

# The *NF1* Locus Encodes a Protein Functionally Related to Mammalian GAP and Yeast *IRA* Proteins

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## Summary

**The von Recklinghausen neurofibromatosis locus, *NF1*, encodes a protein with homology restricted to the catalytic region of the *RAS* GTPase-activating protein, GAP, and with extensive homology to the *IRA1* and *IRA2* gene products of the yeast *S. cerevisiae*. A segment of the *NF1* cDNA gene, expressed in yeast, can complement loss of *IRA* function and can inhibit both wild-type and mutant activated human *H-ras* genes that are coexpressed in yeast. Yeast expressing the *NF1* segment have increased *H-ras* GTPase-stimulating activity. These studies indicate that the *NF1* gene product can interact with *RAS* proteins and demonstrate structural and functional similarities and differences among the GAP, *IRA1*, *IRA2*, and *NF1* proteins.**

## Introduction

von Recklinghausen neurofibromatosis (NF1) is one of the most common autosomal dominant disorders, with an incidence of about 1 in 3000 individuals in all ethnic groups (Crowe et al., 1956; Riccardi and Eichner, 1986). The disorder has a high spontaneous mutation rate, with an estimated 40%–50% of NF1 cases being new mutations. The clinical features of NF1 are quite variable, but most arise in tissues derived from the neural crest. Among these are the characteristic café-au-lait spots, which may be due to abnormal differentiation of melanocytes, and the benign neurofibromas, which give the disorder its name. These latter are composed of a mixture of cell types including Schwann cells, fibroblasts, mast cells, and vascular elements. More serious complications of NF1 can include seizures, learning disabilities, major orthopedic abnormalities, and the development of malignancy, especially neurofibrosarcoma and optic glioma.

No consistent biochemical abnormality has been found in tissues affected by NF1, and this has slowed progress in understanding its biology. Linkage analysis has been used to map the locus to the proximal long arm of chromo-

some 17 (Barker et al., 1987; Seizinger et al., 1987; Goldgar et al., 1989). Subsequent physical mapping efforts (Fountain et al., 1989; O'Connell et al., 1989) narrowed the region where the gene was located to within a few hundred kilobases. These studies culminated in the cloning and identification of the *NF1* gene (Wallace et al., 1990; Viskochil et al., 1990; Cawthon et al., 1990). The initial reports delimited a large locus with a ubiquitously expressed transcript of about 13 kb. Deletions (Viskochil et al., 1990), point mutations (Cawthon et al., 1990), and an insertion (Wallace et al., 1990) were described in NF1 patients that would imply a loss of function of this gene product as the causative mutation. Further evidence for the inactivation hypothesis comes from expression studies of the *NF1* transcript in somatic cell hybrids. The gene was shown to be transcribed from a normal human chromosome 17 in a rodent–human hybrid cell line, but not in somatic cell hybrids derived from two rare NF1 patients containing balanced chromosomal translocations involving 17q11.2 (Wallace et al., 1990). These observations are consistent with the proposal that the *NF1* gene acts as a tumor suppressor (Knudson et al., 1975) and that the mechanism of action is actually recessive at the cellular level.

Further cloning and sequence analysis of the *NF1* transcript (Xu et al., 1990; Buchberg et al., 1990; D. Marchuk et al., unpublished data) demonstrates that the *NF1* gene product has homology with the mammalian *RAS* GTPase-activating protein, GAP (Trahey and McCormick, 1987; Vogel et al., 1988; Trahey et al., 1988), as well as the products of *IRA1* and *IRA2*, two *Saccharomyces cerevisiae* genes that encode functional equivalents of GAP in yeast and regulate *RAS* signal transduction pathways controlling cell growth (Tanaka et al., 1989, 1990a, 1990b). Expression of mammalian GAP can suppress the characteristic heat shock sensitivity phenotype of *ira1*<sup>-</sup> and *ira2*<sup>-</sup> mutants (Ballester et al., 1989; Tanaka et al., 1990a, 1990b), suggesting that the GAP and *IRA* proteins are functionally analogous in their ability to stimulate the GTPase activity of *RAS*. In the work reported here we have made an extensive sequence comparison of the *NF1*, GAP, and *IRA* proteins and analyzed the function of a fragment of the *NF1* protein expressed in yeast.

## Results

### Comparative Sequence Analyses

The results of a multiple alignment analysis of the *NF1*, *IRA1*, *IRA2*, and GAP proteins using the MACAW program (Schuler et al., 1990) are shown in Figure 1. Interestingly, neither the amino-terminal half of GAP nor the first 400 residues of the partial *NF1* sequence have anything in common with the large amino-terminal domains of *IRA1* and *IRA2*. In the remainder of the proteins, 16 to 17 highly conserved sequence motifs or homology blocks common to *NF1*, *IRA1*, and *IRA2* were identified. These motifs cluster into two domains that constitute the central and carboxy-terminal regions of these proteins and are composed of

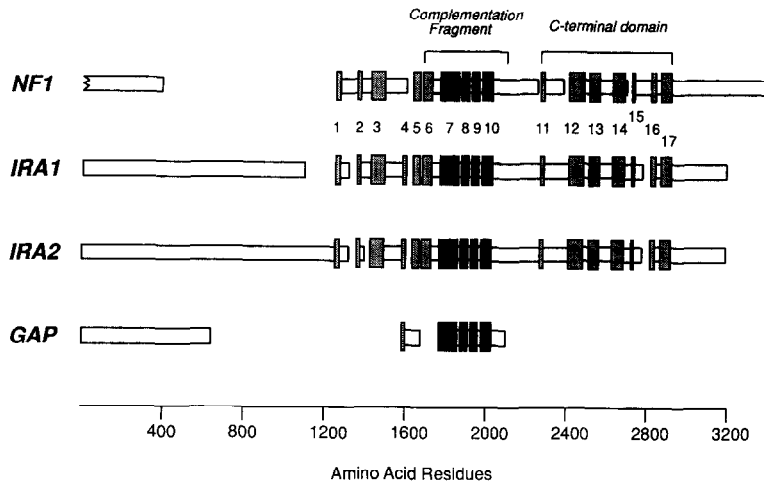


Figure 1. Sequence Domains and Motifs among NF1-Related Proteins

The MACAW program (Schuler et al., 1990) was used to locate, analyze, and assess the statistical significance of ungapped regions of local similarity (also referred to as "blocks") among the NF1, IRA1, IRA2, and GAP protein sequences. All blocks common to at least three of the four sequences, and having chance probabilities  $< \sim 10^{-3}$ , are shown. Unfilled rectangles indicate regions with little or no sequence conservation. Blank spaces between rectangles signify gaps in the multiple alignment. The filled rectangles labeled 1 through 17 denote conserved sequence motifs. The fragment indicated by the bracket, which includes blocks 6 through 10 and corresponds to residues 791-1203 of NF1, was the region used for the complementation experiments as described in the text. Blocks 7 through 10 indicate regions common to all four sequences and are

colored black to indicate this fact. Sources of the sequence data for the four proteins shown are described in Experimental Procedures. The Karlin *p* values (Karlin and Altschul, 1990) associated with each block are as follows: block 1 ( $4.39 \times 10^{-6}$ ), block 2 ( $2.57 \times 10^{-3}$ ), block 3 (0), block 4 ( $1.23 \times 10^{-6}$ ), block 5 ( $9.5 \times 10^{-7}$ ), block 6 (0), block 7 (0), block 8 ( $2.22 \times 10^{-11}$ ), block 9 (0), block 10 ( $3.61 \times 10^{-7}$ ), block 11 ( $9.17 \times 10^{-3}$ ), block 12 (0), block 13 (0), block 14 (0), block 15 ( $5.67 \times 10^{-5}$ ), block 16 ( $1.21 \times 10^{-4}$ ), block 17 ( $1.11 \times 10^{-16}$ ). A *p* value of zero indicates  $p < 10^{-100}$ . For block 4, the statistical significance increases when the GAP segment is removed from the alignment, signifying that the apparent relationship of the GAP segment may be due to chance (MACAW User's Manual, pp. 5-10). For all of the other blocks, the statistical significance decreases if any one sequence is removed from the alignment.

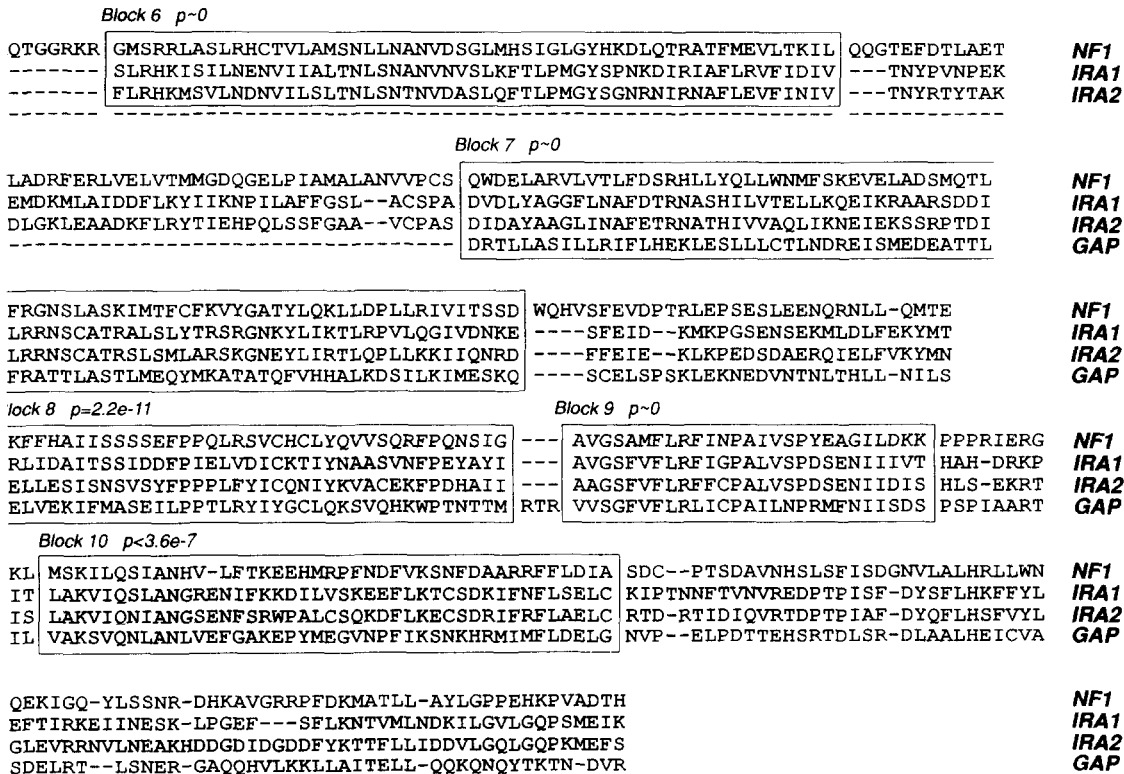


Figure 2. Comparative Sequence Analysis of the NF1 Complementation Fragment

Based on the analysis in Figure 1, subsequences of NF1, IRA1, IRA2, and GAP were selected for a full dynamic programming alignment, with gaps, using the program MSA (Lipman et al., 1989). The optimal multiple alignment is shown. With respect to the highly conserved sequence blocks identified by MACAW (Figure 1), the alignments are identical except for block 5 in which MSA introduced a single-residue deletion in NF1. The probabilities of chance occurrence associated with each block were derived from the MACAW analysis (Figure 1). The subsequences used as input for program MSA correspond to residues 791-1203 for NF1 (following the numbering system of Xu et al., 1990), 1458-1852 for IRA1, 1604-2001 for IRA2, and 745-1044 for human GAP.

blocks 1 through 10 and 11 through 17, respectively. When the GAP sequence is included in the analysis, only four blocks (7–10) remain common to all four sequences. An additional small region (block 4, 24 residues) of questionable significance may be present in the *IRA* sequences and GAP but not NF1 (Figure 1). Thus only a very limited region of 223–232 residues (blocks 7 through 10 in Figure 2) appears to be conserved among all four sequences. This is considerably smaller than previous estimates of the GAP catalytic domain (Tanaka et al., 1989, 1990a, 1990b; Xu et al., 1990).

Once the most highly conserved blocks corresponding to the GAP catalytic domain were mapped using MACAW, these short, approximately equal subsequences were extracted for an optimal global alignment using the MSA program (Lipman et al., 1989). The results are shown in Figure 2. The probability that blocks 6 through 10 are related by chance is vanishingly small despite the fact that only 5%–23% of identical residues are present among all four sequences in these regions. In particular, block 7 contains only four 4-way identities across a 78 residue span, yet the probability that this pattern is ascribable to chance is essentially zero. Using older analytic methods, it would be difficult to attach this degree of significance to similarity so diffuse. In the interblock regions, the sizes and locations of gaps are consistent among the sequences. In contrast, the regions distal to block 10 display much more variability in the sizes and locations of gaps.

#### Design of the NF1 Expression Vector

A region (amino acids 791–1203) spanning the proposed catalytic domain of NF1 (see Figure 1) was amplified by polymerase chain reaction (PCR) from a fetal brain cDNA library (see Experimental Procedures) and cloned into the yeast expression vector pADANS (see Figure 3), creating pADANS-NF1. A single nucleotide difference was observed, at nucleotide position 3407 of the published sequence (Xu et al., 1990). This T to C transition would result in an isoleucine to threonine substitution. This difference was subsequently observed in two out of four independent PCR clones and therefore may reflect a true genetic polymorphism.

The pADANS vector derives from the pADNS plasmid (Colicelli et al., 1989), which is an expression vector designed to express cDNA genes from the alcohol dehydrogenase (*ADH1*) promoter. pADANS also contains the *LEU2* marker and replicates to high copy in yeast extrachromosomally because it contains the  $2\mu$  circle origin of replication. pADANS is used as a control plasmid in the expression studies described below. pADANS differs from pADNS only in that the *ADH1*-driven transcript includes the first 14 codons of the *ADH1* gene. In pADANS-NF1, these codons are in frame with the *NF1* coding sequences. In addition to pADNS and pADANS-NF1, we have also used the plasmid pADGAP, which expresses a full-length human GAP cDNA clone driven by the *ADH1* promoter (Ballester et al., 1989). pADGAP likewise contains the *LEU2* marker and the  $2\mu$  circle origin of replication.

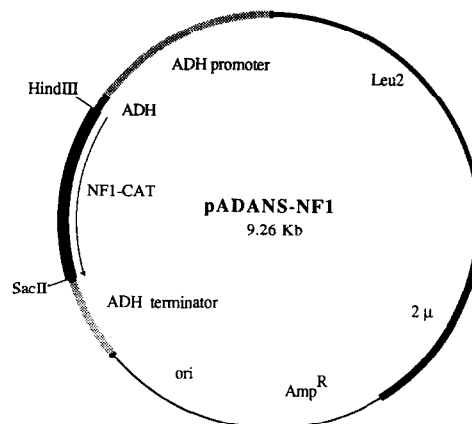


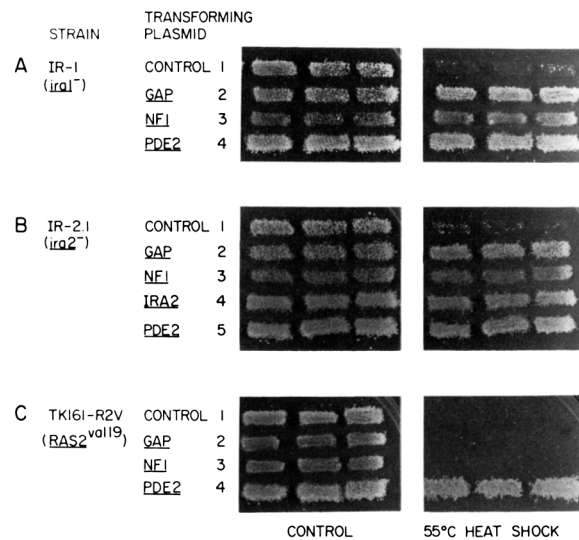
Figure 3. Diagram of the NF1 Expression Vector

The pADANS vector will be described in detail in Colicelli et al. (1991). In addition to bacterial plasmid sequences, pADANS-NF1 contains a selectable marker (*LEU2*) and origin of replication ( $2\mu$ ) for use in yeast. The vector also contains the yeast *ADH1* promoter and first 14 codons of the gene, as well as the *ADH1* terminator. The NF1 catalytic domain (see Figure 2) has been inserted in frame as a fusion peptide with *ADH1*. Further details of the construction of the clone are described in Experimental Procedures.

#### Complementation of *ira*<sup>-</sup> Strains by *NF1*

Disruption of either *IRA* gene leads to a phenotype that resembles that seen in cells containing the mutationally activated *RAS2*<sup>Val-19</sup> (Toda et al., 1985; Sass et al., 1986; Matsumoto et al., 1985; Tanaka et al., 1989, 1990a, 1990b). In particular, *ira1*<sup>-</sup> or *ira2*<sup>-</sup> strains are exquisitely sensitive to heat shock. This phenotype is suppressed by the overexpression of the yeast phosphodiesterase gene, *PDE2*, which also suppresses the phenotype resulting from the activated yeast *RAS2*<sup>Val-19</sup> (Tanaka et al., 1989; Sass et al., 1986). *PDE2* expression undoubtedly counteracts the effects of activated *RAS* protein upon adenylyl cyclase, the main target of *RAS* action in *S. cerevisiae* (Toda et al., 1985; Sass et al., 1986), by lowering cAMP levels. We and others have previously shown that expression of the human *GAP* gene in *ira1*<sup>-</sup> yeast strains suppresses heat shock sensitivity (Ballester et al., 1989; Tanaka et al., 1990a). Expression of the *GAP* gene does not suppress the heat shock sensitivity resulting from the mutant activated *RAS2*<sup>Val-19</sup>. Thus human *GAP* appears to be able to inhibit the function of wild-type yeast *RAS* protein, but not that of mutationally activated *RAS* protein.

We have tested the effect of expressing the human *NF1* gene upon the phenotype of *ira*<sup>-</sup> and *RAS2*<sup>Val-19</sup> strains. In the first of a series of experiments (Figure 4A), the *ira1*<sup>-</sup> strain IR-1 (see Table 1 for strain description) was transformed with either of four plasmids: pADNS, pADGAP, pADANS-NF1, or pPDE2. The plasmid pPDE2 contains the yeast *PDE2* gene, together with the *LEU2* marker and the  $2\mu$  circle origin of replication (Sass et al., 1986). Three independent transformants were picked from each transformation, and patches were tested for heat shock sensitivity by a replica plate method (see Experimental Proce-



**Figure 4. Effect of Expressing NF1 on the Heat Shock Sensitivity of Strains Lacking *IRA1* or *IRA2* and Strains Containing *RAS2<sup>Val-19</sup>***  
Yeast strains were transformed with various plasmids containing the *LEU2* gene and plated onto SC-Leu plates. Independent transformants were patched onto SC-Leu plates, incubated at 30°C for 2 days, replica plated onto SC-Leu plates, and heat shocked (at 55°C) for 10 min (A), 5 min (B and C), or 0 min (A, B, and C) (CONTROL). After heat shock treatment the plates were incubated at 30°C for 2 days. The strains used are IR-1, a strain that contains a disrupted chromosomal *IRA1* gene (A); IR-2.1, a strain that contains a disrupted chromosomal *IRA2* gene (B); and TK161-R2V, a strain that contains the *RAS2<sup>Val-19</sup>* allele (C) (see Table 1 for description of the strains). The different strains were transformed with pADNS, a control plasmid (rows 1); pADGAP, a plasmid containing the human *GAP* gene (rows 2); pADANS-NF1, a plasmid expressing the catalytic domain of the *NF1* gene (rows 3); YepPDE2, a plasmid containing a yeast cAMP phosphodiesterase gene (A, row 4), (B, row 5), (C, row 4); and pYepIRA2, a plasmid containing the *IRA2* gene (B, row 4).

dures). As has been shown previously, expression of human *GAP* or *PDE2* suppresses the phenotype of an *ira1<sup>-</sup>* strain. In addition, expression of the catalytic domain of the *NF1* gene also suppresses the heat shock-sensitive phenotype of IR-1.

This experimental format was repeated with an *ira2<sup>-</sup>* strain, IR-2.1. As before, expression of *GAP*, *NF1*, or *PDE2* suppresses the heat shock-sensitive phenotype (Figure 4B). Suppression of heat shock sensitivity was also demonstrated in *ira1<sup>-</sup>* and *ira2<sup>-</sup>* strains expressing *NF1* in the vector pADNS, which contained the T (isoleucine) polymorphism at position 2047 (data not shown). Finally, the format was repeated in a *RAS2<sup>Val-19</sup>* strain, TK161-R2V. In this strain only expression of the *PDE2* gene suppresses the heat shock-sensitive phenotype (Figure 4C). These results indicate that both *GAP* and *NF1* proteins are capable of inhibiting wild-type yeast *RAS* proteins, but neither can inhibit the mutationally activated *RAS2<sup>Val-19</sup>* protein.

#### Inhibition of Wild-Type and Mutant Mammalian H-ras Protein by NF1

We have previously demonstrated that human *GAP* can inhibit the wild-type human H-ras protein when both are expressed in yeast (Ballester et al., 1989). This is demon-

**Table 1. Yeast Strains**

Strain	Genotype
SP1 <sup>a</sup>	<i>MATα his3 leu2 ura3 trp1 ade8 can1</i>
IR-1 <sup>b</sup>	<i>MATα his3 leu2 ura3 trp1 ade8 can1 ira1::HIS3</i>
IR-2.1 <sup>c</sup>	<i>MATα his3 leu2 ura3 trp1 ade8 can1 ira2::URA3</i>
TK161-R2V <sup>a</sup>	<i>MATα his3 leu2 ura3 trp1 ade8 can1 RAS2<sup>Val-19</sup></i>
TTRB-G1 <sup>b</sup>	<i>MATα his3 leu2 ura3 trp1 ade8 can1 cdc25::URA3 pAHRG-H1<sup>d</sup></i>
TTRB-V2 <sup>b</sup>	<i>MATα his3 leu2 ura3 trp1 ade8 can1 cdc25::URA3 pAHRV-H2<sup>d</sup></i>

<sup>a</sup> Toda et al. (1985).

<sup>b</sup> Ballester et al. (1989).

<sup>c</sup> See Experimental Procedures, Construction of Yeast Strains.

<sup>d</sup> See Experimental Procedures, Plasmids.

strated most readily in strains with a *cdc25<sup>-</sup>* background. The product of the *CDC25* gene appears required for the catalytic activation of wild-type yeast *RAS* proteins (Broek et al., 1987; Robinson et al., 1987; Camonis et al., 1986; Powers et al., 1989). *cdc25<sup>-</sup>* cells are not viable, but *cdc25<sup>-</sup>* strains that express the human H-ras gene are (Marshall et al., 1987). We believe that the human H-ras protein does not require catalytic activation by the *CDC25* gene product in yeast because it is relatively insensitive to the action of the *IRA1* and *IRA2* gene products (Tanaka et al., 1990a). We previously showed that *cdc25<sup>-</sup>* strains that express both the human H-ras and *GAP* genes are temperature sensitive. By contrast, *cdc25<sup>-</sup>* strains that express the human *GAP* and the mutationally activated H-ras<sup>Val-12</sup> gene are not temperature sensitive. We interpreted these results to mean that human *GAP* can down-regulate wild-type H-ras protein but can neither down-regulate nor interfere with the activated H-ras<sup>Val-12</sup> protein (Ballester et al., 1989).

We have repeated these experiments using yeast strains that contain the *NF1* expression vector, pADANS-NF1. The *cdc25<sup>-</sup>* strain expressing human wild-type H-ras, TTRB-G1 (Figure 5B), or the *cdc25<sup>-</sup>* strain expressing the activated H-ras<sup>Val-12</sup>, TTRB-V2 (Figure 5A), was transformed with pADNS, pADGAP, or pADANS-NF1, and eight independent transformants of each were tested in patches for temperature sensitivity by a replica plate method. The results obtained with transformants expressing *GAP* were the same as seen previously. In particular, *cdc25<sup>-</sup>* H-ras strains expressing *GAP* were temperature sensitive, while *cdc25<sup>-</sup>* H-ras<sup>Val-12</sup> strains expressing *GAP* were not. We found that *cdc25<sup>-</sup>* H-ras strains expressing the catalytic domain of *NF1* were also inhibited for growth at elevated temperatures. Unlike *cdc25<sup>-</sup>* H-ras<sup>Val-12</sup> strains expressing *GAP*, however, *cdc25<sup>-</sup>* H-ras<sup>Val-12</sup> strains expressing *NF1* were temperature sensitive. Thus not only does *NF1* inhibit wild-type H-ras protein, but it also appears to be capable of inhibiting the function of the mutant H-ras<sup>Val-12</sup> protein. In this respect, the *NF1* expression vector differs from the *GAP* expression vector.

#### Biochemical Assays of Yeast Expressing NF1

While our genetic experiments clearly indicate that the human *NF1* protein, like *GAP*, interacts with both yeast and mammalian *RAS* proteins, they do not indicate if the *NF1*

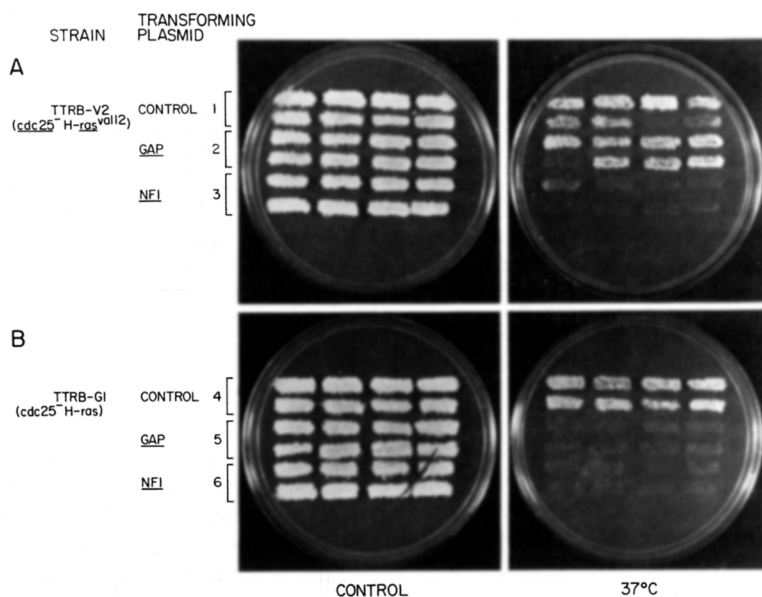


Figure 5. Effect of Expressing NF1 on the Temperature Sensitivity of Strains Lacking *CDC25* and Expressing the Mutant *H-ras*<sup>Val-12</sup> or the Wild-Type Human *H-ras* Proteins

Cells lacking the *CDC25* gene, and expressing (A) the mutant *H-ras*<sup>Val-12</sup> gene (plasmid pAHRV-H2, strain TTRB-V2), or (B) the wild-type *H-ras* gene (plasmid pAHRG-H1, strain TTRB-G1), were transformed with a control plasmid, pADNS (rows 1 and 4), a plasmid expressing *GAP*, pADGAP (rows 2 and 5), or a plasmid expressing the catalytic domain of the *NF1* gene, pADANS-NF1 (rows 3 and 6). Independent Leu<sup>+</sup> transformants were patched onto selective plates and incubated at room temperature for 3 days and then replica plated onto YPD plates. Replicas were incubated at room temperature (CONTROL) or at 37°C for 3 days. Each set shows eight independent transformants.

protein, like *GAP*, has GTPase-stimulating activity. To answer this question, we performed *H-ras* GTPase assays using crude lysates of cells that express human *GAP* or human *NF1*, adding wild-type or mutant *H-ras* proteins purified from *Escherichia coli* expression systems (Gross et al., 1985). This approach is feasible since we have previously observed no *H-ras* GTPase-stimulating activity in lysates from *S. cerevisiae* strains (R. B., unpublished data; Adari et al., 1988). Lysates were prepared from the *ira2*<sup>-</sup> yeast strain (IR-2.1) that had been transformed either with a control vector (pADNS) or vectors expressing human *GAP* (pADGAP), *NF1* protein (pADANS-NF1), or yeast *IRA2* protein (pYep-*IRA2*). *H-ras* protein, which was bound with radiolabeled GTP, hydrolyzed GTP to about the same

extent whether incubated with assay buffer or with lysates from yeast transformed with either vector controls or pYep-*IRA2* (data not shown).

Compared to control lysates, GTP hydrolysis by *H-ras* was greatly accelerated by incubation with lysates from yeast expressing *GAP* (see Figure 6 and Table 2), and hydrolysis was moderately but definitely accelerated when *H-ras* was incubated with lysates from yeast expressing *NF1*. From the stoichiometry of the GTPase-stimulating assays (see Experimental Procedures), we cannot infer that *NF1* acts catalytically on *H-ras*. Neither lysate was capable of accelerating the GTP hydrolysis by the mutant activated *H-ras*<sup>Val-12</sup> protein. The results of three separate experiments with independently prepared lysates are

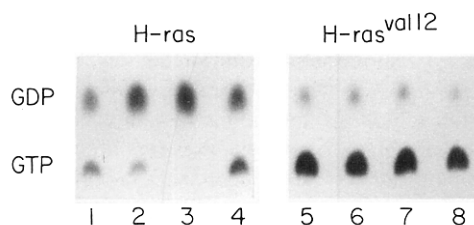


Figure 6. Stimulation of the Human *H-ras* GTPase Activity by Lysates from Yeast Strains Expressing the *NF1* Protein or Human *GAP*

Wild-type human *H-ras* (lanes 1–4) or mutant *H-ras*<sup>Val-12</sup> (lanes 5–8) proteins prelabeled with [ $\alpha$ -<sup>32</sup>P]GTP were incubated for 30 min at room temperature with extracts of the *ira2*<sup>-</sup> yeast strain IR-2.1 transformed with the control plasmid, pADNS (lanes 1 and 5); the plasmid expressing the catalytic domain of the *NF1* gene, pADANS-NF1 (lanes 2 and 6); the plasmid expressing the human *GAP* gene, pADGAP (lanes 3 and 7); and the plasmid expressing the *IRA2* gene, pYep/*IRA2* (lanes 4 and 8). The *H-ras* protein was then immunoprecipitated with the monoclonal antibody Y13-259 and incubated with rabbit anti-rat immunoglobulin and protein A-Sepharose. The labeled nucleotides were eluted and resolved by thin-layer chromatography (see Experimental Procedures for a detailed description). The figure shows the autoradiograph of plates exposed on X-ray film. Results of the quantitation of the plate are shown in Table 2.

Table 2. *H-ras* GTPase Stimulation by Yeast Lysates

Experiment <sup>a</sup>	Proportion of Protein Bound with GDP							
	<i>H-ras</i>				<i>H-ras</i> <sup>Val-12</sup>			
	Control	<i>GAP</i>	<i>NF1</i>	<i>IRA2</i>	Control	<i>GAP</i>	<i>NF1</i>	<i>IRA2</i>
1	0.47	0.81	0.62	0.50	0.05	0.11	0.10	0.11
2 <sup>b</sup>	0.61	0.96	0.82	0.57	0.11	0.14	0.12	0.13
3	0.70	0.97	0.91	0.65	0.20	0.13	0.15	0.13

Human *H-ras* or *H-ras*<sup>Val-12</sup> proteins prebound to [ $\alpha$ -<sup>32</sup>P]GTP were incubated with yeast extracts as described in Figure 6 and Experimental Procedures. The bound nucleotides were eluted and resolved by thin-layer chromatography. The chromatography plates were scanned and analyzed using the PhosphorImager from Molecular Dynamics as described in Experimental Procedures. The results are presented as the proportion of the total counts recovered as GDP from the *H-ras* proteins, after subtracting background. Lysates were prepared from the yeast strain IR-2.1 (*ira2*<sup>-</sup>) transformed with either control plasmids pADNS, or plasmids expressing the indicated proteins. See text for more detail.

<sup>a</sup> Three separate experiments were performed using independently prepared lysates.

<sup>b</sup> The autoradiograph shown in Figure 6 is the result of this experiment.

shown in Table 2. We conclude that, relative to control lysates, lysates from cells containing the NF1 expression vector have increased H-ras GTPase-stimulating activity. Under our assay conditions, NF1 lysates have less activity than lysates from cells expressing GAP.

## Discussion

The *NF1* locus encodes a protein that is structurally related to the *IRA* proteins of yeast and to the GAP protein of mammalian cells (Xu et al., 1990; Buchberg et al., 1990). New software tools (Altschul et al., 1990; Schuler et al., 1990) and statistical methods (Karlin and Altschul, 1990) enabled us to analyze in detail the conserved sequence regions of these proteins and make an assessment of their statistical significance. By these quantitative criteria, the homologies among NF1, *IRA1*, *IRA2*, and GAP proteins are unquestionably of a very high level of significance (Figures 1 and 2). While the similarity of NF1 to GAP is limited to blocks 7–10 in the catalytic domain, the similarity of NF1 to *IRA1* and *IRA2* extends well outside this, in both directions, especially in the C-terminal domain, where blocks 12 through 16 are as highly conserved and significant as those in the catalytic domain. Extensive local and global pairwise comparisons indicate that NF1 is much more similar to the yeast sequences than to GAP, and is closest to *IRA2*, particularly in the C-terminal domain (M. Boguski, unpublished data).

GAP and *IRA* proteins are known to interact with *RAS* proteins. The presumption that the NF1 protein also can interact with *RAS* proteins is confirmed by our studies. We have expressed in yeast the domain of the NF1 protein that corresponds to the common catalytic region of GAP and the *IRA* proteins. Expression of the NF1 catalytic domain, like the expression of GAP, complements *IRA*-deficient yeast and also inhibits the wild-type human H-ras protein expressed in yeast. Unlike GAP, expression of NF1 inhibits even the activated H-ras<sup>Val-12</sup> protein. We have found increases in H-ras GTPase-stimulating activity in lysates of yeast expressing the NF1 catalytic region, although not as much as found in lysates of yeast expressing GAP. These last two observations suggest to us that the expressed NF1 protein fragment might bind more tightly to H-ras proteins than does GAP. Hence, this NF1 protein would display less GAP-like catalytic activity yet would be capable of inhibiting the activated form of H-ras. Further biochemical studies are needed to confirm this idea with the full-length NF1 protein.

We have explored the function of only the common catalytic region of NF1. As the sequence analysis we and others have presented shows (Xu et al., 1990; Buchberg et al., 1990), NF1 is highly related to the yeast *IRA* proteins in carboxy-terminal regions—regions that show no homology with GAP. It seems highly probable to us that these structurally conserved regions have conserved biochemical functions as well. These regions of homology should be subjected to future genetic and biochemical experimentation.

von Recklinghausen neurofibromatosis can be viewed as an autosomal disorder affecting both the cellular

proliferation and differentiation of cells of neuroectodermal origin. Prior to the recent identification of the *NF1* gene by positional cloning methods, efforts to identify a biochemical marker of the disease had failed. While only a few mutations of the *NF1* gene have been identified thus far in patients with the disease, it appears that inactivation of the gene, rather than activation, leads to the appearance of the disorder. This observation would be consistent with a tumor suppressor mechanism, although direct evidence for somatic inactivation of the remaining normal *NF1* allele in tumors has not yet been reported.

The functional linkage between NF1 and *RAS* is highly gratifying since oncoproteins in general, and *RAS* proteins in particular, are known to profoundly affect cellular proliferation and differentiation. Mutant, activated *RAS* genes have been found in neuroblastomas (Shimizu et al., 1983), where, presumably, *RAS* proteins stimulate cellular proliferation. On the other hand, in some cells, such as rat PC12 cells, which are also derived from tumors of neuroectodermal origin, activated *RAS* induces differentiation and causes the cessation of growth (Bar-Sagi and Feramisco, 1985; Noda et al., 1985). In such cells, microinjection of blocking antibodies or transfection with interfering *RAS* genes does not inhibit growth, but rather blocks the differentiation induced by nerve growth factor (Hagag et al., 1986; Szeberenyi et al., 1990). In the absence of information about the role of *RAS* in the neuroectoderm in vivo, and in the absence of knowledge about whether NF1 regulates or mediates *RAS* function, several mechanisms might explain the disorders of proliferation and differentiation found in NF1 patients. In view of the ubiquitous expression of NF1 (Wallace et al., 1990), it is not currently apparent why the disease appears so prominently in neural crest derivatives.

*RAS* proteins, which are found ubiquitously in eukaryotes, bind and hydrolyze GTP, and are in their active configuration when GTP bound (for review see Barbacid, 1987). GAP was discovered as the major factor in cellular lysates that stimulates GTP hydrolysis by *RAS*. GTP hydrolysis by oncogenic forms of *RAS* proteins is generally resistant to stimulation by GAP (Trahey and McCormick, 1987; Adari et al., 1988; Cales et al., 1988), and hence many have proposed that GAP down-regulates wild-type *RAS* proteins. This hypothesis has been strengthened by two sets of observations. First, GAP is homologous to yeast *IRA* proteins (Tanaka et al., 1989, 1990a, 1990b), and GAP expression can complement *IRA*-deficient yeast (Ballester et al., 1989; Tanaka et al., 1990a). In yeast it is amply clear that the *IRA* proteins regulate *RAS*. Second, expression of GAP can inhibit the growth transformation of cultured cells induced by wild-type *RAS* (Zhang et al., 1990).

It is by no means clear that the only function of GAP and *IRA* proteins is to regulate *RAS* proteins. Both GAP and *IRA* proteins, and for that matter the NF1 protein, are very large molecules, but the domain required for stimulating GTP hydrolysis is much smaller (Marshall et al., 1989; Tanaka et al., 1990b). No one has yet properly addressed the issue of whether the *IRA* proteins have additional functions in yeast. Moreover, there is at least one in vitro sys-

tem in which GAP and RAS proteins appear to act in concert (Yatani et al., 1990). Thus it is possible that proteins like GAP, *IRA1*, *IRA2*, and NF1 can mediate some of the effects of RAS proteins.

Our present knowledge of the *IRA* and GAP proteins provides only an incomplete guide for understanding the action of NF1. We cannot confidently anticipate, at present, whether NF1 proteins will act upstream or downstream of RAS, or potentially be capable of both functions. On the other hand, experimental manipulation of cells that lack NF1 protein might illuminate its functional relationship with RAS. By introducing forms of RAS protein that interfere with wild-type RAS function into such cells we may be able to discern if lack of NF1 activates the RAS pathway or blocks it.

## Experimental Procedures

### Comparative Sequence Analyses

Sequence motifs common among NF1-related proteins were identified and their statistical significance was estimated using the Multiple Sequence Alignment Construction and Analysis Workbench, or MACAW, program (Schuler et al., 1990). Multiple alignment is the method of choice for detecting patterns or motifs common to a set of sequences, but there are two approaches to multiple alignment that need to be considered in the context of the sequences under study. Global alignment methods are most appropriate to sequences that are small (<500 residues), approximately equal in length, and share a global, but perhaps distant, relationship (Lipman et al., 1989). Local alignment methods, on the other hand, are preferable for longer sequences that vary greatly in length and may share only isolated regions of similarity separated by variable-length segments of little or no sequence conservation (Postai et al., 1989; Schuler et al., 1990). The NF1-related sequences considered here come under this latter category.

Amino acid sequences used in the analysis include the 2484 residue partial sequence of human NF1 (Xu et al., 1990), the 2938 residue sequence of *S. cerevisiae IRA1* (retrieval code A30135, PIR release 25.0), the 3079 residue sequence of *S. cerevisiae IRA2* (accession number M33779, GenBank/GenPept New Sequences), and the 1044 residue sequence of the human GAP "A" protein (accession number M23379, GenBank release 63.0). The analysis was begun at a threshold of 50 and with the requirement that homology blocks be present in all four sequences. Then the scoring criteria were progressively relaxed until all statistically significant ( $p < 1.0 \times 10^{-3}$ ) motifs common to at least three of the four sequences were identified. Because of this latter requirement, the full extent of the pairwise, global similarity between *IRA1* and *IRA2* is not represented in the analysis.

A major advantage of local alignment methods is that a new theory for estimating the statistical significance of sequence similarities (Karin and Altschul, 1990; Schuler et al., 1990) can be applied to the ungapped homology blocks obtained. However, global alignment methods using dynamic programming are superior for determining the locations and extents of insertion and deletion mutations (Lipman et al., 1989). Also sequence weighting to control for redundancy in the data can be applied (Altschul et al., 1989). The two approaches were combined by computing an optimal multiple alignment of subsequences from the catalytic domain (encompassing the complementation fragment) with the MSA program (Lipman et al., 1989) using a sequence weighting procedure (Altschul et al., 1989) to control for the greater pairwise similarity of *IRA1* and *IRA2*.

### Media, Genetic Manipulations, and Nomenclature

Yeast were grown in YPD (2% peptone, 1% yeast extract, 2% glucose) or in synthetic medium (0.67 g/liter yeast nitrogen base, 2% glucose, and appropriate auxotrophic supplements). Standard yeast genetic methods were followed as described previously (Sherman et al., 1986). The lithium acetate method was used for transformation of yeast cells (Ito et al., 1983).

Wild-type alleles and dominant mutations are denoted by capital letters, recessive mutations by lowercase italicized letters, and gene disruptions by lowercase letters, which represent the disrupted gene, followed by two colons and the auxotrophic gene marker used for the disruption. For example, *ira1::HIS3* indicates the *IRA1* gene was disrupted by the *HIS3* marker. In the text, gene disruptions are abbreviated by lowercase italicized letters representing the gene followed by a superscript minus sign, such as *ira1<sup>-</sup>*.

### DNA Manipulation

DNA manipulations were performed by standard methods (Maniatis et al., 1982). DNA restriction endonucleases, polymerases, and ligases were used under conditions recommended by suppliers (New England Biolabs, Bethesda Research Laboratories, or Perkin-Elmer Cetus). For gene disruption experiments, suitable linear DNA fragments were isolated and used for transformation of yeast cells as described (Rothstein, 1983).

### Plasmids

The cloning vector pADANS (Colicelli et al., 1991) is a derivative of pADNS previously described (Colicelli et al., 1989). It contains the *LEU2* gene, an HpaI-HindIII fragment from the 2 $\mu$  circle including the origin of replication, an SspI-EcoRI fragment containing the ampicillin resistance gene from the plasmid pUC18, an EcoRI-HindIII fragment containing the promoter and the first 14 codons of the *S. cerevisiae ADH1* gene, and a HindIII-BamHI fragment containing the *ADH1* terminator sequences. The promoter and terminator sequences are separated by a polylinker containing unique HindIII and SacII sites.

The plasmid pADANS-NF1 was constructed as follows. A region spanning the proposed catalytic domain of NF1 (Figure 1) was amplified by PCR using DNA from a human fetal brain cDNA library (Stratagene, catalog number 936206) as the template. Primers were 5'-CATCAACCTTGTGCGCAAACAGGTGGCAGGAAAC for the amino terminus of the catalytic domain and 5'-CATGCCGCGTCAGTGTGATCTGCCACAGTTTG for the carboxyl terminus of the domain. These primers included either a HindIII site (amino-terminal) or a SacII site (carboxy-terminal) plus 4 bases at the 5' end to facilitate digestion. The amplified PCR product was digested with HindIII and SacII and ligated into the vector pADANS, cut with the same enzymes. The accuracy of the clone was confirmed by dideoxy sequencing (Sequenase kit, US Biochemical Corp., version 2.0) of the PCR insert encoding the catalytic domain.

The plasmid pADGAP contains the entire coding region of the human GAP gene on a high copy *LEU2* plasmid containing the *ADH1* promoter and terminator sequences, pAD4 $\Delta$  (Ballester et al., 1989). The YepPDE2 plasmid contains the yeast *PDE2* gene on the high copy *LEU2* plasmid, YEp13 (Sass et al., 1986). The *PDE2* gene encodes a high affinity cAMP phosphodiesterase.

The plasmid pYepIRA2 contains the yeast *IRA2* gene on the high copy *LEU2* plasmid Yep13M4. The *IRA2* gene was isolated as a high copy suppressor of the heat shock-sensitive phenotype of a strain, IR-1 (Ballester et al., 1989), with a disruption of the *IRA1* gene. The gene was cloned from a library in the yeast vector Yep13M4 constructed by J. Nikawa (unpublished data). The cloned gene was identified as *IRA2* by sequencing a portion of the gene that codes for amino acids 1374-1446 of the published sequence (Tanaka et al., 1990b) and by restriction endonuclease mapping. The DNA isolated is a partial clone that contains 2 kb 5' of the coding sequence and extends only to the BamHI site 3' of the catalytic domain (see Tanaka et al., 1990b).

The plasmid pAHRG-H1 contains the human H-ras cDNA driven by the *ADH1* promoter in the vector pHV1. pHV1 is a high copy number vector that contains the polylinker from pUC18, the *HIS3* gene, and an HpaI-HindIII fragment from the 2 $\mu$  circle, including the origin of replication. The plasmid pAHRV-H2 is the same as pAHRG-H1 except that it contains the mutant H-ras<sup>Val-12</sup> instead of the wild-type H-ras. The construction of these plasmids has been described previously (Ballester et al., 1989).

### Construction of the *ira2<sup>-</sup>* Strain

The yeast strain IR-2.1 (*MATa his3 leu2 ura3 trp1 ade8 can1 ira2::URA3*) containing a disruption in the *IRA2* gene was constructed as follows: a BamHI DNA fragment from pYepIRA2 was inserted into pUC118 (Vieira and Messing, 1987), which had been digested with BamHI. The

resulting plasmid was digested with *Mlu*I and *Stu*I, filled in with Klenow fragment, and ligated with a filled-in *Hind*III fragment of the *URA3* gene. This results in the deletion of the 5' coding sequences of the gene including approximately 700 bases of the noncoding sequence. The resulting plasmid was used to carry out gene replacement experiments (Rothstein, 1983). A yeast haploid auxotroph, SP1, was transformed with the *Sal*I-digested plasmid containing the deleted *IRA2* gene, and transformants were selected by uridine prototrophy. Southern hybridization analysis was used to verify that the *IRA2* gene was replaced by the disrupted gene.

#### Heat Shock Sensitivity Assay

Heat shock sensitivity was determined as described previously (Toda et al., 1985; Sass et al., 1986). Heat shock was performed by replica plating cells to a plate preheated for 1 hr at 55°C. This plate was then incubated for 5 or 10 min at 55°C and transferred to 30°C for 2 days and photographed.

#### Preparation of Cell Lysates, GAP Assays, and Quantitation

Yeast strains were grown to saturation in selective media and harvested by centrifugation. Cell pellets were resuspended in buffer A (20 mM Tris-HCl [pH 7.5], 50 mM NaCl, 5 mM MgCl<sub>2</sub>) with 1% NP-40, 1 µg/ml each of aprotinin and pepstatin, and 1 mM phenylmethylsulfonyl fluoride. The cells were lysed by vortexing in Eppendorf tubes containing zirconium beads (Biospec Products). The lysates were centrifuged at 4°C for 2 min in an Eppendorf centrifuge, and the supernatant was used in the GAP assays.

GAP assays were performed according to Trahey and McCormick (1987). Purified H-ras p21 (1.5 µg) (Gross et al., 1985) was prebound to GTP by incubation for 30 min at 30°C in buffer B (20 mM Tris-HCl [pH 7.5], 20 mM NaCl, 2.5 mM MgCl<sub>2</sub>) with 3 µM GTP, 20 µCi of [ $\alpha$ -<sup>32</sup>P]GTP (3000 mCi/mmol; New England Nuclear), 1 mM ATP, and 1 mg/ml bovine serum albumin in a volume of 20 µl. The cell lysates, 0.4 mg in 20 µl, were incubated with 2 µl of p21 prebound to GTP for 30 min at room temperature. The samples were then diluted to 200 µl with buffer A and incubated for 30 min at 4°C with 2 µg of monoclonal antibody Y13-259 (Furth et al., 1982) followed by incubation with rabbit anti-rat immunoglobulin and protein A-Sepharose for 1 hr at 4°C. Immunoprecipitates were washed with buffer A, and the nucleotides were eluted from p21 by incubating with 10 µl of 1% SDS and 20 mM EDTA for 5 min at 65°C. Two microliters of eluted nucleotides was resolved by chromatography on polyethyleneimine impregnated cellulose plates in 1 M LiCl.

The chromatography plates were exposed for autoradiography using X-ray film and phosphor imaging plates for quantitation with the PhosphorImager from Molecular Dynamics. The PhosphorImager offers increased sensitivity over X-ray film, and the signal is linear in the range of 10 to 1 × 10<sup>7</sup> disintegrations per mm<sup>2</sup> (Johnston et al., 1990). The chromatography plates were exposed to the storage phosphor screens for 12 hr, and the plates were scanned using the PhosphorImager. The image generated was graphically divided into regions, and the disintegrations were analyzed using the ImageQuant software.

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