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Isolation of *rsp-1*, a Novel cDNA Capable of Suppressing v-Ras Transformation

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Using an expression cloning assay, we have isolated a novel cDNA, referred to as *rsp-1*, which suppresses the v-Ras-transformed phenotype. When introduced into NIH 3T3 fibroblasts under the control of a metallothionein promoter, *rsp-1* confers resistance to v-Ras, but not to v-Mos or v-Src, and inhibits growth of the cells. The *rsp-1* cDNA contains a 831-bp open reading frame encoding a 277-amino-acid leucine-rich protein. The *rsp-1* cDNA exhibits no significant homology to sequences in the DNA data bases. However, searches of the protein data bases revealed that it contains a series of leucine-based repeats which are homologous to the leucine repeats found in the regulatory region of the yeast adenylyl cyclase. *rsp-1* specific RNA is detectable in a wide variety of cell lines and tissues, and the gene is conserved among eukaryotic species. These data suggest that *rsp-1* plays a role in Ras signal transduction.

Activation of the *ras* proto-oncogene is a common mutation in specific human tumors (5). In combination with a mutated p53 gene, activated *ras* is sufficient to transform primary rodent fibroblasts (13). Hence, genes capable of suppressing Ras activity may provide insights into the Ras-mediated events in the transformation process as well as the identities of novel loss-of-function mutations.

Several genes involved in the regulation of Ras function have been identified to date. Noda et al. using an expression cloning strategy isolated the *ras*-related K-*rev-1* gene which, when expressed at high levels, appears to suppress Ki-Ras transformation (15, 22, 35). Similarly, the Ras GTPase-activating protein, Ras GAP, suppresses transformation by both c-*ras* and the *src* oncogenes (9, 24, 34), which may function through the c-Ras pathway(s). The neurofibromatosis locus gene product, NF-1, contains a GAP-related domain which exhibits Ras GAP activity (2, 8, 20, 30), and through this function it may also be involved in the regulation of Ras signal transduction.

The suppression of Ras transformation by Rev-1 describes a single activity for this gene and constitutes only one aspect of its normal function. A role for Rev-1/Rap1 during *Drosophila* eye development has recently been described (12). This demonstrates that Rev-1/Rap1, a gene isolated on the basis of its homology to a *Drosophila ras* homolog (25) and its interaction with v-*ras* in a transformation suppression assay, has biological significance on a wider scale. Therefore, characterization of the interactions between Ras and novel Ras suppressor proteins should provide insights into the mechanism of Ras signalling as well as the role of Ras in development and tumorigenesis.

In this study, we have used suppression of the Ras-transformed phenotype as an expression cloning assay to identify additional genes potentially involved in the regulation of Ras signal transduction pathways. In doing so we have isolated a novel cDNA, referred to as *rsp-1*, which has the property of suppressing v-Ras transformation in both fibroblasts and epithelial cells.

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MATERIALS AND METHODS

Isolation of rsp-1-specific clones. A cDNA library was prepared from CHP9CJ cell polyadeynlated RNA in a plasmid vector-primed system (7). The plasmid vector, p521, contains a Rous sarcoma virus promoter to drive transcription of the cDNA, a simian virus 40 origin to allow recovery of cDNA by fusion to COS cells, and the neomycin resistance gene to allow for selection with neomycin and kanamycin in prokaryotic cells and with G418 in eukaryotic cells. The p521-CHP9CJ cell cDNA library was transfected into DT cells (23) by calcium phosphate precipitation (3, 29) under conditions that would favor the introduction of a few copies or a single copy of plasmid DNA per cell. Following transfection, the cells were selected in G418 (1.2 mg/ml) until colonies were visible and then in oubain (1 mM) to enhance the selection of flat colonies. Over 100 morphologically flat colonies were isolated by screening in this way.

cDNAs recovered from the resulting flat transfectants were tested for their abilities to confer a less-transformed phenotype on DT cells. cDNAs were recovered from one of the flat transfectants, 244-3V, by fusion to COS cells and isolation of Hirt supernatants for transformation of bacteria. Recovery of the 244-3V cDNA-containing plasmids from bacterial colonies yielded some with homologous but variously sized inserts, some of which suppressed the DT cell-transformed phenotype. The cDNA insert from the smallest recovered plasmid with suppressor activity was isolated and used as a probe to screen an NIH 3T3 cell cDNA bacteriophage lambda library. This fragment was subsequently identified as the 5′ 300 bp of the *rsp-1* cDNA. Several positive clones were isolated and compared. The 1.33-kb cDNA clone was sequenced (Fig. 1); it contains the entire *rsp-1* open reading frame. Another partial overlapping clone used for confirmation of the sequence encompassed the 5′ end of the *rsp-1* open reading frame and contained additional 5′ untranslated regions of the major 1.7-kb transcript.

Sequencing of *rsp-1* clones. Specific fragments of the two cDNAs were subcloned into the pBluescript SK and KS

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                                                    MetSerLysSerLeuLysLysLeuVal
1   ATTCCCACCCACCAGGTGTTGCTCGCTGCGAACATGTCCAAGTCACTGAAGAAGCTGGTG

    GluGluSerArgGluLysAsnGlnProGluValAspMetSerAspArgGlyIleSerSer
61  GAGGAGAGCAGGGAGAAGAACCAGCCGGAAGTGGACATGAGTGACAGGGGTATCTCCAGT

    MetLeuAspValAsnGlyLeuPheSerLeuAlaHisIleThrGlnLeuValLeuSerHis
121 ATGTTGGATGTCAACGGCTTGTTCTCTCTAGCCCACATCACACAACTGGTGCTCAGCCAC

    AsnLysLeuThrThrValProProAsnValAlaGluLeuLysAsnLeuGluValLeuAsn
181 AACAAGCTAACAACTGTGCCACCAAATGTAGCGGAACTGAAGAACTTGGAGGTACTAAAC

    PhePheAsnAsnGlnIleGluGluLeuProThrGlnIleSerSerLeuGlnLysLeuLys
241 TTCTTCAACAATCAGATCGAGGAACTGCCTACCCAGATCAGCAGCCTCCAGAAACTCAAA

    HisLeuAsnLeuGlyMetAsnArgLeuAsnThrLeuProArgGlyPheGlySerSerArg
301 CACCTGAACCTTGGCATGAATAGGTTGAACACGCTGCCTCGAGGATTCGGCTCCTCCCGG

    LeuLeuGluValLeuGluLeuThrTyrAsnAsnLeuAsnGluHisSerLeuProGlyAsn
361 CTTCTGGAGGTCCTGGAGTTAACTTACAACAACCTGAATGAACATTCTCTTCCGGGAAAC

    PhePheTyrLeuThrThrLeuArgAlaLeuTyrLeuSerAspAsnAspPheGluIleLeu
421 TTCTTCTACCTCACCACCCTGCGTGCACTCTATCTAAGCGACAACGATTTTGAAATCCTG

    ProProAspIleGlyLysLeuThrLysLeuGlnIleLeuSerLeuArgAspAsnAspLeu
481 CCTCCAGATATTGGGAAGCTCACAAAGTTGCAGATACTCAGCCTCAGGGATAATGACCTG

    IleSerLeuProLysGluIleGlyGluLeuThrGlnLeuLysGluLeuHisIleGlnGly
541 ATCTCACTGCCTAAGGAAATCGGGGAGCTGACCCAGCTGAAGGAGCTCCACATTCAGGGG

    AsnArgLeuThrValLeuProProGluLeuGlyAsnLeuAspLeuThrGlyGlnLysGln
601 AACCGCCTGACCGTTCTGCCTCCAGAGCTTGGCAACTTGGATCTAACTGGTCAGAAGCAG

    ValPheLysAlaGluAsnAsnProTrpValThrProIleAlaAspGlnPheGlnLeuGly
661 GTCTTCAAAGCAGAGAACAACCCCTGGGTTACCCCGATTGCTGACCAGTTCCAGCTTGGC

    ValSerHisValPheGluTyrIleArgSerGluThrTyrLysTyrLeuTyrGlyArgHis
721 GTCTCCCACGTTTTCGAATATATTCGTTCAGAAACTTACAAGTACCTCTACGGCAGACAC

    MetGlnAlaAsnProGluProProLysLysAsnAsnAspLysSerLysLysIleSerArg
781 ATGCAAGCGAACCCAGAACCTCCAAAGAAGAATAACGACAAATCAAAAAAGATCAGCCGG

    LysProLeuAlaAlaLysAsnLysEnd
841 AAACCCCTAGCAGCCAAGAACAAATGAGGAGCAGACACACACACTGGTTTCTGGCCTCCT

901 TCTCCGGCCTTCTTGCTCTGTCCTGCAAATGAATACAGCGTGCTGGTGCCTACTTTTCTT

961 GTTTAAAGAAAATCCTTTTCCCTTTCTCCTCCCGGTGCTTGAACGCAGCCTGACTTTTGC

1021 ACCTTTGAAATACTTGTGGTAGGTGGTGCCGTTTTTATAAAGTCTTACATTCCTTCTCAT

1081 TTGTTTCCTTAAAATCTGCAAAGGCAGAAAACAAAGCTCTCATTCTCCTGCAAATTCCAT

1141 GGTAGGCACAGGTTTCAGTCCCTTGAATAAACGTCACAAGGCTGCTTATTATCAAAAAAT

1201 AATTAAAATCATGTAACCGCTTAAATGTCACAGTTAACACTTTTCATTCTTTCTGTTTAT

1261 TCATATAACTCATTATTGTGCCTTATTAAAAATCTTCCTTCACAGCCAAAAAAAAAAAAG

1321 GAATTC
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FIG. 1. DNA sequence of the 1.33-kb *rsp-1* cDNA clone. The 1.33-kb cDNA clone was isolated as described in Materials and Methods and sequenced; it contains the entire *rsp-1* open reading frame. Another partial overlapping clone used for confirmation of the sequence encompassed the 5′ end of the open reading frame and contained additional 5′ untranslated regions of the major 1.7-kb transcript and the 4-kb RNA. Specific fragments of the two cDNAs were subcloned into the pBluescript SK and KS vectors, and the sequence was determined by using vector-derived primers by the method of Sanger et al. (26). Additionally, sequence was determined by analyzing polymerase chain reactions containing fluorescein-labeled primers on an A.L.F. automated sequencing system (Pharmacia-LKB). Sequence assembly and translation of the sequence were accomplished by using University of Wisconsin GCG software programs.

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Hybridization of Southern and Northern (RNA) blots. Southern and Northern blots were hybridized with *rsp-1*-specific probes in 50% formamide–5× SSPE (1× SSPE is 0.18 M NaCl, 10 mM $NaPO_4$, and 1 mM EDTA [pH 7.7])–1× Denhardt's solution–100 μg of sonicated salmon sperm DNA per ml–0.1% sodium dodecyl sulfate (SDS) at 42°C for 24 h. The blots were washed for 5 min at room temperature in 1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.5% SDS with three buffer changes and then for 1 h at 55°C in 0.1× SSC–0.5% SDS with three buffer changes. The *rsp-1* probes were labeled with [^{32}P]dCTP to a specific activity of 5×10^8 cpm/μg by random priming.

Introduction of *rsp-1* into retroviral vectors. *rsp-1* was introduced into retroviral vectors under the control of internal promoters. *rsp-1* cDNA was inserted either into the retroviral vector pN04 under the control of a cytomegalovirus immediate-early promoter or into p1529 under the control of the mouse metallothionein-1 (MT-1) promoter (21). The pN04-derived plasmid p3V-31 contains the entire *rsp-1* coding sequence and 250 bp of 3′ untranslated sequence in the sense orientation; p3V-29 contains the 1.33-kb *Eco*RI fragment inserted into pN04 in the antisense orientation. The 1.33-kb *Eco*RI fragment containing the *rsp-1* coding sequence and 3′ untranslated region was introduced into p1529 in the sense orientation to produce p3V-36. These recombinant vector DNAs were transfected directly or used to produce retroviral stocks for infection of DT cells.

Transfection and infection of cells with retroviral vectors. pN04 and its derivatives were transfected directly into DT cells by using calcium phosphate precipitation (3, 29). Forty-eight hours after transfection, 2.5×10^4 transfected cells were seeded on monolayer culture in 60-mm dishes in 800 μg of G418 per ml to determine the efficiency of transfection, and 5×10^5 cells were seeded in 0.3% agar medium containing 1,200 μg of G418 per ml. After 10 days of selection, monolayer colonies were fixed, stained, and enumerated, and agar colonies were counted microscopically. Typically, this resulted in the selection of 20 to 100 monolayer colonies per 2.5×10^4 cells and 100 to 1,000 agar colonies per 5×10^5 cells. The plating efficiencies were calculated to correct for cell viability and transfection or infection efficiency and were derived by dividing the number of G418-resistant agar colonies by the number of G418-resistant monolayer colonies per 5×10^5 cells. p1529 and p3V-36 defective retroviral vector stocks were prepared in the ecotropic packaging cell line GP+E86 (19). From 10^2 to 10^3 PFU were used to infect 10^5 DT, NIH 3T3, or NOG8NP4 cells. Forty-eight hours after infection, the cells were plated in G418 medium on monolayers or in agar and selected, and the plating efficiency was determined as described above. p1529 and p3V-36-containing cell lines were derived from individual G418-resistant colonies isolated with cloning cylinders and expanded into cell lines.

The rsp-1-containing cell line, 8-1, and the vector control cell line, 12-1, were infected with ecotropic or amphotropic stocks of acute transforming retroviruses as described previously (32) (Ki-Ras and v-Mos were amphotropic, and Ha-Ras and v-Src were ecotropic) at a concentration of

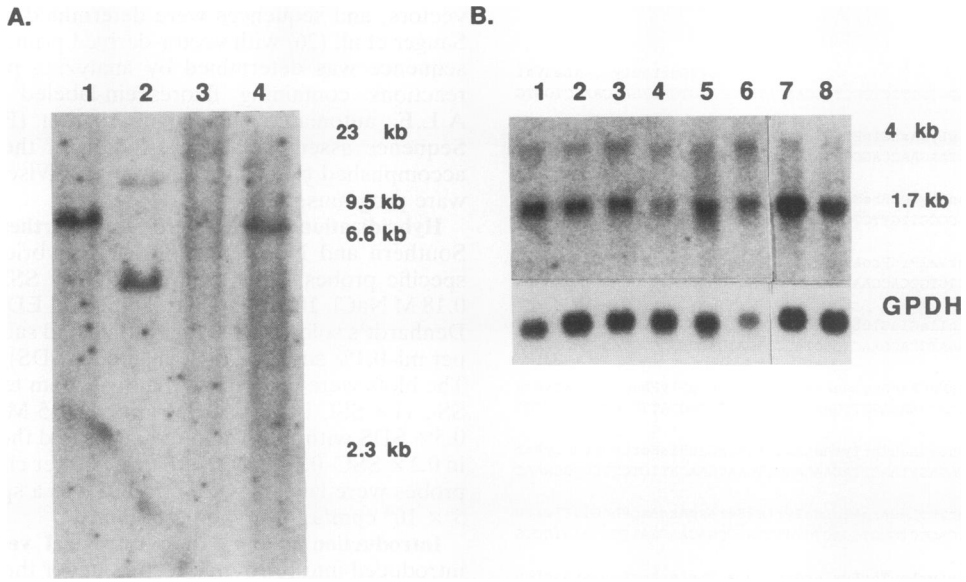


FIG. 2. *rsp-1* specific hybridization to genomic DNA and *rsp-1* specific transcripts in total RNA. (A) Hybridization of an *rsp-1* specific probe to a Southern blot containing 10 μg of *Eco*RI-digested DNA per lane. Lane 1, human placental DNA; lane 2, cat kidney DNA; lane 3, rat liver DNA; lane 4, NIH mouse embryo DNA. The *rsp-1* probe represents the 5′ 300 bp of the cDNA. (B) Hybridization of the 1.33-kb *rsp-1* cDNA to a Northern blot containing 10 μg of total cellular RNA per lane. All transformed cell lines are derived from NIH 3T3 cells. Lanes: 1, NIH 3T3; 2, *ras* transformant DT; 3, *ras* transformant P97-C5; 4, *mos* transformant; 5, *src* transformant; 6, *fms* transformant; 7, *ras* revertant C11 (23); 8, *ras* revertant F2 cell lines (23). Following *rsp-1* hybridization, the blot was hybridized with a glyceraldehyde-3-phosphate dehydrogenase (GPDH) probe to detect loading differences among samples.

100,000 cells per 60-mm dish. Forty-eight hours after infection, the cells were removed from monolayers and seeded in agar at a concentration of 50,000 cells per dish. Colonies were counted after 2 and 3 weeks of incubation. Uninfected cells and cells infected with murine leukemia virus alone did not give rise to any agar colonies. The data are expressed as numbers of colonies per 50,000 cells plated (average of two plates) and percentages of the number of colonies in control plates.

Nucleotide sequence accession number. The sequence of *rsp-1* has been submitted to GenBank and EMBL and has been assigned the accession number X63039.

RESULTS

Expression cloning of sequences that suppress *ras* transformation and determination of the DNA sequence. Our expression cloning assay, which utilized suppression of Ki-Ras transformation, was designed to favor the isolation of dominantly acting cDNAs. The source of RNA for the expression library was a Ras revertant cell line, CHP9CJ, isolated and characterized by Yanagihara et al. (32). A directional, vector-primed CHP9CJ cDNA library (7) was transfected into DT cells, a v-Ras-transformed NIH 3T3 cell line (3, 23, 29). Following selection in G418 and a brief exposure to oubain to inhibit the growth of transformed cells, individual flat transfectants were identified and isolated. The frequency of morphological reversion was <1% during the first round of screening. cDNAs recovered from one of the primary flat transfectants selected in this manner, 244-3V, yielded flat transfectants at a frequency of 10 to 20% when introduced into DT cells. The cDNAs recovered from 244-3V were heterogeneous in size, so the cDNA insert from one of the recovered clones was used as a probe to isolate NIH 3T3 cell cDNA clones (1.33 kb in size), referred to as *rsp-1*, for determination of the complete DNA sequence (Fig. 1). Subsequent sequence comparison of several cDNA-containing plasmids recovered from the 244-3V flat transfectant revealed that they were homologous to the 5′ end of the 1.33-kb NIH 3T3 cell cDNA but that some were truncated, lacking sequences from the 3′ end of the open reading frame (data not shown).

The 831-bp *rsp-1* open reading frame encodes a 277-amino-acid protein. Primer extension and S1 mapping revealed that the open reading frame begins approximately 120 bases from the 5′ end of the major 1.7-kb transcript found in human and rodent cells and that the 5′ untranslated sequences contain no other open reading frames. Three methionine codons are located within the first 30 amino acids, with the first being surrounded by the best consensus sequence for initiation of translation (16). There are no signal peptides or transmembrane regions in the predicted amino acid sequence. The N-terminal two-thirds of the protein consists of a series of leucine repeats, while the C terminus contains a high concentration of basic amino acids. The major predicted modification of the protein is phosphorylation; putative sites include numerous casein kinase II sites, protein kinase C sites, and a cyclic AMP-dependent protein kinase site located near the C terminus.

***rsp-1* is highly conserved and ubiquitously expressed.** Using the 5′ end of the *rsp-1* cDNA as a probe, a single homologous fragment was detected in human, rat, and mouse genomic DNA, indicating that *rsp-1* is a single-copy gene (Fig. 2A). The intensities of the bands on the Southern blot are similar in rodent, cat, and human samples, suggesting that the gene is well conserved. In addition, sequencing of the human *rsp-1* cDNA revealed that the human and mouse genes are >95% conserved in the coding region (28a). Northern blotting indicates that the 1.33-kb cDNA fragment hybridizes to a 1.7-kb RNA which appears to be the major transcript in

TABLE 1. Effect of *rsp-1* cDNA expression on growth of DT cells in agar

Retroviral vector	Plating efficiency in agar[a]			
	Expt 1	Expt 2	Expt 3	Mean (SE)
pN04	0.54 (100%)	0.46 (100%)	0.42 (100%)	0.456 (0.020)
p3V-29	0.57 (105%)	0.38 (83%)	0.43 (101%)	0.467 (0.056)
p3V-31	0.13 (24%)	0.30 (65%)	0.33 (77%)	0.260 (0.062)
p1529	0.47 (100%)	0.88 (100%)		
p3V-36	0.08 (18%)	0.45 (52%)		

[a] *rsp-1* cDNA was introduced into the retroviral vectors under the control of an internal cytomegalovirus promoter, pN04, or the mouse metallothionein 1 promoter, p1529. pN04 and its derivatives were transfected directly into DT cells by using calcium phosphate precipitation. The plating efficiency of the transfected cells in agar was calculated by dividing the number of G418-resistant agar colonies by the number of G418-resistant monolayer colonies per 500,000 cells. The value for pN04 was normalized to 100%, and the percentage of the pN04 value was calculated for the other plasmids. The mean and standard error were calculated and then compared by using Duncan's multiple range test. For p1529 and p3v-36, defective retroviral vector stocks were used to infect 100,000 DT cells. Forty-eight hours after infection, the cells were plated in G418 medium on monolayers or in agar and selected, and the plating efficiency was determined as described above.

TABLE 2. Growth in agar of NOG8 NP4 cells infected with p1529 and p3v-36 defective retroviral stocks[a]

Clone	Growth in agar[b]	
	$-CdSO_4$	$+CdSO_4$
1529-6	3,029 (12.1%)	2,957 (11.8%)
3V-36-7	886 (3.5%)	684 (2.7%)
3V-36-C	813 (3.2%)	208 (0.8%)
3V-36-F	1,380 (5.5%)	294 (1.2%)
3V-36-M	193 (0.8%)	139 (0.5%)

[a] p1529 and p3v-36 defective retroviral stocks were used to infect NOG8 NP4 cells. Following selection in G418, individual colonies were isolated with cloning cylinders and expanded into cell lines. A total of 25,000 cells of each individual line were seeded in 0.3% agar in the absence or presence of 0.5 μM $CdSO_4$, and the colonies were counted after 14 days.

[b] Number of agar colonies and percentage of cells giving rise to agar colonies.

most cell lines (Fig. 2B). A less-abundant 4-kb RNA is also detected in most cell lines, and sequencing of cDNAs from this transcript suggests that it is an incompletely processed precurser (data not shown).

Suppression of the Ras-transformed phenotype by expression of *rsp-1*. Reversion to the nontransformed phenotype in the primary 244-3V transfectant could have resulted from overexpression of the full-length *rsp-1* protein, resulting in dominant negative regulation of the transformed phenotype. Alternatively, it might result from expression of a truncated, competing, or interfering form of the protein, resulting in subsequent disruption of positive regulation of transformation. To distinguish between these two possibilities, the full *rsp-1* coding sequence was introduced into retroviral vectors for transfection and infection into DT cells and subsequent selection in agar containing G418 (Table 1). This assay quantitatively measures the reduction in anchorage-independent growth, a parameter of the transformed phenotype. However, it does not measure the transformation-independent growth inhibitory properties of *rsp-1*, since the plating efficiencies were calculated by normalizing for the number of G418-resistant transfectant or infected cells. The results in Table 1 indicate that introduction of the complete *rsp-1* coding sequence by either transfection, p3V-31, or infection, p3V-36 (Table 1) resulted in a 25 to 75% reduction in agar colony formation. Statistical analysis of the transfection results demonstrates that the plating efficiency of p3V-31 was significantly less than that of pN04 or p3V-29 ($P < 0.05$).

Following the determination of transformation suppressor activity in DT cells, *rsp-1* was tested in a Ki-murine sarcoma virus-infected NOG8 mouse mammary epithelial cell line, NOG8 NP4. Following infection of this nonproducer cell line with the p3V-36 retroviral vector, drug-resistant colonies were isolated and assayed for constitutive and inducible levels of the MT-1 promoter-initiated *rsp-1* RNA (data not shown) as well as for anchorage-independent growth in the presence or absence of the MT-1 promoter inducer $CdSO_4$ (Table 2). All 3V-36-infected cells showed reduced growth on agar compared with the vector control cells (Table 2). Moreover, additional loss of ability to form colonies in soft agar was seen in the presence of $CdSO_4$, which correlated with an inducible increase (up to twofold) in the level of the MT-1-promoted *rsp-1* RNA (data not shown). Collectively, this data and the reduction in agar growth of DT cells upon introduction of the full *rsp-1* coding sequence suggest that *rsp-1* has the capacity to act as a dominant negative regulator of v-Ki-Ras.

Determination of the range of *rsp-1* suppressor activity. Following the determination of transformation suppressor activity in DT cells, *rsp-1* was introduced into NIH 3T3 cells so that the growth inhibitory properties and the range of suppressor activity of the cDNA could more readily be assayed. Following infection with p3V-36, a number of the resulting G418-resistant colonies (approximately 30%) consisted of highly enlarged cells which grew too slowly for the establishment of cell lines, a phenomenon not seen in the vector control infection, suggesting that *rsp-1* exhibited a negative or toxic effect on NIH 3T3 cell growth. A number of *rsp-1*-infected cell lines were established, and Northern blotting of RNA from one, the 8-1 cell line, revealed several *rsp-1*-specific transcripts (Figure 3). In addition to a Cd^{2+}-

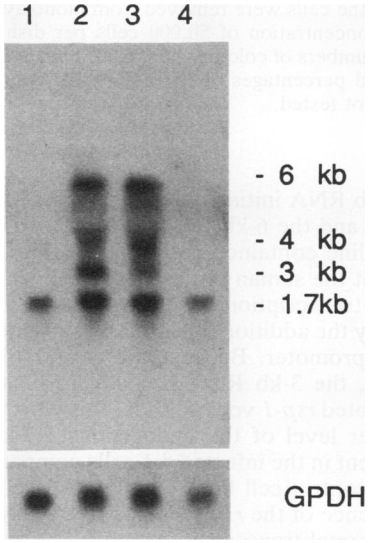
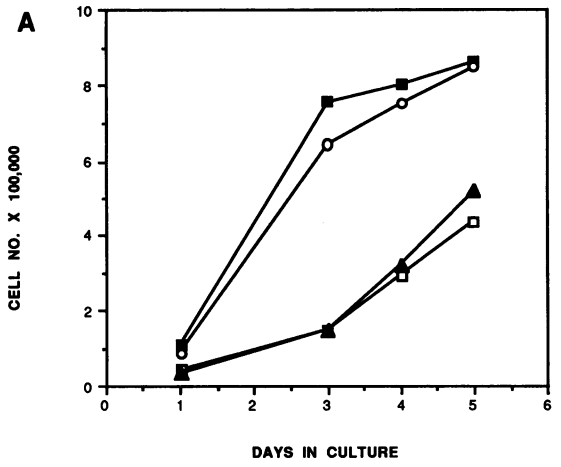


FIG. 3. Northern blot analysis of RNA from *rsp-1*-infected cells. *rsp-1* cDNA probe was hybridized to a Northern blot containing 10 μg of total cellular RNA from *rsp-1*-infected or vector control cell lines. The blot was then hybridized to GPDH probe to control for loading differences. Lanes: 1, 11-1 1529-infected cells; lane 2, 8-1 *rsp-1*-infected cells treated with 1 μM Cd^{2+} for 24 h; lane 3, 8-1 *rsp-1*-infected cells (untreated); 4, uninfected NIH 3T3 cells.

A



B

CELL LINE		VIRAL ONCOGENE			
		v- KI-RAS	v-Ha-RAS	v-SRC	v-MOS
EXP.1	12-1	1162(100%)	2345(100%)	693(100%)	N.T.
	8-1	71(6.1%)	86 (3.6%)	637(91.9%)	N.T.
EXP.2	12-1	N.T.	213(100%)	1252(100%)	835(100%)
	8-1	N.T.	6 (2.8%)	832(74.4%)	375(44.9%)

FIG. 4. Effect of *rsp-1* expression on NIH 3T3 fibroblasts. NIH 3T3 fibroblasts were infected with 3V-36 or 1529 retroviral vector stocks and selected in medium containing G418 (200 μg/ml). Individual colonies were isolated by using cloning cylinders and expanded into cell lines. (A) Effect of *rsp-1* on growth rate. Cell lines were seeded at a concentration of 50,000 cells per 60-mm dish on day 0. On days 1, 3, 4, and 5 cells were removed from the monolayers of duplicate plates with trypsin and counted. The results obtained with two 3V-36-derived *rsp-1*-infected cell lines, 7-1 (▲) and 8-1 (□), and two control cell lines, NIH 3T3 (○) and 1529-derived 12-1 (■), are shown. B. Effect of *rsp-1* on transformation by acute transforming retroviruses. The *rsp-1*-infected cell line 8-1 and the vector control cell line 12-1 were infected with ecotropic or amphotropic stocks of acute transforming retroviruses, and 48 h after infection the cells were removed from monolayers and seeded in agar at a concentration of 50,000 cells per dish. The data are expressed as numbers of colonies per 50,000 cells plated (average of two plates) and percentages of the number of colonies in control plates. N.T., not tested.

inducible 3-kb RNA initiated at the internal MT-1 promoter in the vector and the 6-kb full-length viral RNA, the *rsp-1*-infected cell line contained a 4-kb transcript corresponding to initiation at the simian virus 40 internal vector promoter. Initiation of transcription at this promoter appears to be suppressed by the addition of Cd^{2+} and subsequent initiation at the MT-1 promoter. Because the 6- and 4-kb RNAs are polycistronic, the 3-kb RNA is likely to be the most efficiently translated *rsp-1* vector RNA. It is worth noting that a slightly higher level of the endogenous 1.7-kb *rsp-1* transcript is present in the infected 8-1 cells compared with RNA from vector control cell lines or NIH 3T3 cells, suggesting that the presence of the *rsp-1* vector RNA may alter regulation of the normal transcript.

The *rsp-1*-infected cell lines that were established grew more slowly than vector control cell lines and the NIH 3T3 cell line; a growth curve comparing two of the *rsp-1* lines, 7-1 and 8-1, is shown in Fig. 4A. The *rsp-1*-infected cell line, 8-1, along with the vector control cell line, 12-1, were infected with stocks of acute transforming retroviruses containing v-*ras*, v-*mos*, or v-*src* oncogenes and plated in agar to quantitate the response to the introduction of the oncogenes. The results shown in Fig. 4B indicate that the *rsp-1*-infected cell lines were transformed by v-*src* and v-*mos* but were resistant to transformation by v-Ki-*ras* and v-Ha-*ras*. The sensitivity to v-*src* and v-*mos* transformation suggests that the pathway(s) affected by *rsp-1* in NIH 3T3 cells either is not required for transformation by v-*src* (9, 24) and v-*mos* or is only partially inhibited in these cells. The activity of *rsp-1* in these cells appears to be sufficient and specific for the inhibition of transformation by v-*ras*.

Homology of *rsp-1* to yeast adenlyl cyclases. A mechanism for the negative regulation of Ras by *rsp-1* may be inferred from the results of sequence comparison of *rsp-1* to the known sequences in the protein data bases. While at the level of nucleic acid no significant homologies were found in EMBL or GenBank DNA data bases, the protein data bases indicated homologies to *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* adenylyl cyclase genes (14, 31, 33) (Fig. 5A). Analysis with BESTFIT reported values of 53% similarity and 28% identity between *rsp-1* and adenylyl cyclase regulatory regions (data not shown). The homology is found in an array of leucine repeats located in the regulatory region of the yeast proteins, identified in *S. cerevisiae* as necessary for regulation of adenylyl cyclase by Ras (6, 10, 28). The basic repeat unit in both *rsp-1* and adenylyl cyclase consists of 23 amino acids and is defined by the positions of proline (at position 1), leucine (at positions 7, 10, and 13), asparagine (at position 18), and aliphatic amino acids (at positions 4, 15, 20, and 23) (Fig. 5B). The *rsp-1* protein contains fewer repeats than do the yeast adenylyl cyclases, 7 to 10 versus >20. BLAST analysis (1) revealed that *rsp-1* appears to be most closely related to *S. cerevisiae* adenylyl cyclase; there are regions of complete homology or high similarity to the *S. cerevisiae* adenylyl cyclase repeats concentrated in the *rsp-1* repeat motifs (Fig. 5A), suggesting that these regions may form similar recognition or binding sites in the two molecules.

DISCUSSION

Using an expression cloning assay, we have isolated a novel cDNA, *rsp-1*, which is capable of suppressing the v-Ras-transformed phenotype. The *rsp-1* cDNA, when introduced into Ki-Ras-transformed rodent fibroblasts and epithelial cells under the control of a heterologous promoter, can suppress anchorage-independent growth. When introduced into NIH 3T3 cells, *rsp-1* confers resistance to transformation by v-Ras. In addition, it suppresses the growth of NIH 3T3 cells. An obvious mechanism to explain the growth suppression would be interaction with c-Ras and interference with signal transduction. Several lines of evidence suggest that transformation by v-*src* requires intact c-Ras function (9, 24, 27). Therefore, the absence of v-*src* suppression by *rsp-1* suggests either that the c-Ras signalling pathways are not affected in these cells or that v-*src* does not require *rsp-1*-inhibited c-Ras function(s) for transformation. Unlike the suppressor activity of Ras-GAP, which does not extend to v-Ras (34), *rsp-1* does inhibit v-Ras transformation. This suggests that *rsp-1* does not interact with Ras in a way analogous to the Ras-GAP interaction.

rsp-1 shares homology with the yeast adenylyl cyclases in an array of leucine repeats located in the regulatory region of the yeast proteins and identified in *S. cerevisiae* as necessary for regulation of adenylyl cyclase by Ras (6, 10, 28). Hence, the suppressor property of the gene may be a result of its

```
A.   1  MSKSLKKLVE ESREKNQPEV DMSDRGISSM LDVNGLFSLA HITQLVLSHN KLTTVPPNVA
                                          :++  :+ ++: : :  :  : ++:+
                                       880 NSIMKLS NLTILNLQCN ELESLPAGFE

     61 ELKNLEVLNF FNNQIEELPT QISSLQKLKH LNLGMNRLNT LPRGFGSSRL LEVLELTYNN
        :::::++:+   +:      :   :+   +: + ++:+ :++ + ::++
        ELKNLQLLDL SSNKFMHYPE VINYCTNLLQ IDLSYNKIQS LPQS

    121 LNEHSLPGNF FYLTTLRALY LSDNDFEILP PDIGKLTKLQ ILSLRDNDLI SLPKEIGELT
                  :+ :  :  :++:+   ::   :+:::::   ::+  : :   +: :+++:
              1063 LSFLEKLE LNQNNLTRLP QEISKLTKLV FLSVARNKLE YIPPELSQLK

    181 QLKELHIQGN RLTVLPPELG NLDLTGQKQV FKAENNPWVT PIADQFQLGV SHVFEYIRSE
         :+ : +++:  :      :  ::+::+
        SLRTLDLHSN NIRDFVDGME NLELTS

    241 TYKYLYGRHM QANPEPPKKN NDKSKKISRK PLAAKNK
```

```
B.   1        M S K S L K K L V E E S R E K N Q
    18    P E V D M S D R G I S S M L D V N G L F S L
    40                  A H I T Q L V L S H N K L T T V
    57  P P N V A E L K N L E V L N F F N N Q I E E L
    79  P T Q I S S L Q K L K H L N L G M N R L N T L
   102  P R G F G S S R L L E V L E L T Y N N L N E H S L
   127  P G N F F Y L T T L R A L Y L S D N D F E I L
   150  P P D I G K L T K L Q I L S L R D N D L I S L
   173  P K E I G E L T Q L K E L H I Q G N R L T V L
   196  P P E L G                N L D L T G Q K Q V F K A

        P X X a X X L X X L X X L N/D a S X N X a X X a

        LEUCINE REPEATS   Sc. pompe, Sa. cerevisiae  adenylyl cyclase
```

FIG. 5. Alignment of the *S. cerevisiae* adenylyl cyclase amino acid sequence to the *rsp-1* translated sequence. Searches of the Genbank and EMBL protein data bases, accomplished by using University of Wisconsin GCG tFASTA, revealed that yeast adenylyl cyclases are homologous to the *rsp-1* translated sequence. (A) Two regions of the *S. cerevisiae* adenylyl cyclase translated sequence (bottom line), identified by using the BLAST program (1), are aligned to the *rsp-1* translated sequence (top line). The amino acid number of the 5′ end of the aligned adenylyl cyclase sequence is identified at the start of the aligned region. Identical amino acids (:) and conservative substitutions (+) are indicated. (B) Comparison of the leucine repeat motifs found in *rsp-1* with the consensus leucine motifs of yeast adenylyl cyclase. The 5′ end of the *rsp-1* amino acid sequence is shown aligned in 23-amino-acid leucine repeats. The consensus leucine repeat motifs for *S. cerevisiae* and *S. pombe* adenylyl cyclases (14, 31, 33) is shown at the bottom. All amino acids are represented by the single-letter code; a lower-case a represents an aliphatic residue.

ability to associate with Ras p21 in a manner analogous to the association between Ras and adenylyl cyclase in *S. cerevisiae*. While there is no direct biochemical evidence to confirm this interaction in *S. cerevisiae*, genetic studies clearly point to an association between the two molecules. A number of proteins with diverse functions contain similar but not identical leucine-rich repeat motifs that lack one or more of the defining elements present in the adenylyl cyclase and *rsp-1* repeats (11, 17). While this type of leucine-based repeat may be the unit of a general structure, i.e., an amphipathic beta sheet (17), the conservation of amino acids at key positions in the repeat likely confers specificity on the structure. Several studies have demonstrated that Ras does not regulate adenylyl cyclase in higher eukaryotes (4, 18), suggesting that the homology represents a structural similarity rather than the identification of an analogous component of the same pathway. The regions of homology between adenylyl cyclase and *rsp-1* may characterize a novel family of Ras-interactive proteins which ultimately may be useful in defining a Ras recognition sequence(s).

It is not clear whether suppression of c-Ras activity is the normal function of *rsp-1*. Constitutive expression of *rsp-1* in

our assay may prevent binding of Ras p21 to a downstream target, but it may act differently in the context of normal regulation in the cell. In fact, *rsp-1* may itself be a c-Ras target. *rsp-1* appears to be a conserved gene which is ubiquitously expressed in tissue and cell lines at the level of RNA. However, additional information on the regulation of the *rsp-1* protein in normal and Ras-transformed cells, along with in vitro analysis of Ras-*rsp-1* interaction, will be necessary to clarify the role of *rsp-1* in normal cell function.

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