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New Human Transforming Genes Detected by a Tumorigenicity Assay

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We have developed a sensitive bioassay for transforming genes based on the tumorigenicity of cotransfected NIH3T3 cells in nude mice. The assay differs substantially from the NIH3T3 focus assay. Using it, we have detected the transfer of three transforming genes from the DNA of MCF-7, a human mammary carcinoma cell line. One of these is *N-ras*, which is amplified in MCF-7 DNA. The other two, which we have called *mcf2* and *mcf3*, do not appear to be related to known oncogenes. We cannot detect their transfer by using the NIH3T3 focus assay. We do not yet know whether either *mcf2* or *mcf3* is associated with genetic abnormalities in MCF-7 cells.

The transfer of genomic DNA into NIH3T3 cells has led to the discovery that genes present in some tumor cells are capable of inducing foci of morphologically transformed NIH3T3 cells (24, 27, 31, 33, 40). Most of the transforming genes which have been detected this way have now been identified as members of the *ras* gene family, either H-, K-, or *N-ras* (8, 17, 29, 33, 36, 43). The transforming *ras* genes detected by the NIH3T3 focus assay have structural gene mutations that account for their transforming activity (34, 48, 50, 51, 55). Since the great majority of tumor DNAs tested fail to induce transformed foci (24, 31, 33), we have speculated that the focus assay has a bias for *ras* genes containing structural mutations. Therefore, we have begun a series of experiments to explore alternative assays for transforming genes present in NIH3T3 cells after DNA transfer. We describe here some preliminary results with an assay system that detects new oncogenes. The system is a modification of the one described by Blair and co-workers (2). Like theirs, our assay also relies on the ability of transformed NIH3T3 cells to form tumors in nude mice, but incorporates methods of cotransfection (54) to heighten sensitivity. Using this assay, we have transferred three human transforming genes from the DNA of MCF-7, a human breast carcinoma cell line. One of these is *N-ras*. The other two appear to be unrelated to previously known transforming genes. The *N-ras* gene is amplified in MCF-7 cells but does not appear to contain structural mutations. We have not yet established whether the other two genes, which we have called *mcf2* and *mcf3*, are associated with any genetic abnormality in MCF-7.

MATERIALS AND METHODS

Preparation and source of DNA. High-molecular-weight DNA from various cell lines was prepared as described previously (31). MCF-7 was from the Human Tumor Cell Line Bank of the Sloan Kettering Institute, Rye, N.Y. (44). Preparation of DNA from solid tumors was done as follows. Tumors (1 to 2 g of frozen tissue) were finely ground by using a pestle in a mortar containing liquid N₂. The resulting powder was added to 40 ml of 400 mM NaCl-10 mM EDTA-20 mM Tris-hydrochloride (pH 7.5)-0.5% sodium dodecyl sulfate prewarmed at 68°C and incubated at this temperature

for 20 min. An additional incubation at 37°C in the presence of proteinase K at the final concentration of 200 µg/ml was carried out with very slow agitation for 24 to 48 h. Phenol extraction and subsequent steps were as described previously (31). Plasmid and phage DNAs were prepared by published methods (21, 49). Many plasmids that were used in this study were generously provided by the following scientists: pKOneo, D. Hanahan; psrc-11, G. Cooper; pBR-Y73, N. Kitamura; pAB-sub3, S. Goff; pGA-FeSV, C. Sherr; pRCII-1B, H. Hanafusa; pBR-UR2, H. Hanafusa; pAE-PvuII, M. Bishop; pHB-11, E. Scolnick; pKBE-2, E. Scolnick; pm-1, D. Blair; pMCV38, M. Bishop; pVM-2, M. Bishop; pFBJ-2, I. Verma; pSSV-11, S. Aaronson; pREV-T3, H. Temin; pvski-1, E. Stavnezer; pMT-2.5, H. Varmus; pSM-FeSV, C. Sherr; and blur 8, C. Schmid. Phage λ *myc4.1.1* was from M. Cole.

DNA transfections and nude mice assay. All DNA transfers used a modified calcium phosphate precipitation method (15, 54) with NIH3T3 cells as recipients (19, 31). Focus assays were performed as previously described (31). For the nude mouse experiments, 30 µg of cellular DNA plus 300 ng of pKOneo plasmid, constructed by D. Hanahan (unpublished data), were added as a calcium phosphate precipitate (volume, 1 ml) to each 100-mm culture dish containing 5 × 10⁵ cells in 10 ml of Dulbecco medium and 10% calf serum (D10). After incubation for 8 h, the precipitate was removed and replaced by 10 ml of fresh D10. After an additional 6 h, the cells in each plate were trypsinized and seeded into four 100-mm dishes containing D10 plus 0.4 g of the antibiotic G418 (GIBCO Laboratories) per liter (as calculated for 100% active antibiotic). This time schedule is critical, since the antibiotic efficiently selects cells expressing the resistance marker only when seeding is done at low cell density. Refeeding of the cultures was done at intervals of 3 days, using the same medium. The plates, each containing about 300 colonies, reached confluency after 16 to 18 days. At this time, the four plates of cells were trypsinized, pooled, centrifuged at 700 rpm for 5 min, and resuspended in 0.25 ml of D10. Approximately 10⁷ cells were injected subcutaneously over the right shoulders of athymic, 4- to 8-week-old male or female Nu-Nu mice of Rex-Trembler origin which had been back-crossed to Swiss three times. One deviation from this protocol is described in the footnote to Table 1.

Tumor growth was monitored weekly, and the mean

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diameter was calculated as the cubic root of the product of its three major diameters. Before death from tumor growth, the mice were sacrificed, and the tumor tissue (1 to 3 g) was frozen in liquid N₂ to obtain DNA at a later time.

Southern filter-blot hybridization. DNA samples were digested with restriction endonucleases and subjected to agarose gel electrophoresis and filter-blot transfer by the method of Southern (45). Filter-blot DNAs were hybridized with nick-translated probes (26) under stringent conditions described previously (43). Nonstringent hybridization conditions entailed use of essentially the same components at 60°C instead of 74°C. When total human or mouse DNA was nick translated and used as a probe, its concentration during hybridization was 0.03 µg/ml. For hybridization with *ras* probes, we nick translated these DNAs: plasmid p6aI, a partial cDNA clone of the human *N-ras* gene (50); the 3.0-kilobase-pair (kbp) *EcoRI* fragment containing coding exon II of the human *K-ras* gene (41); and the 0.7-kbp *NarI-AvaI* fragment from plasmid RS6 (11), a cDNA clone of the human *H-ras* gene containing most of the coding sequences.

Isolation of *mcf1*, *mcf2*, and *mcf3*. Genomic libraries of MCF-7 DNA and the secondary tumor DNAs (MCF-7-1-1, MCF-7-2-2, and MCF-7-3-4) were constructed in λ charon 4a (4, 18) from DNA partially digested with *EcoRI* and fractionated over a sucrose gradient as described previously (31). To obtain clones of the *mcf* genes, we screened 5 × 10⁵ phages from libraries of secondary tumor DNAs for human sequences by the method of Benton and Davis (1) using nick-translated human placental DNA as a probe (16). To obtain *mcf* clones from MCF-7 DNA, we screened that library with repeat-free fragments of the respective genes. The filter hybridization was performed in 6 × SSC-1 × Denhardt (1 × SSC is 0.15 M NaCl plus 0.015 M sodium citrate) at 68°C for 20 h followed by five washings in 2 × SSC at 68°C.

RESULTS

Assays with MCF-7 DNA. In brief, the new assay is performed as follows. NIH3T3 cells are cotransfected with cellular DNA and DNA of the pKOneo plasmid, which renders animal cells resistant to the antibiotic G418 (46). In

each experimental group, about 10³ G418-resistant colonies containing about 10⁴ cells are present after 2 to 3 weeks. These are pooled and injected into one nude mouse. The time of the first appearance of tumors and their subsequent growth is noted. As a positive control, one set of mice is inoculated with NIH3T3 cells exposed to genomic DNA from a1-1, an NIH3T3 transformant containing a high copy number of the transforming human *H-ras* gene of T24 bladder carcinoma cells (14, 31). As a negative control, one set of mice is inoculated with cells exposed to genomic DNA from NIH3T3 cells. Mice are maintained for up to 3 months.

Our first experiments with the tumorigenicity assay were performed with DNA from a human breast carcinoma cell line, MCF-7. Lane et al. had previously reported MCF-7 DNA to be positive in the NIH3T3 focus assay (25). However, we had consistently failed to observe any induced foci when using this DNA as donor. Nevertheless, we did obtain positive results using MCF-7 DNA in the nude mouse assay (Tables 1 and 2). In two experiments, three independent tumors were obtained. There was a low tumor incidence in the negative control group (see tables) as well as in mice injected with NIH3T3 cells exposed to human placental DNA (data not shown). There is, therefore, a background of tumor induction when testing normal DNA, a point to which we will return later. With MCF-7 DNA, as with many other tumor DNAs we have since examined, tumor incidence was higher than with control DNAs, but this may not be statistically significant. We therefore do not assume that the positive results with MCF-7 DNA reflect a genetic abnormality in MCF-7 cells. The assay is, however, clearly responsive to high copies of strongly transforming genes since tumor incidence is markedly increased with the positive control groups, consisting of nude mice injected with NIH3T3 exposed to DNA from a1-1.

We call tumors resulting from cells exposed to MCF-7 DNA primary tumors MCF-7-1, MCF-7-2, and MCF-7-3. DNAs prepared from the primary tumors were used again in the tumorigenicity assay to obtain secondary tumors. The lineages of the relevant tumors are given in Table 3. In each case, DNA from the primary tumor was significantly more

TABLE 1. Tumorigenicity assay of MCF-7 DNA^a

Donor DNA	Group	Mean tumor diam (mm) on day:								Tumor designation
		14	21	28	35	42	49	56	63	
NIH3T3	A	0	0	0	0	Tr	14.5	17.0	20.0	
		0	0	0	0	Tr	6.8	3.4	0	
		0	0	0	0	Tr	6.6	0	0	
MCF-7	B	0	9.1	18.8	23.3					MCF-7-1a
		0	Tr	14.6	22.3					MCF-7-1b
		0	Tr	12.2	21.5					MCF-7-1c
		0	Tr	8.4	9.2	18.4				MCF-7-1d
		0	Tr	13.6	18.3					MCF-7-1e
		0	Tr	9.0	16.7					MCF-7-1f
		0	Tr	6.6	13.6					MCF-7-1g
a1-1	C	21.9								
		20.2								
		15.1								

^a Tumorigenesis assays were performed essentially as described in the text. NIH3T3 cells were exposed to both pKOneo and DNA from the indicated cell line and then selected for G418 resistance. Two weeks later, surviving colonies were trypsinized, pooled, and injected into animals. Each group represents a pool of G418-resistant cells. Each line represents a single nude mouse injected with ca. 5 × 10⁶ cells from the equivalent of four plates. a1-1 is an NIH3T3 transformant with a high copy number of a transforming *H-ras* gene (14, 31). MCF-7 is a human breast cancer cell line (44). Tumors designated MCF-7-1a through MCF-7-1g all derived from the same initial transformed NIH3T3 cell since they display identical Southern blot patterns when probed from human repeat sequences (data not shown). This is consistent with their origin from a single pool of transformants. These tumors are referred to collectively as MCF-7-1. In subsequent experiments, we avoided this problem by injecting each mouse with an independent pool of transformed cells. See text for more details.

TABLE 2. Tumorigenicity assay of MCF-7 and primary tumor DNAs^a

Donor DNA	Group	Mean tumor diam (mm) on day:								Tumor designation
		13	20	27	34	41	48	55	61	
NIH3T3	A1	0	0	0	0	0	0	0	0	
	A2	0	0	0	0	0	0	0	0	
	A3	0	0	0	0	0	0	0	0	
	A4	0	0	0	0	0	0	0	0	
	A5	0	0	0	0	0	0	0	0	
	A6	0	0	0	0	0	0	0	0	
	A7	0	0	0	0	0	0	0	0	
MCF-7	B1	0	0	0	0	0	0	0	0	
	B2	0	0	0	0	0	0	0	0	
	B3	0	0	Tr	9.7	12.1	15.4			MCF-7-2
	B4	0	0	0	0	0	0	0	0	
	B5	0	0	0	0	0	0	0	0	
	B6	0	0	0	0	0	0	0	0	
	B7	0	0	0	Tr	14.7	22.4			MCF-7-3
MCF-7-1	C1	0	0	0	Tr	6.3	10.6	18.6		MCF-7-1-2
	C2	0	0	0	0	0	Tr	6.6	12.1	MCF-7-1-7
	C3	0	0	0	0	Tr	8.1	15.4		MCF-7-1-3
	C4	0	0	0	5.1	11.0	17.2			MCF-7-1-1
	C5	0	0	0	0	0	5.8	10.4	9.6	MCF-7-1-5
	C6	0	0	0	0	Tr	7.9	16.5		MCF-7-1-4
	C7	0	0	0	0	0	4.5	7.4	12.5	MCF-7-1-6
a1-1	D1	14.4	22.8							
	D2	10.2	20.8							
	D3	13.1	25.9							

^a See Table 1, footnote a, and the text. Each group represents an independent pool of transformants and each line a nude mouse inoculated with ca. 10⁷ cells.

active at inducing tumorigenic NIH3T3 than the original MCF-7 DNA itself (see, for example, Table 2). This probably results from the amplification of the transforming gene in primary transformants (see below). Despite their higher activity in the nude mouse assay, DNAs from primary tumors still cannot induce foci of morphologically altered NIH3T3 cells. This reflects, we think, an intrinsic difference between the present assay and the focus assay.

DNAs from primary and secondary tumors were prepared and analyzed by Southern blotting for the presence of human repetitive sequences (Fig. 1). This powerful method allows the comparison of our putative transforming genes before their actual isolation (27, 31). All secondary tumors derived from the same primary tumor DNA contained a common set of human repetitive sequences. However, these sets differed among secondary tumors derived from different primary tumor DNAs. From this observation we conclude that the nude mouse assay appears to detect three different transforming genes in the DNA of MCF-7 which we called *mcf1*, *mcf2*, and *mcf3*. This conclusion was confirmed by subsequent experiments, described below.

Relation of *mcf* genes to the known *ras* genes. DNAs from the three primary tumors were analyzed for the presence of the three human *ras* genes, N-, H-, and K-*ras*, by Southern analysis. Neither MCF-7-2 nor MCF-7-3 contained these genes. Later studies showed that indeed the *mcf2* and *mcf3* genes are not even weakly related to members of the *ras* gene family (see below).

DNA from MCF-7-1, however, contained 30 to 100 copies of the human N-*ras* gene (Fig. 2). Moreover, all secondary tumors derived from MCF-7-1 DNA contained a similar number of copies of the human N-*ras* gene. Therefore, *mcf1* was N-*ras*. Although *EcoRI* fragments with homology to N-

ras are seen in the MCF-7-1 secondary DNAs, they are not the expected size. We cloned the N-*ras* gene from one of these DNAs (MCF-7-1-1) and analyzed its structure. It contained the 9.2-kbp fragment, which corresponds to the intact 5' end of the N-*ras* gene (50), but had undergone rearrangement in sequences which flanked the 3' end of the gene. We next examined the possibility that the N-*ras* gene of MCF-7 was itself rearranged. We performed Southern blot analysis of MCF-7 DNA with an N-*ras* probe and observed that this gene was not rearranged, at least within the 16.3-kbp region containing a functional N-*ras* gene. This was definitely established by the cloning of the N-*ras* region from MCF-7 DNA. We conclude that rearrangement of the

TABLE 3. Lineages of tumors grown in nude mice^a

Tumor designation	Induced by DNA from:
MCF-7-1	MCF-7
MCF-7-1-1	MCF-7-1
MCF-7-1-2	MCF-7-1
MCF-7-1-3	MCF-7-1
MCF-7-2	MCF-7
MCF-7-2-1	MCF-7-2
MCF-7-2-2	MCF-7-2
MCF-7-3	MCF-7
MCF-7-3-1	MCF-7-3
MCF-7-3-2	MCF-7-3
MCF-7-3-4	MCF-7-3

^a The tumors to which we refer by name in the text are listed, together with the cell or tumor DNA which was used to induce them.

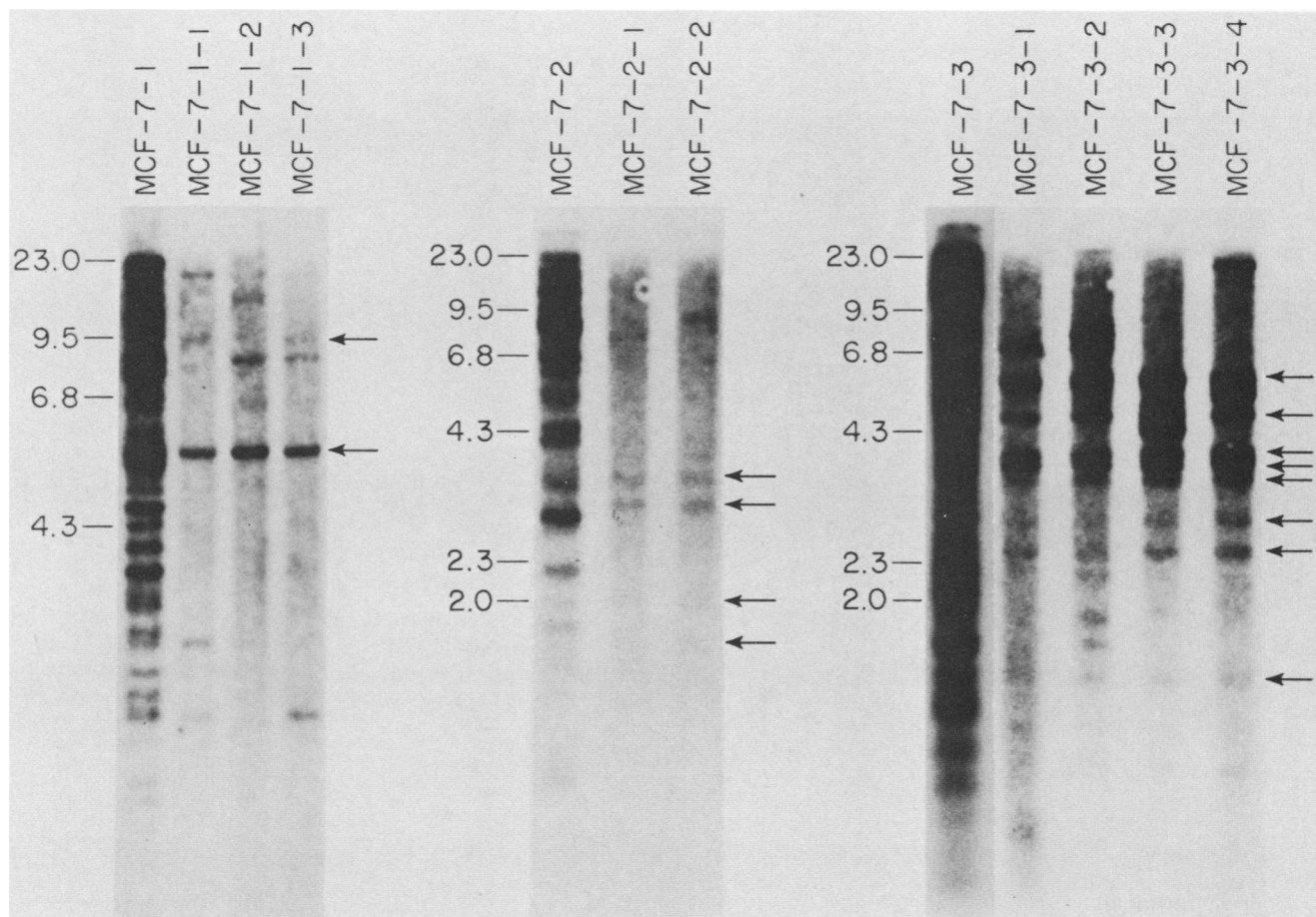


FIG. 1. Human repeat sequences in transformants. DNAs (10 μ g each) from the indicated tumors were digested with *Eco*RI and analyzed by Southern hybridization for the presence of human repeat sequences under conditions of high stringency. Total nick-translated human DNA was used as a probe. Numbers on the left are molecular weight markers in kilobases. Arrows indicate restriction endonuclease fragments with human repeat sequences which have been consistently transferred into secondary transformants.

N-ras gene in NIH3T3 transformants therefore occurred during or after gene transfer. Such rearrangements are common events during gene transfer (14, 30).

These same studies, however, did indicate that the *N-ras* gene of MCF-7 is present at several times the copy number found in normal human diploid cells (Fig. 2). These blot hybridization experiments were repeated several times with

the same results. We carefully monitored the amount of DNA loaded per slot by ethidium bromide staining and UV illumination of the agarose gel before DNA transfer onto nitrocellulose. After hybridizing nitrocellulose filters with nick-translated *N-ras* probes and performing an autoradiographic exposure, we monitored the amount of DNA transferred from gels by rehybridizing these filters with nick-

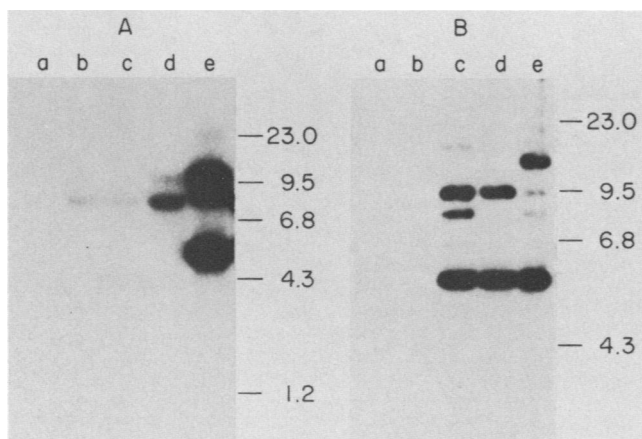


FIG. 2. Presence of *N-ras* in transformants and MCF-7. Various DNAs (10 μ g each) were digested with *Eco*RI and analyzed by Southern hybridization for the presence of the *N-ras* gene. The partial cDNA clone p6a1 (50) was nick translated and used as a probe. This probe hybridizes more strongly with the 3' *Eco*RI fragment of *N-ras* than with the 5' *Eco*RI fragment (50). (A) Lane a, NIH3T3 DNA; lane b, DNA from a normal human fibroblast cell line, GM2998; lane c, DNA from a human placenta; lane d, DNA from MCF-7 cells; lane e, DNA from the primary tumor MCF-7-1. Nitrocellulose filters were exposed for 24 h at -70°C with intensifying screens. (B) Lane a, DNA from primary tumor MCF-7-2; lane b, DNA from primary tumor MCF-7-3; lane c, DNA from primary tumor MCF-7-1; lane d, DNA from secondary tumor MCF-7-1-2; lane e, DNA from secondary tumor MCF-7-1-3. The hybridizations were done at high stringency. Exposure was for 12 h. Numbers on the right are DNA molecular weight markers in kilobases.

translated total human DNA and performing a second autoradiographic exposure. These studies indicated that *N-ras* was present in MCF-7 cells at 5 to 10 times the levels seen in cultured diploid human fibroblasts and human placental cells.

Induction of tumors with *ras* genes encoding normal proteins. We were surprised to find that DNA from MCF-7-1 could not induce morphologically altered foci in NIH3T3 cells. When the *N-ras* gene was cloned from a tumor induced by MCF-7-1 DNA, it too was unable to induce foci in NIH3T3 cells. Since the focus assay is sensitive to activated *ras* genes containing structural mutations, we tentatively concluded that the *N-ras* gene of MCF-7 encodes a normal protein product and, further, that the tumorigenicity assay, unlike the focus assay, is sensitive to cellular genes with the potential to induce tumorigenicity by increased expression of unaltered protein products.

We directly tested this hypothesis by assaying an activated *H-ras* gene cloned from T24 bladder carcinoma cells (51), a wild-type *H-ras* gene cloned from human placental cells (51), an activated *N-ras* gene cloned from SK-N-SH cells (50), and a wild-type *N-ras* gene cloned from human placental cells (50) (Table 3). The results are summarized in Table 4. The structurally mutated, transforming *H-ras* and *N-ras* genes efficiently induced tumors. The *H-ras* and *N-ras* genes cloned from human placental DNA were much less active but did nevertheless efficiently induce tumors in nude mice. We attribute this to the presence of multiple copies of these genes in NIH3T3 cells after DNA transfer, a hypothesis consistent with the work of others (5), who have shown that cells expressing high levels of the human *H-ras* gene product are tumorigenic.

It was still possible that the *N-ras* gene of MCF-7 contained mutations which made it more active in the tumorige-

TABLE 4. Tumorigenicity assays of normal and mutant *ras* genes^a

Donor DNA (ng)	Group	Mean tumor diam (mm) on day:						
		7	14	21	28	35	43	50
NIH3T3	A1	0	0	0	0	0	0	0
	A2	0	0	0	0	0	0	0
	A3	0	0	0	0	0	0	0
NIH3T3 + <i>H-ras</i> -pT24 (10)	B1	7.8	21.5					
	B2	7.3	18.7					
	B3	8.4	22.8					
NIH3T3 + <i>H-ras</i> -pP3 (10)	C1	0	0	0	4.9	12.4	19.5	20.8
	C2	0	0	0	0	0	8.4	16.5
	C3	0	0	0	4.5	11.4	18.8	19.5
NIH3T3 + <i>H-ras</i> -pP3 (100)	D1	0	4.2	13.2	17.7			
	D2	0	4.9	14.3	20.8			
	D3	0	5.3	8.9	16.5			
NIH3T3 + <i>N-ras</i> -λNS-35 (10)	E1	7.3	27.3					
	E2	5.6	23.2					
	E3	6.8	20.8					
	E4	6.5	22.8					
NIH3T3 + <i>N-ras</i> -λNP-12 (10)	F1	0	0	0	0	0	0	8.4
	F2	0	0	0	Tr	5.1	12.0	15.3
	F3	0	0	0	6.2	10.2	15.8	20.3
	F4	0	0	0	0	0	0	0
NIH3T3 + <i>N-ras</i> -λNP-12 (100)	G1	0	0	0	9.6	15.3		
	G2	0	0	5.7	11.6	20.7		
	G3	0	0	5.8	11.2	20.0		
	G4	0	0	3.9	8.9	15.6		
NIH3T3 + <i>N-ras</i> -λMN-317 (10)	H1	0	0	0	0	0	0	0
	H2	0	0	0	0	0	0	0
	H3	0	0.	0.	0.	0.	4.0	8.4
	H4	0	0	0	0	0	7.9	18.1
NIH3T3 + <i>N-ras</i> -λMN-317 (100)	J1	0	0	0	8.4	16.5	24.6	
	J2	0	0	0	7.6	12.5	20.8	
	J3	0	0	0	Tr	7.2	15.8	
	J4	0	0	0	8.4	15.3	20.5	
a1-1	K	10.4	18.7					

^a In this experiment, each plate of NIH3T3 cells was exposed to the usual concentration of pKOneo and 30 μg of NIH3T3 DNA (groups A through J) or a1-1 DNA (group K). Moreover, each plate (groups B through J) received the following additions: group B, 10 ng of the transforming human *H-ras* gene cloned from T24 DNA, called plasmid pT24; groups C and D, 10 and 100 ng, respectively, of the wild-type *H-ras* gene cloned from human placental DNA, called plasmid pP3; group E, 10 ng of the transforming human *N-ras* gene cloned from SK-N-SH neuroblastoma cells, called λNS-35; groups F and G, 10 and 100 ng, respectively, of the wild-type *N-ras* gene cloned from human placental DNA, called λNP-12; groups H and J, 10 and 100 ng, respectively, of the human *N-ras* gene cloned from MCF-7-1-1, called λMN-317.

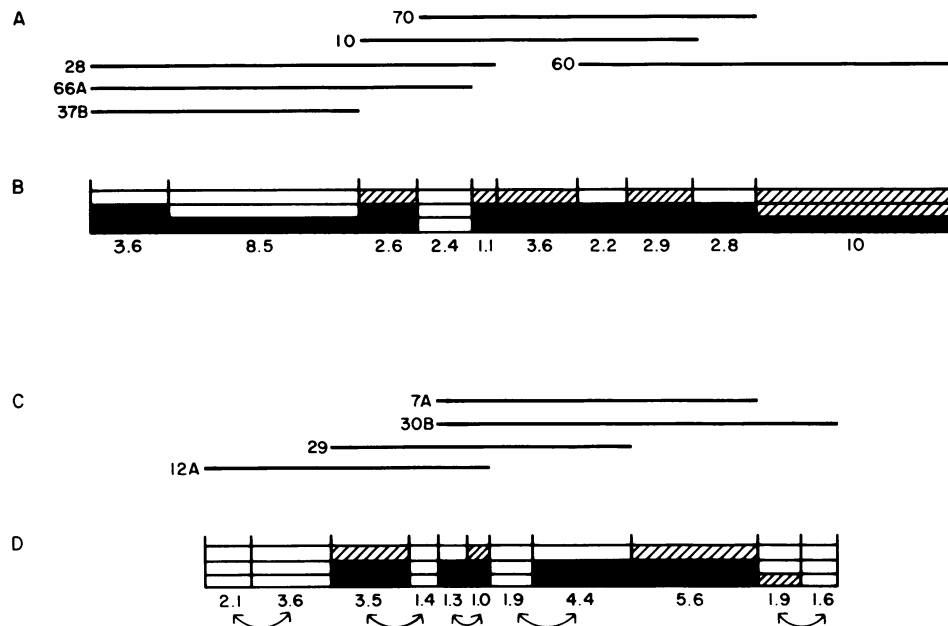


FIG. 3. *EcoRI* restriction fragments of clones of *mcf2* and *mcf3*. (A and C) *EcoRI* Inserts of independent λ charon 4a clones of *mcf2* and *mcf3*, respectively. Numbers to the left refer to the λ clone designation (for example, clone λ *mcf2*-10 or λ *mcf3*-30B, etc.). (B and D) Composite *EcoRI* restriction endonuclease map of these genes. Vertical lines indicate *EcoRI* sites. The relative position of the *EcoRI* fragments was determined from digests of phages with overlapping inserts. Not all such phages are shown. Ambiguities in relative position are indicated by arrows. Numbers below the fragments are their length in kbp. For each fragment, boxes indicate hybridization signal with total human DNA (lower box), human *alu* clone blur 8 (20) (middle box), or total mouse DNA (upper box). Solid boxes indicate a strong hybridization signal, slashed boxes indicate a very weak signal, and empty boxes indicate no observable hybridization signal. See Fig. 4 for typical blot hybridization data.

nicity assay but not in the focus assay. However, the *N-ras* gene cloned from MCF-7-1-1 was no more efficient at inducing tumorigenicity than the *N-ras* gene cloned from human placental DNA (Table 4). A similar result was obtained when we assayed the *N-ras* gene cloned directly from MCF-7 DNA (data not shown). These results suggest that the *N-ras* gene of MCF-7 does not contain significant structural mutations.

Preliminary characterization of the *mcf2* and *mcf3* genes. We have cloned portions of the two other genes detected by our assay of MCF-7 DNA. We used human repetitive sequences to isolate these genes from λ phage libraries we constructed using DNA from secondary tumors MCF-7-2-2 and MCF-7-3-4. In retrospect, this isolation was greatly facilitated by the high copy number of these genes present in induced mouse tumors (see Fig. 5). From overlapping λ phage inserts we determined preliminary *EcoRI* maps of these genes (Fig. 3). We also determined the presence of mouse or human repeat sequences on these genes (Fig. 3 and 4). None of our clones have shown strong hybridization to total nick-translated mouse DNA (Fig. 4D), but we observed weak hybridization between some fragments and mouse DNA upon long autoradiographic exposures (Fig. 4C). We attribute this to weak homology between human and mouse sequences since sequences which were cloned directly from the human cell line MCF-7 also showed this weak hybridization (Fig. 4, lane h). We present this data to facilitate the subsequent unambiguous identification of new oncogenes as they become isolated by others. However, we caution that the human DNA which we have isolated may contain rearrangements. Since none of our phage clones is biologi-

cally active in the nude mouse assay, these genes may be too large to clone in a biologically active form as a λ phage insert, and we are currently isolating cosmid clones.

By blotting our cloned DNAs with total human or mouse DNA, we identified relatively repeat-free restriction endonuclease fragments useful for Southern hybridization to genomic DNAs. We then were able to examine the copy number of these genes in our various transformants, in MCF-7 cells themselves, and in transformed NIH3T3 cells obtained from G. Cooper which he obtained by using MCF-7 DNA in a focus assay (Fig. 5). Evidently, the *mcf2* and *mcf3* genes are not related to each other. They are present in high copy numbers in the primary tumors MCF-7-2 and MCF-7-3, respectively, and in the respective secondary tumors derived from them. However, neither gene appears to be amplified in MCF-7 DNA itself. Significantly, neither gene appears to be related to the gene that Lane et al. (25) reported to transfer from MCF-7 DNA to NIH3T3 cells using an NIH3T3 focus assay (Fig. 5, lanes h and i).

We finally used our cloned DNA as probes to search for homology to known oncogenes. This was done by low-stringency blot hybridization to complete or nearly complete oncogene clones. For probes, we nick translated a total of 39.7 and 28.3 kbp from *mcf2* and *mcf3*, respectively. No homology was seen to the clones of oncogenes shown in Table 5 (blot data not shown). Since this survey includes most of the known cellular oncogenes, we conclude that *mcf2* and *mcf3* are previously uncharacterized transforming genes. This conclusion must be taken with caution, since we do not know if the cloned portions of the *mcf* genes include coding sequences.

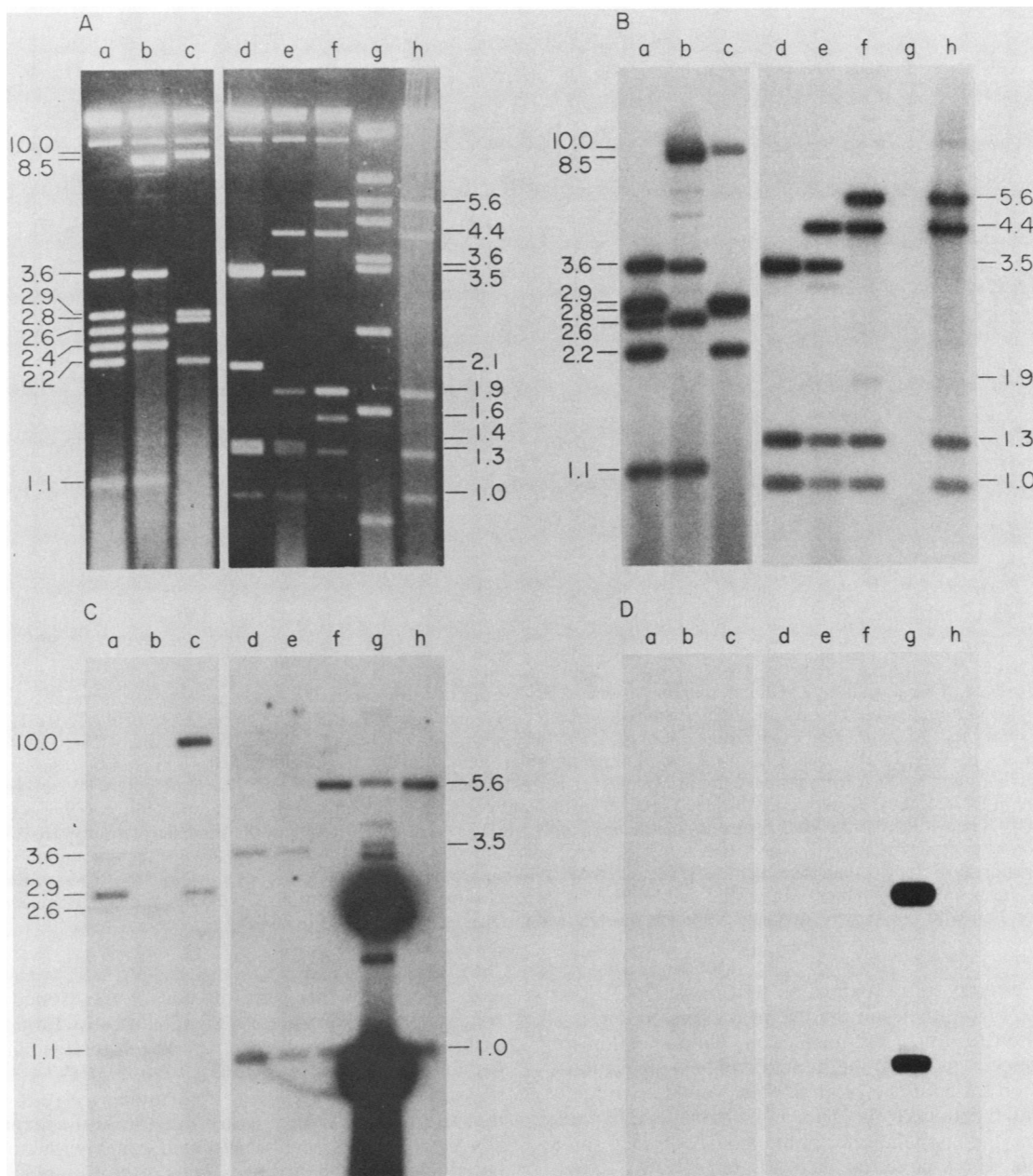


FIG. 4. Hybridization of λ phage clones of the *mcf2* and *mcf3* genes with human and mouse repetitive sequences. (A) Ethidium bromide staining pattern of a 1% agarose gel loaded with 1.5 μ g of *Eco*RI digests of DNA from the following λ clones: λ *mcf2*-10, -28, and -60 (lanes a, b, and c, respectively) from the secondary tumor MCF-7-2-2; λ *mcf3*-12A, -29, and -30B (lanes d, e, and f, respectively) from the secondary tumor MCF-7-3-4; λ *mcf3*-7A (lane h) from the human cell line MCF-7, which was isolated with an *mcf3*-specific probe. Lane g shows an *Eco*RI-*Bam*HI digest of λ *myc*4.1.1, a cloned *myc* gene from mouse plasmacytoma MOPC315 (39) used as a positive control for mouse repeat sequences. The length of the inserts, indicated except for lane g, is given in kbp. (B, C, and D) Southern blot hybridization of the gel shown in (A) with nick-translated total human DNA after 2 h of exposure (B) and with nick-translated total mouse DNA after either 4 days (C) or 2 h (D) of exposure. The hybridizations were done at high stringency (see the text), and exposure was at -70°C with an intensifying screen.

DISCUSSION

The decision to use tumorigenicity in nude mice rather than focus formation to assay transforming genes was based on two considerations. First, it was clearly at least theoretically possible for altered NIH3T3 cells to be tumorigenic yet unable to form a recognizable focus of morphologically altered cells under conditions of in vitro cultivation. Second,

morphologically altered foci of a certain type are sometimes induced in cultures of NIH3T3 in response to the mere physical presence of calcium phosphate-precipitated DNA (31). This background of spontaneous foci potentially obscured detection of certain transforming genes. A similar approach to detecting transforming genes has been described by Blair and co-workers (2), who injected NIH3T3 cells into

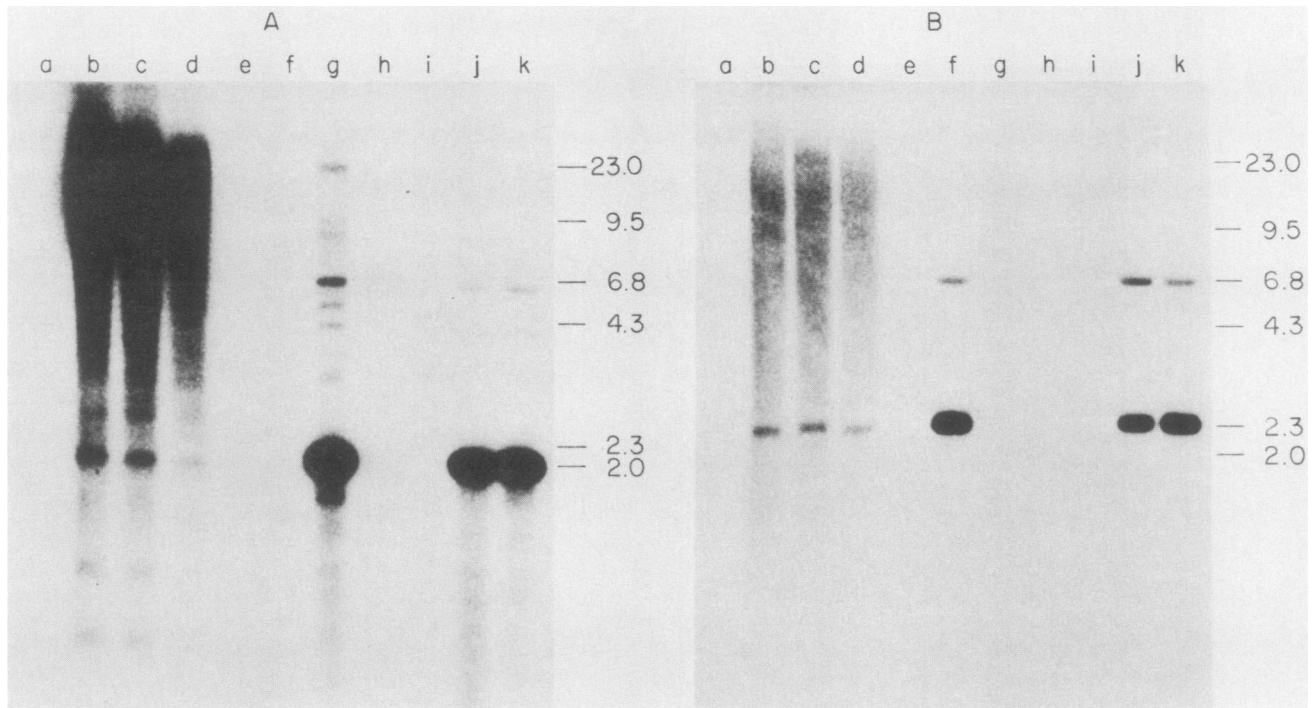


FIG. 5. Presence of *mcf2* and *mcf3* genes in human DNA and DNA from transformants. DNAs (10 μ g each) from various sources were cleaved with *Eco*RI and analyzed by the method of Southern (45) for DNA sequences homologous to *mcf3* (A) or *mcf2* (B). Hybridization probes used were: the 1.9-kbp *alu*-free *Eco*RI fragment of *mcf3* and the 2.4-kbp *alu*-free fragment of *mcf2*, respectively (Fig. 3). DNAs were as follows. (A) Lane a, NIH3T3 DNA; lane b, DNA from human fibroblast cells (GM2998); lane c, DNA from human placental cells; lane d, DNA from MCF-7; lane e, DNA from primary tumor MCF-7-1; lane f, DNA from primary tumor MCF-7-2; lane g, DNA from primary tumor MCF-7-3; lane h, DNA from Hu1, a primary NIH3T3 focus transformant obtained from G. Cooper which reportedly contains a transforming gene from MCF-7; lane i, DNA from Hu2, a second, but independent, NIH3T3 focus transformant of the same derivation as Hu1; lane j, DNA from the secondary tumor MCF-7-3-1; lane k, DNA from the secondary tumor MCF-7-3-2. In this experiment, the amount of MCF-7 DNA loaded was proportionately less than the other lanes, as judged by ethidium bromide staining. (B) Lanes a through i were as in (A); lanes j and k were DNAs from secondary tumors MCF-7-2-1 and MCF-7-2-2, respectively. The hybridizations were done at high stringency. Exposure times were for 24 h with intensifying screens. Numbers on right are DNA size markers in kbp.

nude mice directly after exposure to tumor DNA. Our method differs from theirs in the use of cotransfection.

The decision to utilize cotransfection, with selection for G418 resistance, was based on the following considerations. First, selection for a biochemical marker preselects for cells competent in the uptake of DNA (54) and effectively removes from the population spontaneously transformed NIH3T3 cells which have not incorporated DNA. Second, from previous studies we know that cells which incorporate a selectable marker are likely to have incorporated, on average, 3×10^3 kbp from the coprecipitated DNA (32). A total of 10^3 G418-resistant colonies would likely have incorporated in total, therefore, about 3×10^6 kbp of coprecipitated DNA, or about one genomic equivalent. Third, by allowing each G418-resistant colony to grow to a size of 10^4 cells, we could in principle detect tumor formation even from weakly tumorigenic cell populations.

Using cotransfer and tumorigenicity in nude mice, we can detect the transfer of three different genes from MCF-7 DNA into NIH3T3 cells which we cannot detect using the focus assay with this DNA as donor. One of the genes which we have transferred from MCF-7 DNA is *N-ras*. To determine if there are mutations associated with the *N-ras* gene, we cloned this gene from both MCF-7 and the secondary tumor MCF-7-1-1. These *N-ras* genes are no more potent in the tumorigenicity assay than an *N-ras* gene cloned from human placental cells. Although this does not rule out alterations

associated with this gene outside of the cloned region, it suggests that there are no significant structural mutations within coding regions. Since the *N-ras* gene is highly amplified in MCF-7-1-1 and since DNA from MCF-7-1-1 has a high transforming potential in the nude mouse assay, we conclude that the tumorigenicity assay may serve to identify tumor DNAs containing highly amplified genes with transforming potential, even when those genes do not contain structural gene mutations. The tumorigenicity assay differs from the focus assay in this respect, and in fact we have shown that, in a dose-dependent manner, DNAs containing high copy numbers of normal *ras* genes do induce tumorigenicity.

Our blot hybridization data indicate that the *N-ras* gene is amplified in MCF-7 cells. In a survey of 10 other human tumors and tumor cell lines (42; unpublished studies) we have not previously observed amplification of the *N-ras* gene. MCF-7 cells thus have a genetic abnormality associated with *N-ras*. Amplification of other *ras* genes, however, has been observed in some tumors and tumor cell lines (14, 37; our unpublished studies).

Two of the genes we have transferred from MCF-7, *mcf2* and *mcf3*, appear to be unrelated to any of the previously identified oncogenes which we have examined. Moreover, an oncogene in MCF-7 cells, detectable after transfer by the NIH3T3 focus assay, was previously reported by Cooper and co-workers (25). Neither the *mcf2* nor the *mcf3* gene appears to be related to the gene reported by those workers.

TABLE 5. Molecular clones of oncogenes

Gene designation	Origin	Plasmid	Reference
<i>abl</i>	Abelson murine leukemia virus	pAB-sub3	13
<i>erb</i>	Avian erythroblastosis virus	pAE-PvuII	52
<i>fes</i>	GA feline sarcoma virus	pGA-FeSV	12
<i>fos</i>	FBJ osteosarcoma virus	pFBJ-2	7
<i>fms</i>	SM feline sarcoma virus	pSM-FeSV	9
<i>fps</i>	PRCII avian sarcoma virus	pRCII-1B	Hanafusa ^a
<i>H-ras</i>	Harvey murine sarcoma virus	pHB-11	10
<i>int-1</i>	Mouse mammary carcinoma	pMT-2.5	28
<i>K-ras</i>	Kirsten murine sarcoma virus	pKBE-2	10
<i>mos</i>	Moloney murine sarcoma virus	pm-1	3
<i>myb</i>	Avian myeloblastosis virus	pVM-2	23
<i>myc</i>	Avian myelocytomatosis	pMCV38	53
<i>N-ras</i>	Human neuroblastoma	p6al	50
<i>rel</i>	Reticuloendotheliosis virus	pRev-T3	6
<i>ros</i>	UR2 avian sarcoma virus	pBR-UR2	Hanafusa ^a
<i>sis</i>	Simian sarcoma virus	pSSV-11	35
<i>ski</i>	Avian SKV770 virus	pvski-1	Stavnezer ^a
<i>src</i>	Rous sarcoma virus	psrc-11	38
<i>yes</i>	Y73 sarcoma virus	pBR-Y73	22

^a Personal communications. Avian SKV770 virus is described in reference 47.

A more definitive conclusion, however, must await the identification of *mcf2* and *mcf3* coding regions.

We do not know if the *mcf2* and *mcf3* genes of MCF-7 cells have genetic abnormalities. On the one hand, it would not be particularly surprising to find multiple weakly transforming genetic lesions in MCF-7 cells or, for that matter, most tumorigenic cells. On the other hand, neither would it be surprising if either or both *mcf2* and *mcf3* are indeed normal genes which can induce tumorigenicity by overexpression after gene transfer into NIH3T3 cells. To answer these questions, it will be necessary to clone these genes in a biologically active form both from MCF-7 and from normal human DNA to compare their transforming activities.

In summary, we have described a tumorigenicity assay for transforming genes which differs considerably from the NIH3T3 focus assay. We do not fully understand the basis of this difference, nor do we know if the genes we detect with the assay will usually reflect genetic abnormalities in the DNA being tested. Indeed, our results strongly suggest that, with some frequency, normal genes with transforming potential will sometimes be detected. Nevertheless, we appear to have uncovered a previously unknown genetic abnormality in MCF-7 cells and have probably discovered two previously unknown human oncogenes.

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