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Dominant transformation by mutated human *ras* genes *in vitro* requires more than 100 times higher expression than is observed in cancers

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ABSTRACT **The gene-mutation-cancer hypothesis holds that mutated cellular protooncogenes, such as point-mutated proto-*ras*, "play a dominant part in cancer," because they are sufficient to transform transfected mouse cell lines *in vitro* [Alberts, B., Bray, D., Lewis, J., Raff, M., Roberts, K. & Watson, J. D. (1994) *Molecular Biology of the Cell* (Garland, New York)]. However, in cells transformed *in vitro* mutated human *ras* genes are expressed more than 100-fold than in the cancers from which they are isolated. In view of the discrepancy between the very low levels of *ras* transcription in cancers and the very high levels in cells transformed *in vitro*, we have investigated the minimal level of human *ras* expression for transformation *in vitro*. Using point-mutated human *ras* genes recombined with different promoters from either human metallothionein-IIA or human fibronectin or from retroviruses we found dominant *in vitro* transformation of the mouse C3H cell line only with *ras* genes linked to viral promoters. These *ras* genes were expressed more than 120-fold higher than are native *ras* genes of C3H cells. The copy number of transfected *ras* genes ranged from 2–6 in our system. In addition, nondominant transformation was observed in a small percentage (2–7%) of C3H cells transfected with *ras* genes that are expressed less than 20 times higher than native C3H *ras* genes. Because over 90% of cells expressing *ras* at this moderately enhanced level were untransformed, transformation must follow either a nondominant *ras* mechanism or a non-*ras* mechanism. We conclude that the mutated, but normally expressed, *ras* genes found in human and animal cancers are not likely to "play a dominant part in cancer." The conclusion that mutated *ras* genes are not sufficient or dominant for cancer is directly supported by recent discoveries of mutated *ras* in normal animals, and in benign human tissue, "which has little potential to progress" [Jen, J., Powell, S. M., Papadopoulos, N., Smith, K. J., Hamilton, S. R., Vogelstein, B. & Kinzler, K. W. (1994) *Cancer Res.* 54, 5523–5526]. Even the view that mutated *ras* is necessary for cancer is hard to reconcile with (*i*) otherwise indistinguishable cancers with and without *ras* mutations, (*ii*) metastases of the same human cancers with and without *ras* mutations, (*iii*) retroviral *ras* genes that are oncogenic without point mutations, and (*iv*) human tumor cells having spontaneously lost *ras* mutation but not tumorigencity.**

All directly oncogenic retroviruses carry dominant cancer genes, termed oncogenes (1–5). These oncogenes are genetic hybrids that consist of strong retroviral promoters linked to coding sequences transduced from cellular genes, termed protooncogenes (2, 6). Retroviral promoters enhance transcription of protooncogene coding regions at least 100-fold compared with those of cellular protooncogenes (7–11). This 100-fold higher transcription of viral oncogenes compared with native cellular protooncogenes is the key to their oncogenic function (2, 7, 11).

In view of the coding sequence that cellular protooncogenes share with retroviral oncogenes, it has been proposed that these cellular genes are convertible to dominant cancer genes by mutation, e.g., a point mutation of human proto-*ras* is thought to convert this gene to an oncogene (1, 6, 12). According to this gene-mutation-cancer hypothesis, or oncogene hypothesis, the cell contains "about 60 protooncogenes . . . ; each of these can be converted into an oncogene that plays a dominant part in cancer of some sort or another" (1). In the light of this hypothesis, numerous mutations of protooncogenes have been identified in cancer, above all in *ras* genes (1). But practically none of these mutations ever elevates the low native levels of protooncogene transcription including that of proto-*ras* (2, 11, 13–15).

The protooncogene-mutation-cancer hypothesis derives support from two kinds of observations: (*i*) Mutations of proto-*ras* ond other protooncogenes are more frequent in cancer than predicted from the spontaneous incidence of mutation (1, 16). (*ii*) Point-mutated proto-Harvey- and proto-Kirsten-*ras* DNA from various nonviral human and animal cancer cells is able to dominantly transform the mouse 3T3 cell line—just like the *ras*-containing Harvey and Kirsten sarcoma viruses do (1, 16). This appeared to be functional support for the oncogene hypothesis (17–19).

But other facts challenge the gene-mutation hypothesis.

Several experimental observations indicate that mutated *ras* genes are not necessary for cancer: (*i*) Typically only a minority of a given cancer contains a specific protooncogene mutation, as, for example, mutated proto-*ras.* The majority of histologically and clinically indistinguishable cancers lack *ras* mutations (2, 14–16, 20–23). (*ii*) Some cancer patients carry metastases with and without *ras* mutations (24, 25). (*iii*) Human cancer cells that have lost mutated *ras* remain tumorigenic (26). (*iv*) Retroviruses from which *ras* (7, 27–29), *src* (9, 10), and *myc*-oncogene mutations (8, 30) have been removed remain oncogenic. Thus point mutation of proto-*ras* is not necessary for carcinogenesis.

According to the literature the level of *ras* transcription in mouse 3T3 cells and rodent embryo cells transformed *in vitro* with mutated *ras* genes is about 100-fold higher than in untransfected cells (2, 14, 31–43). Likewise the level of *ras* transcription in animal tumors formed with synthetic retroviruses, carrying human proto-*ras* coding regions (28, 29, 44), is as high as that in tumors caused by Harvey sarcoma virus (HaSV) (7), the virus in which *ras* was originally identified (45). Thus dominant transforming function of mutated human *ras* genes *in vitro* appears to depend on 100-fold elevated transcription, which is not seen in the cancers from which these genes are isolated.

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Abbreviations: HaSV, Harvey sarcoma virus; LTR, long terminal repeat; SV40, simian virus 40; FN, fibronectin; MN, metallothionein.

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The elevated transcription of human proto-*ras* in cells transformed *in vitro* appears to derive from two sources: (*i*) Heterologous promoters acquired by recombination with either plasmid DNA, cellular DNA used as carrier in transfection, or retroviral or DNA tumor viral helper genes that must be added to *ras* genes to transform rodent embryo cells (2, 31, 37, 38, 40–43); and (*ii*) the introduction of multiple *ras* copies into the same cell (2).

Both concatenation and recombination of DNAs are known artifacts of transfection (46–49).

Thus enhanced *ras* transcription in cells transformed *in vitro* by transfection with mutated cellular *ras* genes is an artifact of the transfection assay (2, 11). It follows that the *in vitro* assays may create, via artificial overexpression, dominant transforming genes from point-mutated proto-*ras* genes that have no transforming function at their native levels of expression.

In view of the discrepancy between the low, normal levels of transcription of mutated *ras* genes in natural cancers (2, 11, 13–15) and the very high levels in cells transformed *in vitro*, we have investigated here which level of transcription is necessary for dominant transformation by point-mutated cellular *ras* genes. For this purpose we have synthetically recombined mutated human proto-*ras* coding regions with heterologous promoters from human metallothionein IIA (MN) and fibronectin (FN) (50, 51) and from retroviruses. And we have analyzed the ability of these recombinant human *ras* genes to dominantly transform the mouse C3H cell line upon transfection. It was found that efficient, dominant transformation depends on at least 100-fold enhanced *ras* transcription compared with the expression in the natural tumors from which the *ras* DNAs were isolated. This result calls into question the hypothesis that mutated, but normally expressed, human *ras* genes of natural cancers play a dominant role in carcinogenesis.

MATERIALS AND METHODS

Recombinant *ras* Plasmid Constructs. *pHa#8 (pLTR/v-ras).* pHa#8 is a pBR322-derived plasmid carrying an infectious HaSV flanked by redundant viral sequences extending from a proviral *Mst*II (4317) site 5′ of the 5′-long terminal repeat (LTR) and to a *Pvu*I (253) site 3′ of the 3′-LTR (6). This circularly permutated provirus was cloned into the *Eco*RI (4361) and *Pvu*II (2064) sites of pBR322 (52) (Fig. 1*B*, part 1).

pLTR/proto-ras^m. The coding region of the mutated human proto-Ha-*ras*-1 gene was derived from plasmid construct proto-ras^m (ATCC, no. 41000), which contains a 6.6-kb *Bam*HI–*Bam*HI *ras* fragment from the human T24 bladder carcinoma cell line mutated at *ras* codon 12 (Gly-12 to Val-12). A 2.35-kb *Cel*II–*Sac*I fragment containing the full-length T24 proto-Ha-ras^m coding sequence (exons 2 to 5) and the poly(A)-addition signal, but without the native cellular promoter of proto-Ha-*ras*, was cut from the proto-ras^m plasmid, blunt-ended, and ligated with a blunt-ended *Sac*II (940)–*Nco*I (3739) vector fragment from pHa#8. The resulting plasmid is termed pLTR/proto-ras^m (Fig. 1*B*, part 2).

pFN-SV40/proto-ras^m and pMN-SV40/proto-ras^m. The vectors carrying human FN and MN promoters (pFN-6 and pMN-10) were constructed by Shelley Blam (Lawrence Berkeley Laboratory, Berkeley, CA), by replacing the cytomegalovirus promoter of the pSV2CMV plasmid with either the human FN or human MN-IIA promoter (50, 51, 53) (Fig. 1*A*). These two vectors also contain, in the opposite transcriptional orientation, the neomycin-resistance gene (*Neo*^{R}) driven by the simian virus 40 (SV40) early promoter/enhancer region. The 2.35-kb *Cel*II–*Sac*I mutated proto-Ha-*ras* coding region was blunted and joined with *Eco*RI linkers, and inserted into the *Eco*RI site of pFN-6 and pMN-10 vectors. The resulting two plasmids were termed pFN-SV40/proto-ras^m (Fig. 1*B*, part 3) and pMN-SV40/proto-ras^m (not shown in Fig. 1) (53).

pFN/proto-ras^m and pMN/proto-ras^m. The SV40 promoter/enhancer region and neomycin coding region were deleted from plasmid vectors pFN-6 and pMN-10 with *Pvu*II digestion, resulting in plasmid vectors pFN-6/dSV40 and pMN-10/dSV40. The 2.35-kb *Cel*II–*Sac*I mutated proto-Ha-*ras* coding region joined with *Eco*RI linkers (see above) was inserted into the *Eco*RI site of pFN-6/dSV40 and pMN-10/dSV40 vectors to generate pFN/proto-ras^m (Fig. 1*B*, part 4) and pMN/proto-ras^m (not shown in Fig. 1) (53).

pFN/v-ras and pMN/v-ras. These two plasmids were made by the following strategy: the v-Ha-*ras* coding sequence between the *Bam*HI (409) and *Nae*I (5720) sites of pHa#8 was first cloned into the *Bam*HI (375) and *Nae*I (1283) sites of pBR322, followed by *Xba*I digestion to partially delete the LTR downstream of the v-Ha-*ras* coding region (corresponding to the *Xba*I sites of 2023 and 5359 in pHa#8). The resulting plasmid, called pBR322/v-ras/dx, then was cut in the pBR322

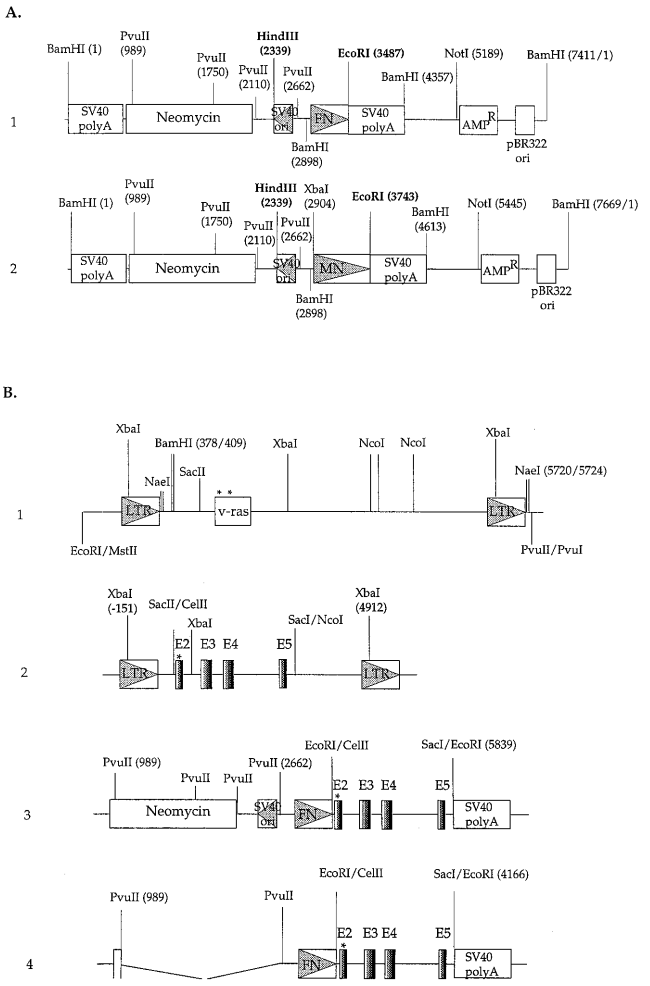

FIG. 1. (*A*) Genetic structures of plasmid vectors carrying the human FN promoter pFN-6 and the human MN promoter pMN-10. pFN-6 (part 1) and pMN-10 (part 2) are composed of DNA fragments from various sources: the origin (ori) of replication and the β-lactamase gene (Amp^R) of pBR322; the promoters of human FN or human MN genes; the neomycin-resistance gene; the origin of DNA replication of SV40 (SV40 ori) containing the SV40 early promoter/enhancer region, which is in the opposite transcriptional orientation to the FN or MN promoter; and the SV40 poly(A)-addition sequences. (*B*) Genetic structures of human proto-Ha-*ras* and v-Ha-*ras* constructs. Part 1, pLTR/v-ras (pHa#8), part 2, pLTR/proto-ras^m, part 3, pFN-SV40/proto-ras^m, and part 4, pFN/proto-ras^m. Construction of and origin of the complete plasmids are described in *Materials and Methods*. Three letter symbols identify restriction enzyme sites. Numbers following some v-*ras* restriction sites refer to the sequence position of HaSV (19). LTR is the retroviral promoter, and *ras* is the coding region of HaSV. E2, E3, E4, and E5 are the coding exons of human proto-Ha-*ras*.

sequence with *Eco*RI and *Nae*I to generate a 2,352-bp *ras*-containing fragment that was inserted into the pFN-6/dSV40 and pMN-10/dSV40 vectors. The vectors were prepared by cutting pFN-6/dSV40 and pMN-10/dSV40 with *Not*I, end-filling with Klenow enzyme, followed by digestion with *Eco*RI. The larger *Eco*RI–*Not*I vector fragments containing the FN or MN promoter were purified and ligated to the 2,352-bp *Eco*RI–*Nae*I v-Ha-*ras* insert, resulting in two new plasmids termed pFN/v-ras and pMN/v-ras (not shown in Fig. 1) (53).

RESULTS

Dominant Transformation with Mutated *ras* Coding Regions. To determine the role of expression on dominant, *in vitro* transformation by mutated proto-Ha-*ras* genes from human cancers, *ras* coding regions artificially recombined with promoters of varying strength were examined. The constructs tested included the mutated human proto-*ras* coding region linked with the promoters of HaSV (pLTR/proto-ras^m), of human FN with or without an added SV40 enhancer (pFN-SV40/proto-ras^m and pFN/proto-ras^m), or of human MN with or without an added SV40 enhancer (pMN-SV40/proto-ras^m and pMN/proto-ras^m). In addition constructs were tested in which the *ras* coding region of HaSV, naturally linked with promoters of HaSV (pLTR/v-ras), was linked with promoters of human FN (pFN/v-ras) and MN (pMN/v-ras) (Fig. 1, Table 1). About 10 μg of the plasmids carrying these *ras* constructs were transfected with 20 μg of salmon sperm carrier DNA and 1 μg of pSV2neo if the plasmids did not contain a neomycin resistance gene (47, 54).

Transforming function of these *ras* genes was tested in mouse C3H cells instead of 3T3 cells. Based on simultaneous working experience with both cell lines for more than 12 years (7, 27, 47), we have determined that spontaneous transformation of C3H cells is much lower than that of 3T3 cells. Transformed and untransformed C3H colonies (Fig. 2) appeared in the presence of 600 μg/ml geneticin G418 (a neomycin derivative) about 1–2 weeks after transfection as described previously (28, 53). The efficiency of morphological transformation by *ras* genes compared to the efficiency of conferring neomycin resistance by the neomycin-resistance plasmid varied from 80% for LTR-*ras* to <1% for FN-promoted proto-*ras* (Table 1).

Based on quantitative hybridization of total cellular RNA bound to nitrocellulose with ^{32}P-labeled 564-bp *Hin*dIII (1088)–*Dra*III (1650) v-Ha-*ras* probe, derived from HaSV-provirus pHa#8 (*Materials and Methods*), *ras* was expressed in transformants generated by LTR-promoted *ras* genes 120- to 200-fold higher than in untransfected cells (Table 1 and Fig. 3) (53, 55). By contrast, transformants generated by *ras* genes with cellular FN and MN promoters, even with additional SV40 viral enhancers, expressed *ras* only 10- to 20-fold higher than untransformed cells (Table 1).

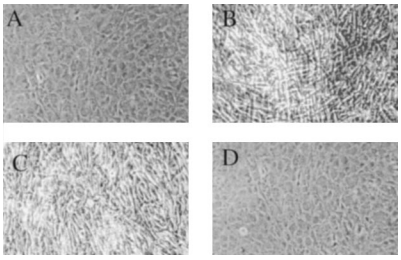

FIG. 2. Morphology of mouse C3H cells transfected with mutated human proto-*ras* and viral *ras* constructs. (*A*) Untransfected normal C3H cells. (*B*) pLTR/proto-ras^m-transformed C3H clone. (*C*) pFN-SV40/proto-ras^m-transformed C3H clone. (*D*) Proto-ras^m-transfected, untransformed C3H clone. (Cell pictures were taken at 100× magnification.)

Table 1. Survey of the structure, transforming efficiency, and transcriptional activity of various human, mutated proto-Ha-*ras*, and Harvey sarcoma viral-*ras* constructs under heterologous promoters

	Construct	% of transfected cells transformed	*ras* copy number	*ras* over-expression factor
1	LTR v-ras LTR	~80%	3	170-200
2	LTR * LTR	~11%	3	120
3	FN v-ras	2%	5	15
4	MN v-ras	1.8%	2-3	15
5	SV40 Enh. FN *	7%	4-6	20
6	SV40 Enh. MN *	5%	4	15
7	FN *	<1%	20/4-6	25/10
8	MN *	-	4-6	10
9	pRAS *	-	4	2

Constructs: 1, pLTR/v-ras (pHA#8); 2, pLTR/proto-ras^m; 3, pFN/v-ras; 4, pMN/v-ras; 5, pFN-SV40/proto-ras^m; 6, pMN-SV40/proto-ras^m; 7, pFN/proto-ras^m; 8, pMN/proto-ras^m; and 9, proto-ras^m.

The level of *ras* expression of untransfected C3H cells was too low to be detected by this method with total cell RNA (Fig. 3*A*). To estimate normal proto-*ras* expression, poly(A)-selected mRNA from untransfected C3H cells was compared with poly(A)-selected mRNA from cells transfected with mutated proto-*ras* (Fig. 3*B*). On this basis it was estimated that expression in untransfected cells is two times lower than in cells transfected with native, mutated *ras* genes from human cancers and 120–200 times lower than in cells transfected and transformed with *ras* genes with viral promoters (compare Fig. 3 *A* and *B*).

Nondominant *in Vitro* Transformation with Mutated *ras* Genes. To determine whether the untransformed, neomycin-resistant colonies that had been transfected with *ras* genes linked to cellular promoters had in fact not received *ras* DNAs, the genomic DNAs of several untransformed neomycin-resistant clones were analyzed. The presence of transfected *ras* DNA with FN and MN promoters, plus or minus SV40 viral enhancers, in one group of five neomycin-resistant cultures, of which three were transformed (T) and two were untransformed (NT), is shown in Fig. 4*A*, *Left*. The presence of transfected *ras* DNA in another group of six clonal cultures, all of which were untransformed, is shown in Fig. 4*A*, *Right*. In each untransformed (NT) culture either the predicted 3.8-kb *Bam*HI-resistant fragment from the proto-*ras* plasmids containing the FN promoter or the predicted 4.07-kb *Bam*HI-resistant fragment from the proto-*ras* plasmids containing the MN promoter was detected. The predicted *ras* fragments are made up from the central 2.35-kb human proto-*ras* region flanked by *Cel*II and *Sac*I, and about 1.5 kb of pFN or pMN Bam-flanked vector elements (Fig. 1*A*). Based on the radiographic intensities there were about 4–6 copies of transfected DNA per each C3H genomic proto-*ras* copy of 3.4 kb, regardless of whether the cell was transformed or untransformed (Fig. 4*A*, *Right*). However, one transformed colony carried about 20 copies of human proto-*ras* under a FN promoter, pFN/proto-ras^m (Fig. 4*A*, lane 5). Barring this one exception, there was no difference between transformed and untransformed cells with regard to the presence and even copy number of *ras* genes with cellular promoters.

Thus differential expression was theoretically the only variable left for *ras* to exert a dominant role in transformation. But because the copy numbers of transfected *ras* genes in trans-

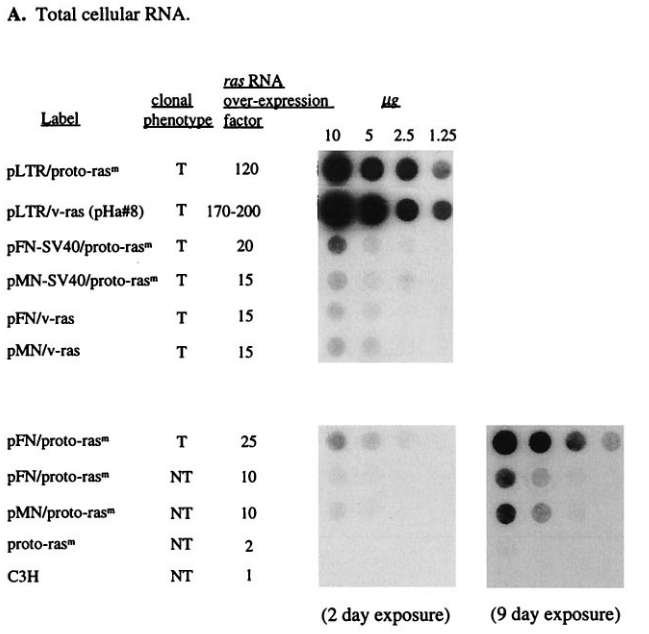

FIG. 3. Expression level of *ras* RNA in mouse C3H cells transfected with mutated human proto-*ras*- and HaSV-derived constructs. Total cellular RNA (*A*) or poly(A)-selected mRNA (*B*) was isolated with the guanidinium isothiocyanate method (55) from transformed (T) and nontransformed (NT) clonal cultures of G418-resistant C3H cells transfected with either proto-*ras* or viral *ras* constructs or from untransfected C3H control cells. The RNA was quantitated by spectrophotometry, equal amounts of RNA were dotted onto nitrocellulose membranes for each dilution and hybridized with the ^{32}P-labeled *ras*-DNA probe described in the text. The blot in the lower left of *A* shows no detectable *ras* expression in untransfected C3H control cells and in proto-ras^m transfected C3H cells after 2 days of exposure. After exposure for 9 days, the same blot showed barely detectable levels of *ras* expression in proto-ras^m-transfected C3H cells, but not in untransfected C3H control cells (*A*, *Lower Right*).

formed and untransformed cells were the same, and because the input promoters were also the same, the chances for significant differences in expression were small. Nevertheless, *ras* expression was tested in four transformed and 12 untransformed clonal cultures transfected with proto-*ras* or viral *ras* under human cellular promoters, e.g., those of MN, FN, or proto-*ras* itself.

Indeed, Fig. 5 shows that *ras* expression was the same both in transformed and untransformed cells transfected by the same *ras* construct. Although expression levels in cultures transfected with *ras* genes linked to cellular promoters exceeded normal C3H *ras* expression levels up to 20-fold (Fig. 5), they were 5–10 times lower than in cells transfected with dominantly transforming viral LTR-linked *ras* genes (Fig. 3). Fig. 5 also shows that *ras* expression of nontransformed control cells, transfected with *ras*-free plasmid vectors, was not elevated.

Because only a small percentage of cells expressing transfected *ras* at relatively low levels are transformed (Table 1), the corresponding *ras* constructs are not sufficient, and thus not dominant transforming genes.

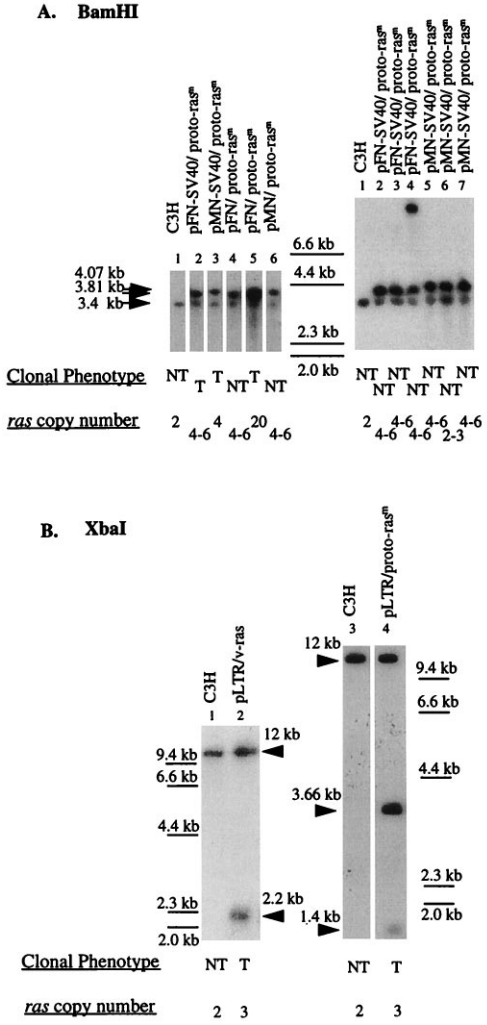

FIG. 4. Identification of transfected and endogenous *ras* DNAs in G418-resistant C3H cells transfected with proto-Ha-*ras* or v-Ha-*ras* under human FN or MN promoters. About 20 μg cellular DNAs were digested with *Bam*HI (*A*) or *Xba*I (*B*), resolved on a 0.8% agarose gel, transferred to positively charged nylon membrane by the alkaline transfer method (55), and hybridized with ^{32}P-labeled *ras* DNA from HaSV (see text). The positions of *Hin*dIII-resistant fragments of lambda DNA were used as molecular weight standards and are indicated between the two filters in *A* and on each side of the two filters in *B*. (*A*, *Left*) Lane 1, untransfected C3H; lane 2, pFN-SV40/proto-ras^m-transformed C3H clone; lane 3, pMN-SV40/proto-ras^m-transformed C3H clone; lane 4, pFN/proto-ras^m-transfected, untransformed C3H clone; lane 5, rare pFN/proto-ras^m-transformed C3H clone; and lane 6, pMN/proto-ras^m-transfected, untransformed normal C3H clone. (*A*, *Right*) Lane 1, untransfected C3H; lanes 2–4, pFN-SV40/proto-ras^m-transfected, untransformed C3H clones 1–3; and lanes 5–7, pMN-SV40/proto-ras^m-transfected, untransformed C3H clones 1–3. The 3.4-kb *Bam*HI-resistant *ras* fragment represents the genomic proto-Ha-*ras*-1 gene of C3H mice. The 4.07-kb (*A*, *Left*, lanes 3 and and *A*, *Right*, lanes 4–7) and 3.81-kb (*A*, *Left*, lanes 2, 4 and 5 and *A*, *Right*, lanes 2–4) *Bam*HI-resistant *ras* fragments are diagnostic of the plasmids pFN-SV40/proto-ras^m and pMN-SV40/proto-ras^m, respectively (see Fig. 1). (*B*) Lane 1, untransfected C3H; lane 2,: pLTR/v-ras (pHa#8)-transformed C3H; lane 3, untransfected C3H; and lane 4, pLTR/proto-ras^m-transformed C3H. The ≈12-kb *Xba*I fragment represents the genomic mouse proto-Ha-*ras*-1 gene cut with *Xba*I. The 2.2-kb *Xba*I-resistant *ras* fragment (lane 2) is diagnostic of the pLTR/v-ras plasmid; and the 3.66-kb and 1.4-kb *Xba*I-resistant *ras* fragments (lane 4) are diagnostic of the pLTR/proto-ras^m plasmid.

Role of *ras* Copy Number on Transformation of C3H Cells. The copy number of transfected *ras* genes in transformed cells was 2- to 6-fold higher than that of the endogenous proto-Ha-*ras* standard of C3H cells (Fig. 4 and Table 1). For example, cells

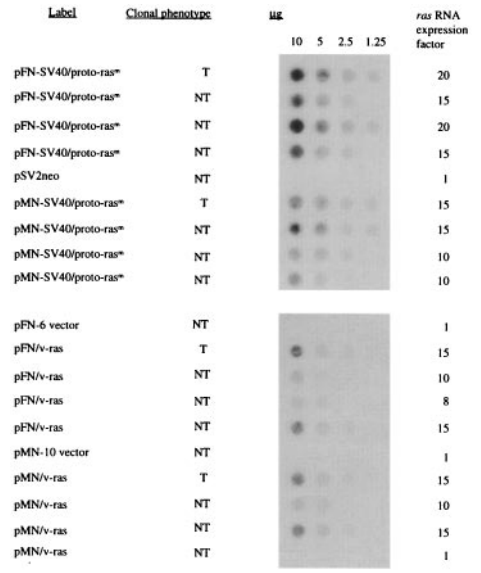

FIG. 5. Expression level of *ras* RNA in transformed and untransformed, G418-resistant clonal cultures of mouse C3H cells that were transfected with *ras* constructs in which human proto-Ha-*ras*- and viral *ras*- coding regions are linked to human MN and FN promoters. Equal amounts of RNA were dotted onto nitrocellulose membranes for each dilution and hybridized with the ^{32}P-labeled *ras*-DNA probe as described in Fig. 3.

transformed by mutated human proto-*ras* linked to the promoter of HaSV (pLTR/proto-ras^m) contained about four copies of the 3.66-kb *Xba*-resistant fragment of transfected pLTR/proto-ras^m per genomic *ras* (Fig. 4*B*, lane 4). Likewise cells transfected with cloned HaSV DNA (pLTR/v-ras) contained about four copies of a 2.2-kb *Xba*-resistant fragment of that plasmid (Fig. 4*B*, lane 2; other data not shown). Cells transfected by mutated human proto-*ras* linked to FN or MN promoters (pFN and MN/proto-*ras*^{m}) also contained 2–6 *ras* copies regardless whether they were transformed or untransformed (Fig. 4*A* and Table 1). It follows that the level of *ras* expression in *in vitro* transfection is increased not only by artificial promoters, but also by the copy number of transfected *ras* genes.

DISCUSSION

Dominant Transformation *in Vitro*. Our results show that dominant transformation of C3H mouse cells by point-mutated human proto-*ras* genes requires at least 100-fold overexpression, compared with the levels observed in untransfected C3H cells. This overexpression is achieved in our system by two factors: artificial retrovirus-derived promoters and artificially increased copy numbers of transfected *ras* genes. Surprisingly, not even the strong cellular promoters of human FN and MN were sufficient by themselves to confer dominant transforming function to mutated human proto-*ras* genes (Table 1).

Our evidence for the essential role of overexpression in dominant transformation with mutated proto-*ras* genes confirms and extends the results obtained by transfection of mouse 3T3 cells (2, 14, 32–36), and rodent embryo cells (2, 31, 37, 38, 40–43), and by infection of animals with *ras*-containing retroviruses (28, 29, 44). Cells previously transformed by transfection *in vitro* also contained multiple transfected *ras* genes (34, 35, 38, 39, 56). Thus earlier studies confirm overexpression and overdosage of *ras* genes in cells transformed by transfection, but considered point mutation as the critical requirement for dominant transformation, rather than enhanced expression. However, experiments by ourselves and others with proto-*ras* genes whose natural mutations had been reverted *in vitro* have shown since 1986 that point mutations are not necessary for transforming function of *ras* genes (7, 27–29).

Nondominant Transformation *in Vitro*. Only 2–7% of C3H cells transfected with recombinant *ras* genes driven by cellular FN or MN promoters were transformed. Unexpectedly, these cells expressed *ras* at the same level as untransformed cells transfected with the same *ras* constructs. These *ras* constructs were expressed less than 20-fold over the level of untransfected C3H cells. Because 93–98% of cells expressing this level of *ras* were untransformed, transformation of a few cells must have followed one of two nondominant mechanisms: cooperation of *ras* with another mutated gene or *ras*-independent transformation. However, this nondominant transformation is not necessarily relevant to the hypothetical role that point-mutated proto-*ras* genes play in cancer, because transfection with recombinant *ras* genes, particularly those that include an SV40 virus enhancer, may artificially activate unknown cellular genes via downstream promotion.

Relevance of Point-Mutated *ras* to Natural Carcinogenesis. Because mutated *ras* genes are not overexpressed and are not amplified in most human and animal cancers (see above), and because dominant transformation by mutated *ras* genes depends on at least 100-fold overexpression, we conclude that point-mutated *ras* genes do not "play a dominant part in cancer" (1).

Several experimental and theoretical arguments support the conclusion that mutated proto-*ras* is not dominant in, or sufficient for, natural carcinogenesis.

Point-mutated proto-*ras* genes have been identified in normal animals (57, 58) and in benign human tissue "which has little potential to progress" (59).

As demonstrated here, point-mutated human proto-*ras* with native human and heterologous human FN and MN promoters do not transform C3H mouse cells.

All humans can be calculated to contain at least 10^5 cells with mutated proto-*ras* genes. This calculation is based on the facts that the spontaneous mutation rate of human and nonhuman cells is 1 in 10^9 nucleotides per cell division (1, 2, 11, 16), and that humans and most mammals contain about 10^9 nucleotides per cell (1, 2, 11). Thus 1 in 10^9 cells contains a mutation in any specific nucleotide of the human genome. Because humans contain about 10^{14} cells, each person contains at least 10^5 cells with point-mutated proto-*ras*. Because several point mutations convert *ras* genes to 3T3 cell-transforming genes, and because there are several families of *ras* genes (1, 6), even more than 10^5 cells should be cancer cells at any given time if point-mutated *ras* genes were dominant cancer genes.

In view of this it has been argued that point-mutated proto-*ras* genes are not sufficient, but necessary for carcinogenesis, and that carcinogenesis depends on the cooperation of multiple mutated genes (20, 31). However, according to several studies by ourselves and others point mutation of proto-*ras* is not even necessary for carcinogenesis.

We have shown that oncogenic retroviruses from which *ras* (7, 27–29), *src* (9, 10), and *myc*-oncogene mutations (8, 30) have been removed remain oncogenic.

Others have shown that typically only a minority of a given cancer contains a specific protooncogene mutation, as, for example, mutated proto-*ras*. The majority of histologically and clinically indistinguishable cancers lack *ras* mutations (2, 14–16, 20–23).

Some cancer patients have metastases with and without *ras* mutations (24, 25).

Human cancer cells that have lost mutated *ras* remain tumorigenic (26).

Moreover it is unlikely that the same *ras* protein that plays a dominant, or sufficient, role in transformation by transfection and by retrovirus infection, also would function as a cofactor of other gene(s) in a completely different mechanism of carcinogenesis. Thus mutated proto-*ras* under its native

promoter, as it is in most human and animal cancers, appears neither sufficient nor necessary for carcinogenesis.

Finally, it appears that the dominantly transforming host range even of highly expressed retroviral oncogenes, including *ras* genes, does not include human cells (62).

This leaves open the possibility that point-mutated, normally expressed, *ras* genes play an indirect role in cancer. For example, mutated proto-*ras* could stimulate cell proliferation, and enhanced proliferation is a known cancer risk (60, 61). This hypothesis would provide a plausible explanation for the unexpectedly high, but not consistent, presence of *ras* mutations in certain cancers.

Perhaps aneuploidy, which according to Hollstein *et al.* (63) is "almost always found in human cancers," including those with mutated *ras* genes, is the cause of these cancers (11).

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