ras Gene Amplification and Malignant Transformation

SIMONETTA PULCIANI,[†] EUGENIO SANTOS,[‡] LINDA K. LONG, VINCENZO SORRENTINO,[§] AND MARIANO BARBACID^{*}

Developmental Oncology Section, Basic Research Program, Frederick Cancer Research Facility, Frederick, Maryland 21701

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Morphologic transformation of NIH 3T3 mouse cells occurs upon transfection of these cells with large amounts ($\geq 10 \mu\text{g}$) of recombinant DNA molecules carrying the normal human *H-ras-1* proto-oncogene. We provide experimental evidence indicating that transformation of these NIH 3T3 cells results from the combined effect of multiple copies of the *H-ras-1* proto-oncogene rather than from spontaneous mutation of one of the transfected *H-ras-1* clones (E. Santos, E. P. Reddy, S. Pulciani, R. J. Feldman, and M. Barbacid, *Proc. Natl. Acad. Sci. USA* 80:4679-4683, 1983). Levels of *H-ras-1* RNA and p21 expression are highly elevated in the NIH 3T3 transformants, and in those cases examined, these levels correlate with the malignant properties of these cells. We have also investigated the presence of amplified *ras* genes in a variety of human carcinomas. In 75 tumor biopsies, we found amplification of the human *K-ras-2* locus in one carcinoma of the lung. These results indicate that *ras* gene amplification is an alternative pathway by which *ras* genes may participate in the development of human neoplasia.

Cellular oncogenes can be generated by alterations that broadly fall into two categories, i.e., alteration of coding potential and increased levels of expression. Single-point mutations leading to substitution of critical amino acid residues in the *ras* gene-coded p21 proteins are sufficient to confer transforming properties to these oncogenes (21, 29, 30). Mutated *ras* oncogenes have been identified in about 15% of the most common forms of human cancers (for a review, see reference 32), as well as in a high percentage ($\geq 70\%$) of tumors in several carcinogen-induced animal tumor systems (4, 15, 28). Increased expression of otherwise unaltered proto-oncogenes is also a frequent mechanism of oncogene activation. Blair et al. first demonstrated that the mouse *mos* proto-oncogene can transform NIH 3T3 cells when placed under the control of retroviral regulatory elements (6). Similar results have subsequently been obtained with other proto-oncogenes such as *H-ras-1* (7, 9) and *sis* (8, 12).

Several proto-oncogenes, including *c-abl*, *c-myb*, *c-myc*, *N-myc*, *N-ras*, and *K-ras*, have been found to be amplified in a variety of tumor cell lines as well as in some primary neoplasias (for reviews, see references 1 and 32). In those cases examined, gene amplification was always associated with increased expression of the corresponding proto-oncogene product (1, 32). There are two caveats to firmly establishing that gene amplification is an alternative mechanism of oncogene activation. In addition to the oncogene locus, many other sequences are usually amplified in the tumor cells (2, 19, 24). Moreover, the tumorigenic properties of the amplified proto-oncogene cannot be directly assayed. In the present report, we demonstrate that the combined effect of multiple copies of the human *H-ras-1* proto-

oncogene induces malignant transformation of NIH 3T3 cells. We also report that amplification of *ras* genes can be observed in human tumors, although at a relatively low frequency.

Morphologic transformation of NIH 3T3 cells by the human *H-ras-1* proto-oncogene. It has been previously reported that the human *H-ras-1* proto-oncogene did not induce malignant transformation upon transfection of NIH 3T3 mouse cells (21, 29, 30). However, in those experiments, the amount of cloned proto-oncogene used never exceeded $1 \mu\text{g}$ of DNA per 2×10^5 transfected cells. We now report that under experimental conditions in which large amounts of cloned *H-ras-1* DNA were utilized to transfect NIH 3T3 cells, discrete areas of morphologic transformation could be observed after 16 to 20 days of incubation.

In a typical experiment, 15 to 30 μg of λ bc-N DNA, a recombinant Charon 4A λ phage that carries a 6.6-kilobase-pair (kbp) *Bam*HI DNA fragment containing the normal human *H-ras-1* locus (20), induced the appearance of 20 to 50 foci of morphologically altered cells per 2×10^5 transfected NIH 3T3 cells. Similar results were obtained with linearized pbc-N1, a pBR322 derivative in which this 6.6-kbp *Bam*HI DNA fragment was subcloned (20), as well as with the purified 6.6-kbp *Bam*HI DNA insert alone. No foci could be detected in plates transfected with 5 μg or less of λ bc-N or pbc-N1 DNAs. Thirty-two independent foci of morphologically altered NIH 3T3 cells were selected from four separate experiments in which cultures were transfected with either λ bc-N, pbc-N1, or the purified 6.6-kbp *Bam*HI fragment DNAs. Foci were picked with the aid of a cloning cylinder and grown in semisolid agar. These clonal cells exhibited a rather elongated morphology, were moderately refractile, and piled up in a parallel fashion instead of in the characteristic criss-crossing manner of NIH 3T3 cells transformed by the *H-ras-1* oncogene (Fig. 1).

Morphologically altered NIH 3T3 cells were analyzed for the presence of *H-ras-1* sequences. Clonal cells were grown to mass culture, and their DNAs were subjected to Southern blot analysis. Each of the 32 independent clones contained multiple copies of the human *H-ras-1* proto-oncogene. Quantitative dot blot analysis indicated a range of from 30 to more

* Corresponding author.

[†] Present address: Department of Virology, Istituto Superiore Di Sanita, 00161 Rome, Italy.

[‡] Present address: Laboratory of Molecular Microbiology, National Institute of Allergy and Infectious Diseases, Bethesda, MD 20205.

[§] Present address: Memorial Sloan-Kettering Cancer Center, Cornell University Graduate School of Medicine, New York, NY 10021.

FIG. 2. Number of integrated human *H-ras-1* gene copies in NIH 3T3 cells (A) transformed with 10 to 30 μ g of λ bc-N, pbc-N1, or the 6.6-kbp *Bam*HI DNA fragment that carries the normal human *H-ras-1* proto-oncogene, (B) morphologically normal, transfected with 10 μ g of pbc-N1 and 1 μ g of pSV2neo and selected in the presence of G418 (500 μ g/ml); and (C) transformed with 20 μ g of pT24-C3, a plasmid that carries the human T24 *H-ras-1* oncogene (23). Several dilutions of the corresponding genomic DNAs were dot blotted and fixed to nitrocellulose paper. Hybridization was performed as previously described (22), utilizing as 32 P-labeled probe the internal 3.0-kbp *Sac*I DNA fragment of the human *H-ras-1* proto-oncogene (23). The nitrocellulose filter was exposed to XAR Kodak film for 24 h in the presence of intensifier screens.

than 80 copies of the human *H-ras-1* locus per NIH 3T3 genome (Fig. 2). These results suggest that the phenotypic alteration of NIH 3T3 cells was the direct consequence of the integration of multiple copies of the *H-ras-1* proto-oncogene in their genomes. However, it could be argued that the observed morphologic alteration of NIH 3T3 cells was due to one or a few altered *H-ras-1* DNAs rather than to the combined affect of the multiple proto-oncogene copies. Three control experiments were designed to exclude this possibility. First, we cotransfected NIH 3T3 cells with pbc-N1 and pSV2-neo (25). G418-resistant NIH 3T3 colonies exhibiting a flat, normal morphology were selected, and their DNAs were subjected to dot blot and Southern blot analyses. Although the number of integrated *H-ras-1* proto-oncogene DNA copies varied, none of the clones contained more than 15 copies (Fig. 2).

In the second control experiment, we examined whether the consistently high copy number of *H-ras-1* proto-oncogenes in these NIH 3T3 clones was a requirement for the morphologic transformation of the clones or was a consequence of the large amounts of cloned DNA utilized in these experiments. NIH 3T3 cells were transfected with comparable amounts (20 μ g) of λ T24-15A (20) or linearized pT24-C3 (23). These recombinant molecules, derived from λ

FIG. 1. NIH 3T3 cells. (A) Normal; (B) transformed by multiple copies of the human *H-ras-1* proto-oncogene (clone 115-16); (C) transformed by a single copy of the human (T24) *H-ras-1* oncogene (clone 109-31). Transfection assays were carried out by the calcium phosphate precipitation technique (14).

TABLE 1. Properties of NIH 3T3 cells transformed by the normal *H-ras-1* proto-oncogene and its transforming allele, the *H-ras* oncogene

Cell line	Transfected <i>H-ras</i> gene	No. of gene copies	Tumorigenicity			Level of expression of:	
			Phenotype	Growth in soft agar	Tumor induction ^a	<i>H-ras</i> RNA ^b	p21 <i>ras</i> ^c
134-51	spontaneously activated oncogene ^d	1	Transformed	+	5/5	1× ^e	1× ^e
109-31	T24 oncogene	1	Transformed	+	5/5	10×	20×
115-11	Proto-oncogene	30	Transformed	+	3/10	20×	20×
115-14	Proto-oncogene	>50	Transformed	+	10/10	80×	50×
115-16	Proto-oncogene	>50	Transformed	+	10/10	80×	50×
136-41	Proto-oncogene	>50	Transformed	+	10/10	ND ^f	ND
NIH 3T3			Normal	—	0/10	0.3×	0.4×

^a NFS/N weanlings were subcutaneously injected with 10⁶ cells. Only nonregressing tumors were scored.

^b See Fig. 3 for a representative experiment.

^c Based on immunoprecipitation analysis of ³⁵S-labeled proteins. See Fig. 4 for a representative experiment.

^d See reference 23.

^e 134-51 cells are taken as reference because they contain the lowest level of oncogene expression of all NIH 3T3 transformants tested in our laboratory, thus representing the minimal amount of *H-ras-1* oncogene expression necessary to transform NIH 3T3 cells.

^f ND, Not determined.

Charon 9A and pBR322, respectively, carry the *H-ras-1* oncogene present in T24 bladder carcinoma cells. Both of these DNAs exhibit transforming activities of over 10⁴ focus-forming units per µg when assayed in NIH 3T3 cells. Twelve independent transformants were selected as indicated above and analyzed for the presence of human *H-ras-1* sequences. Each of these transformants contained a wide range of integrated DNA copies; however, only one transformant exhibited a high (≥30) copy number (Fig. 2). Finally, we ruled out the possibility that morphologically altered NIH 3T3 clones contained a mutated transforming *H-ras-1* locus by failing to transmit their transformed phenotype in additional cycles of transfection (22). These findings, taken together, established that the combined effect of multiple copies of a normal *H-ras-1* proto-oncogene was responsible for the morphologic transformation of NIH 3T3 cells.

Tumorigenicity of NIH 3T3 cells morphologically transformed by the human *H-ras-1* proto-oncogene. The malignant properties of NIH 3T3 cells containing multiple copies of the human *H-ras-1* proto-oncogene were examined (Table 1). Four representative NIH 3T3 clones containing about 30 (clone 115-11) or more (clones 115-14, 115-16, and 136-41) *H-ras-1* DNA copies were selected for this study. The injection of 10⁵ clone 115-11 cells led to tumor formation in 3 of 10 injected animals in two separate experiments. However, the injection of 10⁵ cells of the remaining three clones (115-14, 115-16, and 136-41) into newborn NIH Swiss mice led to the development of tumors at the site of injection followed by death in 100% of the animals injected (10 animals per cell line). No tumor induction was observed with morphologically normal NIH 3T3 clones containing several (1 to 10) copies of the *H-ras-1* proto-oncogene (Table 1). These results demonstrate the tumorigenic properties of NIH 3T3 cells morphologically transformed by multiple *H-ras-1* proto-oncogene copies.

Expression of the amplified *H-ras-1* proto-oncogene in transformed NIH 3T3 cells. To determine the mechanism by which the human *H-ras-1* proto-oncogene induces malignant transformation of NIH 3T3 cells, we examined the levels of transcription and translation of the *H-ras-1* sequences. Poly(A)-containing RNAs were extracted from representative transformants and subjected to dot blot analysis. As probe we used the 3.0-kbp *SacI* DNA internal fragment of the human *H-ras-1* locus which contains the four coding

exons and their corresponding intervening sequences (23). For comparison, we utilized NIH 3T3 cells transformed by a single copy of the *H-ras-1* oncogene. These cells exhibit a wide range of oncogene expression levels. We selected for this study the two NIH 3T3 transformants that expressed the lowest (134-51 clone; see reference 22) and highest (109-31 clone; see reference 20) levels of *H-ras-1* transcripts among those transformants previously characterized in our laboratory.

Transcription of *ras* gene sequences in NIH 3T3 cells transformed by the human *H-ras-1* proto-oncogene was directly proportional to the number of integrated copies (Fig. 3). Clone 115-11 cells, which contain about 30 copies of the *H-ras-1* proto-oncogene, exhibited high levels of poly(A)-containing *H-ras-1* RNA, about 2-fold higher than the high-expression 109-31 cells and about 20-fold higher than 134-51

FIG. 3. Northern blot analysis of human *H-ras-1* gene expression in NIH 3T3 cells transformed by the normal *H-ras-1* proto-oncogene and its transforming allele. Five micrograms of poly(A)-containing RNA (3) was extracted from each of the following: *H-ras-1* oncogene-transformed 134-51 cells (lane a); T24 *H-ras-1* oncogene-transformed 109-31 cells (lane b); *H-ras-1* proto-oncogene-transformed 115-16 (lane c), 115-14 (lane d), and 115-11 (lane e) cells; and control NIH 3T3 cells (lane f), and electrophoresed in 1.1% agarose gels containing 5 mM methyl mercury hydroxide, blotted to nitrocellulose filters (31), and hybridized to a ³²P-labeled probe obtained by nick translation of the 3.0-kbp *SacI* DNA fragment of pbc-N1 as previously described (23). Filters were exposed to XAR Kodak film for 24 h at -70°C in the presence of intensifier screens.

FIG. 4. p21 *ras* gene protein expression in NIH 3T3 cells transformed by the human *H-ras-1* proto-oncogene and its transforming allele. Exponentially growing cells (about 10^6 cells per immunoprecipitation reaction) including NIH 3T3 cells transformed by a single copy of human *H-ras-1* oncogenes (134-51 [lane a] and 109-31 [lane b]), NIH 3T3 cells transformed by multiple copies of the normal human *H-ras-1* proto-oncogene (115-16 [lane c], 115-14 [lane d], and 115-11 [lane e] cells), and NIH 3T3 control cells (lane f) were metabolically labeled with [35 S]methionine as previously described (5) and immunoprecipitated with 5 μ l of anti-p21 monoclonal antibody Y13-259 (11). Immunoprecipitates were collected with the aid of protein A bound to Sepharose 4B beads and disrupted in the presence of 1% sodium dodecyl sulfate–2% 2-mercaptoethanol at 95°C for 2 min. Solubilized proteins were analyzed by electrophoresis (18 h at 100 V) in sodium dodecyl sulfate–polyacrylamide (14%, wt/vol) gels. Electrophoresed gels were dried, fluorographed, and exposed to Kodak XAR films for 24 h at -70°C . Coelectrophoresed molecular weight markers included bovine serum albumin (69,000), ovalbumin (43,000), carbonic anhydrase (29,000), lactoglobulin (18,000), and lysozyme (14,000). Arrows indicate migration of the three types of p21 *ras* proteins immunoprecipitated in this experiment, including p21^{Asp12} (lane a) (23), p21^{Val12} (lane b) (21), and p21^{Gly12} (lanes c through f) (21).

cells (Table 1). Both clone 115-14 and 115-16 cells, which contain 80 to 100 copies of the *H-ras-1* proto-oncogene (Table 1), expressed even higher levels of *H-ras-1* RNA. In addition to the previously reported 1.2-kbp human *H-ras-1* RNA species, the *H-ras-1* proto-oncogene-induced NIH 3T3 transformants expressed a 3.0-kbp RNA species, presumably a precursor form (Fig. 3). This RNA species was not detected in the *H-ras-1* oncogene-transformed cells, including the 109-31 cell line which expressed almost as much 1.2-kbp RNA as did clone 115-11. Whether this apparently different RNA processing in the proto-oncogene-induced transformed NIH 3T3 cells had any biological significance remains to be determined.

Finally, we examined the levels of expression of p21

proteins. Postmicrosomal extracts of metabolically labeled cells were immunoprecipitated with monoclonal antibodies against the *H-ras-1* p21 protein (kindly provided by M. Furth; see reference 11). Again, a direct correlation between the levels of p21 expression and *H-ras-1* gene copy number was observed (Table 1). *H-ras-1* proto-oncogene transformants exhibit levels of p21 expression between 20-fold (115-11 cells) and 50-fold (115-14 and 115-16 cells) higher than do *H-ras-1* oncogene-transformed 134-51 cells (Fig. 4). The low levels of expression of transforming p21 protein in 134-51 cells (comparable to background levels present in control NIH 3T3 cells) illustrated that minimal amounts of mutated p21 molecules are sufficient to induce malignant transformation (Fig. 4). In contrast, the high levels of p21 expression present in all NIH 3T3 cells transformed by multiple copies of the *H-ras-1* proto-oncogene are additional evidence to support the concept that increased levels of a normal p21 protein can lead to malignant transformation.

Amplification of *ras* genes in human tumors. The demonstration that multiple copies of the human *H-ras-1* proto-oncogene can induce malignant transformation of NIH 3T3 cells prompted us to investigate whether *ras* gene amplification occurs in unmanipulated human solid tumors. A total of 75 human tumors or human tumor biopsies were analyzed (Table 2). DNAs were extracted from carcinomas of the lung (35 samples), colon (22 samples), rectum (9 samples), and bladder (9 samples). In only one case, a lung carcinoma, were we able to identify a significant amplification (50- to 60-fold) of the *K-ras* locus (Fig. 5). Parallel studies with DNAs isolated from preneoplastic tumors (13 colon polyps) as well as normal lymphocytes (36 samples) failed to reveal any *ras* gene amplified sequences (Table 2). These results indicate that *ras* gene amplification occurs in unmanipulated tumor biopsies and, therefore, is not a consequence of *in vitro* establishment of cell lines.

Biological implications of *ras* gene amplification. *ras* proto-oncogenes acquire the property of transforming cells in culture by single-point mutations within their coding sequences (21, 29, 30). However, quantitative changes in the

TABLE 2. *ras* gene amplification in human solid tumors

Source of DNA	No. of samples tested	<i>ras</i> gene amplification	
		No. of occurrences	Amplified locus
Normal lymphocytes	36	0	
Bladder carcinoma	9	0	
Colon polyps	13	0	
Colon carcinoma	22	0	
Lung carcinoma	35	1	<i>K-ras</i>
Rectal carcinoma	9	0

FIG. 5. *K-ras* gene amplification in a human lung carcinoma. DNAs (20 μ g) isolated from human normal lymphocytes (lanes a) and lung carcinoma no. 1888 (lanes b) were digested with *Eco*RI and subjected to Southern blot analysis. 32 P-nick-translated probes were derived from pHiHi 3 (10). These probes contained sequences corresponding to the first (A) and second and third (B) exons of the human *K-ras-2* locus. Nitrocellulose filters were exposed to Kodak XAR films for 6 h at -70°C with the help of intensifier screens.

expression of normal p21 *ras* gene-coded proteins also have marked biological consequences. Increased expression of normal *ras* p21 molecules, driven either by retroviral regulatory elements (7, 9) or, as shown in the present studies, by the combined effect of multiple copies of the normal *H-ras-1* proto-oncogene, leads to the morphologic transformation of NIH 3T3 cells. A limited agar growth of NIH 3T3 cells expressing elevated levels of normal *H-ras-1* p21 protein has also been reported by Taparowsky et al. (30). Increased expression of mutated (transforming) *ras* p21 proteins, achieved either by placing the *ras* oncogenes under the regulatory control of strong promoters (27) or by transduction into retroviruses (16, 18), allows these proteins to transform not only established cell lines, but also primary embryo cells. Increased expression of the normal p21 product also leads to immortalization of early-passage rodent cells (27). Thus, it appears as if *ras* genes will have a full range of biological effects depending on the progressive addition of quantitative and qualitative changes.

Spandidos and Kerr have observed increased levels of *ras* gene transcription in certain preneoplastic tumors (colon polyps) as well as in fully developed colon adenocarcinomas (26). More recently, Schlom and co-workers have identified enhanced p21 expression in the majority of human colon and mammary carcinomas (17). These observations might be interpreted as evidence for a possible involvement of increased expression of *ras* proto-oncogenes in the development of human malignancies, far beyond the 10 to 15% of cases in which transforming (mutated) *ras* genes have been identified by gene transfer assay. Whereas this interpretation should not be ruled out, we must keep in mind that increased *ras* gene expression has also been observed in regenerating rat liver (13). Thus, it is possible that the moderate increase in *ras* gene expression so frequently found in human tumors is a consequence of the active stage of cell proliferation of the tumor cells compared with the state of surrounding normal tissue. Involvement of quantitative changes of *ras* gene expression in the development of neoplastic processes must await careful definition of the threshold that separates those levels of *ras* p21 expression needed for normal cell proliferation from those that trigger malignant transformation.

Proto-oncogene amplification has been implicated in the development of several human malignancies (1, 32). Although the evidence is still circumstantial, it is very likely that increased amounts of normal proto-oncogene proteins may alter the basic regulatory controls of cell proliferation. In the case of the *ras* genes, low levels of a mutated protein appear to confer more malignant properties than do the combined effects of multiple copies of the normal proto-oncogene (Table 1; references 7 and 27). These observations may explain why *ras* genes are more frequently activated by point mutations than by gene amplification. Nevertheless, the presence of an amplified *K-ras* gene in Y1 mouse adrenocortical tumor cells (24), along with the present results obtained with human tumor biopsies, supports the concept that gene amplification is an alternative pathway by which *ras* genes can also induce malignant transformation.

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