms other than the AP-1 autoregulatory loops in these human cell lines.

In several murine cell lines, some adhesion molecules are regulated by endogenous AP-1. For example, CD44 is positively regulated (31) whereas E-cadherin is negatively regulated (32). In all human cell lines examined here, however, SupJunD-1 did not alter expression of CD44 or E-cadherin (Fig. 5, right panel). Using murine cell lines in which *p53* and/or c-*jun* were disrupted, it was suggested that c-Jun activates cellular growth by reducing *p53* gene expression (33). Therefore, we tested whether p53 expression is elevated by SupJunD-1. In most of the human tumor cell lines, p53 levels were unaffected by SupJunD-1 induction. However, slight reduction of p53 expression was detected in U2-OS and A549 (Fig. 5, right panel). This result indicates that the lengthy doubling time observed in the tumor cell lines expressing high levels of


A
NIH3T3/Neo
NIH3T3/Ras
Saos-2
A549
H1299
SupJunD-1:
band A
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B
control virus
SupJunD-1 virus
anti-Fos
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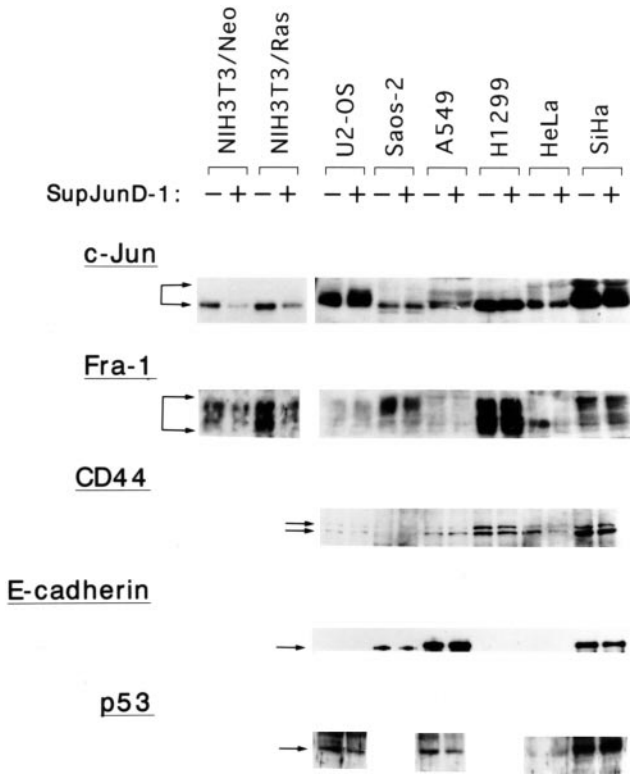

FIG. 5. Expression of AP-1 targets in control virus- or SupJunD-1 virus-transduced cell lines (MOI of 10). Cell lysates were prepared under denaturating conditions, separated by SDS–PAGE (30 μg per lane) and detected by Western blotting with anti-c-Jun, anti-Fra-1, anti-CD44, anti-E-cadherin, or anti-p53 immune serum. Saos-2 and H1299 are null mutants of p53.

SupJunD-1 is not due to the elevation of p53 expression.

DISCUSSION

Endogenous AP-1 has been suggested to be involved in the tumorigenicity in many human tumors. The cell lines examined here include lines originating from osteosarcomas, non-small cell lung carcinomas and HPV-induced cervical carcinomas and there is evidence supporting the involvement of Fos and Jun family proteins. Transgenic mice expressing a high level of exogenous c-*fos* develop osteosarcomas or osteogenic tumors (34). In non-small cell lung carcinomas, AP-1 components are abundant and these cells often display significant levels of functional AP-1 DNA-binding activity (35, 36). HPV16 and HPV18 are frequently associated with cervical carcinomas and E6 and E7 genes are sufficient for transformation (37). AP-1 binding sites are present in the LCRs of both HPV16 and HPV18 and these sites are necessary for transcription of E6 and E7 genes.

Here, we demonstrated that, by a single inoculation with SupJunD-1 virus at high MOI introduced *supjun*D-1 into nearly the entire cell population and almost completely restored anchorage-dependent growth of six human tumor cell lines as well as Ras-transformed NIH3T3 cells (Fig. 1, Table 1). This indicates that endogenous AP-1 functions are essential to maintain oncogenicity of cell lines originating from a wide variety of human solid tumors. Thus, inhibition of endogenous AP-1 activity by SupJunD-1 may provide an attractive gene therapy for cancer originating from a broad spectrum of solid tumors.

Expression of SupJunD-1 reduced functional, endogenous AP-1 activity by forming dimers between SupJunD-1 and endogenous Fos family proteins (Fig. 4). According to studies of murine cells transformed by oncogenes, several target genes regulated by AP-1 are suggested to encode AP-1 members, adhesion molecules, cytoskeleton components, matrix-degrading enzymes and cell cycle regulators, all of which may influence anchorage-independent growth (27, 31, 32, 38, 39). But we did not detect significant changes in c-*jun*, *fra*-1 (AP-1 components), CD44, E-cadherin (adhesion molecules) or *p53* (cell cycle regulators) gene expression in any of the human tumor cell lines examined here. Therefore, we conclude that these genes are not related to the acquisition of anchorage-independent growth displayed by these human tumor cell lines.

Making use of dose-dependent transduction of VSV-G pseudotyped retrovirus, we were able to regulate the cellular proviral copy number of *supjun*D-1 from 1 to approximately 10 and subsequently examine how suppression of endogenous AP-1 function affected the growth characteristics of six human tumor cell lines. In all of the human cell lines examined as well as Ras-transformed NIH3T3 cells, the level of endogenous AP-1 that was essential for anchorage-independent growth was much higher than that sufficient to support full growth in monolayer cultures (Fig. 3, Table 1). In U2-OS cells and or A549 cells that were inoculated

FIG. 4. AP-1 DNA-binding activity in nuclear extracts from control virus- or SupJunD-1 virus-transduced cell lines. The AP-1-binding activity present in nuclear extracts was determined by gel shift assays using ^{32}P-labeled DNA probe containing a single collagenase gene AP-1-binding site. (A) Gel shift assay of nuclear extracts from NIH3T3/Neo, NIH3T3/Ras, Saos-2, A549, or H1299 cells that were transduced with either control virus or SupJunD-1 virus at an MOI of 10. (B) Gel shift assay of nuclear extracts isolated from NIH3T3/Ras cells (top panel) or A549 cells (bottom panel) that were inoculated with either control virus or SupJunD-1 virus. The sequence specificity of DNA binding was determined by competition assays with a 30-fold molar excess of unlabeled oligonucleotide (+) or mutant oligonucleotide (m). Some of the extracts were treated with anti-Fos (cross-reactive to all the Eos family proteins), anti-Jun (cross-reactive to all the Jun family proteins) or anti-JunD antiserum as well as preimmune serum.

at an MOI of 3, anchorage-independent growth was efficiently suppressed, whereas growth in monolayer cultures was unaffected. In contrast to our findings, MCF7, AZ224, SKOV3 and OVCAR3 cells stably transfected with the dominant negative c-Jun mutant TAM67 displayed reduced growth rates in monolayer culture (40, 41). These differing results may be related to techniques used for introducing dominant negative mutants and/or to the type of cell lines employed for each study. By a single transduction of high titer retrovirus vector, we were able to introduce *supjun*D-1 and its biological effects on the entire population were analyzed. DNA transfection method used for TAM67, however, required drug selection of transfectants before biological analysis.

The importance of AP-1 in cell growth has been demonstrated by other techniques. For example, microinjection of anti-Fos family or anti-Jun family antibodies inhibits cell cycle progression (42) and c-$jun^{-/-}$ or c-$fos^{-/-}$ $fosB^{-/-}$ fibroblasts have a defect in proliferation (43, 44). These earlier findings and ours indicate that only a low level of expression of AP-1 is sufficient to support cell growth in monolayer cultures, whereas significantly higher levels are necessary for oncogenicity. In summary, we demonstrated that AP-1 plays important roles in maintaining oncogenicity in human tumor cell lines. We further found that a high level of AP-1 expression supports oncogenic growth, whereas a low level of AP-1 expression supports attachment-dependent growth. VSV-G-pseudotyped retrovirus vector carrying SupJunD-1 may also be a powerful tool for human cancer gene therapy because it can suppress oncogenicity of tumor cell lines efficiently by a single inoculation *in vitro*. In combination with other technologies such as DNA microarray systems, this vector may be useful for screening large numbers of genes potentially responsible for anchorage-independent growth.

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