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Simultaneous Transfer of Tumorigenic and Metastatic Phenotypes by Transfection With Genomic DNA From a Human Cutaneous Squamous Cell Carcinoma

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High-molecular-weight genomic DNA isolated from a human cutaneous squamous cell carcinoma (AS) was assayed for its ability to induce tumorigenic transformation of NIH 3T3 cells. Subcutaneous injection of NIH 3T3 cells cotransfected with DNAs from AS tumor and pSV2-*neo* plasmid not only induced tumors at the site of injection, but also metastasized spontaneously to the lungs in 100% of nude mice injected. DNA isolated from a representative primary tumor and a metastasis was again used in a second round of transfection. Injection of secondary transfectants into nude mice again resulted in induction of both subcutaneous tumors and spontaneous long metastases. Southern blot hybridization with *ras*-specific probes revealed that DNA from both primary tumors and metastases induced by AS tumor DNA contained highly amplified Ha-*ras* oncogene. Furthermore, DNAs from secondary tumors and metastases induced by DNA from a primary tumor and a metastasis also contained similar highly amplified Ha-*ras* oncogene. These results suggest that the amplified Ha-*ras* oncogene may be responsible for induction of both tumorigenic and metastatic phenotypes in NIH 3T3 cells transfected with DNA from AS tumor.

Key words: tumorigenicity, metastasis, ha-*ras*, oncogene, gene transfer, skin cancer

Numerous studies have shown that cellular DNA from a variety of human and rodent tumors contain oncogenes capable of inducing morphologic and tumorigenic transformation when introduced into NIH 3T3 cells by DNA-mediated gene transfer [1–12]. Many normal cells also express oncogenes, which in this case are termed protooncogenes; these genes may play a role in the growth and differentiation of normal embryonic and adult tissues [13,14]. However, exposure of cells to carcinogenic agents may activate the normal genes into pathologic ones by causing them to

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produce higher levels of their normal protooncogene product or by inducing structurally aberrant oncogene products [15–19]. A majority of the oncogenes so far identified in solid tumors belong to members of the *ras* gene family (designated Ha-*ras*, Ki-*ras*, and N-*ras*) [20–25]. Although the cellular *ras* protooncogenes are normally not active in the NIH 3T3 transforming assay, mutations that cause a single amino acid change in the 12th, 13th, or 61st codon or that increase the level of expression of unmutated *ras* can result in transformation of NIH 3T3 cells [17,18,26–30].

Although many different types of human tumors and tumor cell lines have been examined for the presence of activated oncogenes, most of the tumors originated in lung, colon, bladder, or other internal organs. We have found no published reports on oncogenes activated in nonmelanoma human skin cancers that originated on sun-exposed body sites. Epidemiological and clinical studies indicate that ultraviolet (UV) radiation present in sunlight is responsible for the induction of most skin cancers in humans [31,32]. Since cancers of the skin are the most prevalent form of human cancer, it is important to determine whether they contain specific transforming genes. Therefore, we began a series of experiments to determine whether DNA from fresh human skin cancers occurring on sun-exposed body sites contain oncogenes capable of inducing tumorigenic transformation when introduced into NIH 3T3 cells by DNA-mediated gene transfer. We report here that DNA isolated from a human primary squamous cell carcinoma induced tumorigenic transformation of NIH 3T3 cells. Interestingly, we also found that NIH 3T3 cells transfected with this human skin cancer DNA not only induced subcutaneous tumors at the site of injection, but also metastasized spontaneously to the lungs in 100% of mice injected. Both subcutaneous tumors and lung metastases induced in nude mice contained highly amplified Ha-*ras* oncogene.

MATERIALS AND METHODS

Tumor

The primary tumor used in this study originated on the left temple of a 67-year-old male Caucasian patient. The tumor (designated "AS") was diagnosed as a poorly differentiated squamous cell carcinoma. Untreated by prior chemotherapy or radiation, it was obtained at the time of surgical resection and immediately frozen at −70°C until processed for DNA extraction.

Preparation of Cellular DNA

High-molecular-weight genomic DNA was isolated by the phenol extraction method described by Wigler et al [33], with slight modifications. The frozen tumor tissue was minced thoroughly with dissection scissors on ice and washed twice with ice-cold phosphate-buffered saline. The minced tissue was resuspended in buffer A (150 mM NaCl:10 mM Tris-HCl (pH 8.0):5 mM EDTA (pH 8.0)) (1 ml/100 mg tissue); proteinase K was added to a final concentration of 200 μg/ml; and the cells were lysed by adding sodium dodecyl sulfate (SDS) to 1%. The lysed preparation was heated at 65°C for 15 min to inactivate endogenous nucleases and then incubated at 37°C overnight with gentle shaking. An equal volume of 650 mM NaCl:10 mM Tris-HCl (pH 8.0):10 mM EDTA solution was added to the lysate and then extracted twice with an equal volume of buffer-saturated phenol. The aqueous phase was again extracted once with a mixture of phenol, chloroform, and isoamyl alcohol (25:24:1)

and once with a mixture of chloroform and isoamyl alcohol (24:1). The DNA was precipitated from the aqueous phase by adding two volumes of cold absolute ethanol. The DNA precipitate was removed with a Pasteur pipette and washed successively with two changes of 70% ethanol and two changes of 100% ethanol. The precipitate was air-dried and dissolved in sterile 10 mM Tris-HCl (pH 8.0):1 mM EDTA. The cellular DNA was sized by gel electrophoresis in 0.8% agarose gels. Uncut and *Hind*III restriction enzyme-cut lambda DNAs were used as size markers.

DNA from pSV2-*neo* plasmids was isolated according to the procedure described by Maniatis et al [34]. Supercoiled plasmid DNA was purified on a CsCl gradient.

DNA Transfection

NIH 3T3 cells were used as recipients for DNA transfection. For best results, the cells were kept subconfluent by twice weekly subculturing in Dulbecco's medium (Grand Island Biological Co., Grand Island, NY) containing 10% calf serum (Hyclone). A cotransfection protocol [10] using pSV2-*neo* DNA [35], which confers resistance to the antibiotic G418, was employed to select cells that had taken up the foreign DNA. The calcium phosphate precipitate, which contained 120 μg of tumor DNA plus 2 μg of pSV2-*neo* DNA, was transfected onto four 60-mm plates containing 4×10^5 NIH 3T3 cells/plate. As controls, NIH 3T3 cells were transfected with pSV2-*neo* DNA alone or cotransfected with human placental DNA and pSV2-*neo* DNA. Twenty-four hours after transfection, cells from each dish were trypsinized and transferred to two 100-mm dishes. After another 16–20 hr, G418 was added to the medium at a concentration of 400 μg/ml. The cells were fed with fresh medium containing G418 every 3 or 4 days. The colonies, of which there were about 250 on each plate, reached confluency in 14–16 days.

Tumorigenicity Assay

G418-resistant colonies from sets of two 100-mm plates corresponding to one original 60-mm transfected plate were trypsinized, pooled, and centrifuged at 1,000 rpm for 5 min. The cell pellet was resuspended in Hanks' balanced salt solution and 5×10^6 cells were injected s.c. into 5–6-week-old, athymic nude Nu/Nu mice. Tumor growth was monitored weekly. When the tumor reached 15–20 mm in diameter, the mice were sacrificed and the tumor tissue was resected, established as cell lines, and subjected to G418 selection to eliminate contaminating host cells (which should be G418 sensitive). All tumor-bearing mice were examined for lung, liver, and lymph node metastasis following surgical resection of s.c. tumors. Visible colonies were excised and established as cell lines as described above.

Southern Blot Hybridization

DNA isolated from various tumors and metastases induced in nude mice were digested with restriction endonucleases and electrophoresed through a 0.8% agarose gel. DNA fragments that had been separated according to size by electrophoresis were denatured, blotted onto nitrocellulose filters, and immobilized by the method of Southern [36]. The DNA attached to the filter was prehybridized and then hybridized for about 24 hr with ^{32}P-labeled, nick-translated probes under stringent conditions according to manufacturer's instructions. The labeled Ha-*ras*, Ki-*ras*, and *alu* probes were obtained from Oncor, Inc. (Gaithersberg, MD). After hybridization, the filters

were washed four times for 5 min each at room temperature with 2× standard saline citrate (SSC) (300 mM sodium chloride, 30 mM sodium citrate)-0.5% SDS and then three times for 20 min each at 56°C with 0.1% SSC-0.1% SDS. After being blot-dried, the filters were exposed at −70°C for 24–72 hr to Kodak XAR-5 film with intensifying screens.

RESULTS

High-molecular-weight DNA isolated from a primary human squamous cell carcinoma (AS) was assayed for transforming activity by cotransfection of NIH 3T3 cells. About 2 weeks after transfection, G418-resistant colonies were pooled and injected s.c. into nude mice. The time of first appearance of tumors and their subsequent growth were noted. The results shown in Table I indicate that all four mice injected with NIH 3T3 cells transfected with AS tumor DNA developed tumors within 4 weeks after injection. In contrast, untransfected NIH 3T3 cells or NIH 3T3 cells transfected with pSV2-*neo* DNA alone did not induce tumors. However, two of four mice injected with NIH 3T3 cells transfected with human placental DNA developed tumors 6–8 weeks after injection. Thus, the NIH 3T3 "tumor assay" employed by us and by Fasano et al [10] for detecting transforming genes has an intrinsic background of tumor induction when tested with normal DNA.

When the mice were autopsied following surgical resection of s.c. tumors, we were surprised to find lung metastasis in all four mice injected with AS DNA transfectants. Although these lung colonies were few (1–14), they were very large (Fig. 1). Contrarily, the two mice in the control group that had tumors induced by human placental DNA transfectants did not have any macroscopic or microscopic lung colonies. The primary tumors and lung metastases induced by AS DNA transfectants were designated as AS-1T, AS-2T, AS-3T, AS-4T and AS-1M, AS-2M, AS-3M and AS-4M, respectively.

DNAs prepared from primary tumor and metastasis cell lines were analyzed by Southern blotting for the presence of human repetitive sequences. All the primary

TABLE I. Tumorigenicity of DNA-Transfected NIH 3T3 Cells*

Donor DNA	Group	Tumor diameter (mm) on week 1	2	3	4	Metastasis	Tumor designation[a]
AS	1	0	0	4.5	17.0	Yes	AS-1T, AS-1M
	2	0	0	7.5	15.0	Yes	AS-2T, AS-2M
	3	0	7.0	18.0	25.5	Yes	AS-3T, AS-3M
	4	0	7.0	16.0	20.5	Yes	AS-4T, AS-4M
Human placenta	1	0	0	0	0	—	—
	2	0	0	0	0	—	—
	3	0	0	0	0	—	—
	4	0	0	0	0	—	—
None	1	0	0	0	0	—	—
	2	0	0	0	0	—	—
	3	0	0	0	0	—	—
	4	0	0	0	0	—	—

*About 2 weeks after transfection, G418-resistant colonies were pooled and injected s.c. into nude mice. Each group represents a pool of G418-resistant colonies and each line a nude mouse injected with 5 × 10^6 cells from two plates.

[a]Subcutaneous tumors are designated with a suffix "T" and lung metastases with an "M."

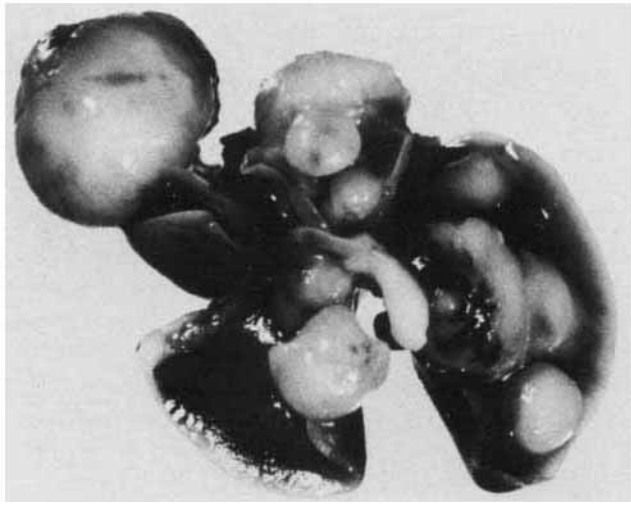

Fig. 1. Spontaneous lung metastases induced in nude mice by injection of NIH 3T3 cells transfected with AS DNA.

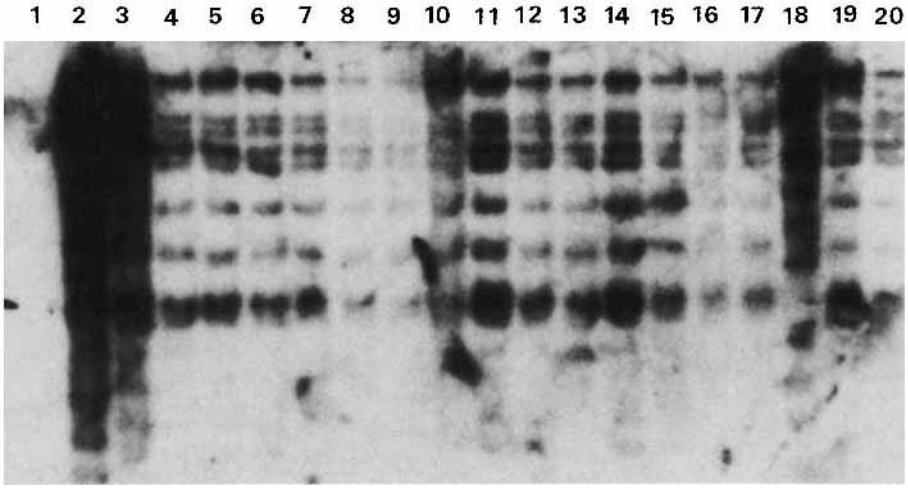

Fig. 2. Detection of human *alu* sequences in primary tumors and metastases induced in nude mice by injection of NIH 3T3 cells transfected with AS tumor DNA. DNAs (10 μg each) from the indicated tumors and metastases were digested with *Eco*RI and analyzed by Southern hybridization to ^{32}P-labeled, nick-translated *alu* probe (Palu.1, Oncor, Inc.). **Lane1:** NIH 3T3. **Lane 2:** AS-2T. **Lanes 3–7:** Individual lung colonies of AS-2M. **Lane 8:** AS-1T. **Lane 9:** AS-1M. **Lane 10:** AS-3T. **Lanes 11–16:** Individual lung colonies of AS-3M. **Lane 17: AS-4T. Lanes 18–20:** Individual lung colonies of AS-4M.

tumors and metastases induced by AS DNA contained human *alu* sequences (Fig. 2). Interestingly, all the primary tumors and metastases induced by AS DNA exhibited a common set of human repetitive sequences. In contrast, the two tumors induced by NIH 3T3 cells transfected with human placental DNA contained very little or no human sequences (data not shown).

In order to determine whether the primary tumors and metastases contained specific oncogenes, their DNAs were analyzed for the presence of human Ha- and

Ki-*ras* genes by Southern analysis. None of the primary tumors and metastases induced by AS DNA contained the human Ki-*ras* gene (data not shown). The hybridization pattern observed with the Ki-*ras* probe in primary tumors and metastases was similar to that of untransfected NIH 3T3 cells. However, when duplicate blots were hybridized with the Ha-*ras* probe, all the primary tumors and metastases induced by AS DNA exhibited additional bands besides the mouse endogenous Ha-*ras* sequences (Fig. 3). In contrast, the two tumors induced by transfection with human placental DNA did not contain the human Ki- or the Ha-*ras* gene (data not shown). These data, together with the observation that the two tumors induced by human placental DNA transfectants contained very little or no human *alu* sequences, suggests that these two tumors were induced by spontaneous transformation.

Interestingly, DNA from AS tumor contained amplified copies of the Ha-*ras* gene compared with the Ha-*ras* gene present in human placental DNA. Similarly, the Ha-*ras* gene in all the primary tumors and metastases, with the exception of AS-1T, was further amplified compared with the Ha-*ras* present in AS tumor. In addition, *Bam*HI fragments homologous to the Ha-*ras* gene present in most primary tumors and metastases were different from the 6.6-kilobase (kb) fragment found in AS tumor DNA and in human placental DNA. Whereas DNAs from AS-1T, AS-1M, and AS-4M contained only the 6.6-kb *Bam*HI fragment of the Ha-*ras* gene, DNAs from AS-2T, AS-2M, and AS-3M contained, in addition to the 6.6-kb fragment, a novel 9.4-kb *Bam*HI fragment homologous to the Ha-*ras* gene. On the other hand, DNAs from AS-3T and AS-4T contained only the 9.4-kb and not the 6.6-kb *Bam*HI fragment of the Ha-*ras* gene. The newly acquired 9.4-kb *Bam*HI fragment of the Ha-*ras* gene present in some tumors and metastases could be attributable to the loss of a restriction site on integration of the donor DNA into the recipient cell DNA. In addition, these

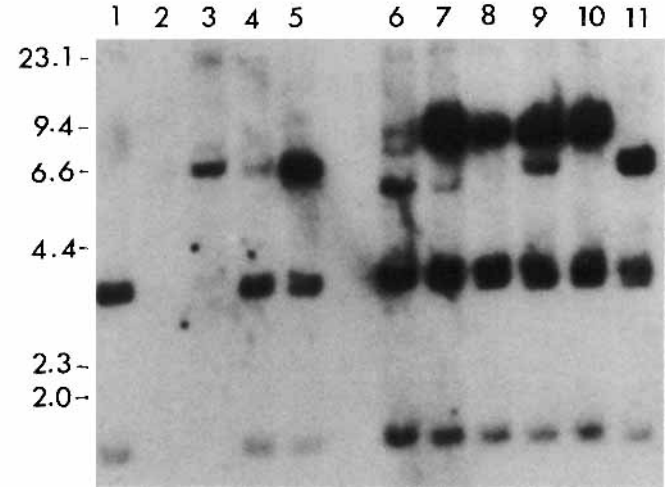

Fig. 3. Detection of Ha-*ras* sequences in primary tumors and metastases induced in nude mice by injection of NIH 3T3 cells transfected with AS DNA. Various DNAs (10 μg each) were digested with *Bam*HI and analyzed by Southern blot hybridization to ^{32}P-labeled, nick-translated Ha-*ras* probe (*Pras*.1, Oncor, Inc.) under conditions of high stringency. **Lane 1:** NIH 3T3. **Lane 2:** Human placenta. **Lane 3:** AS tumor. **Lane 4:** AS-1T. **Lane 5:** AS-1M. **Lane 6:** AS-2T. **Lane 7:** AS-2M. **Lane 8:** AS-3T. **Lane 9:** AS-3M. **Lane 10:** AS-4T. **Lane 11:** AS-4M. Numbers on the left are *Hind*III-digested lambda phage DNA molecular weight markers.

results also demonstrate the heterogeneous nature of the transfected cells. Since these tumors were induced in mice by injection of pooled G418-resistant colonies, it is likely that they were made up of a heterogeneous population of cells.

Genomic DNAs from a representative primary tumor (AS-3T) and a metastasis (AS-2M) was used again in a second round of transfection. Both AS-3T and AS-2M secondary transfectants not only induced tumors in nude mice at the site of injection but also metastasized spontaneously to the lungs (Table II). The lung colonies induced by the secondary transfectants were similar in number (2–14) and size to those induced by the primary transfectants.

DNAs from secondary tumors and metastases were analyzed for the presence of human *alu* and Ha-*ras* sequences. All the secondary tumors and lung metastases tested contained a common set of human *alu* sequences (Fig. 4). In most cases, there were only one or two discrete bands, which suggests that these sequences may be tightly linked to the Ha-*ras* oncogene. Southern blot analysis of DNAs from secondary

TABLE II. Tumorigenicity of Secondary Transfectants*

Donor DNA	Group	Tumor diameter (mm) on week— 1	2	3	4	5	Metastasis	Tumor Designation
AS-3T	1	0	0	0	4.0	9.5	Yes	AS-3T-1T, AS-3T-1M
	2	0	0	3.0	6.0	11.0	Yes	AS-3T-2T, AS-3T-2M
AS-2M	3	0	0	4.0	8.0	17.0	Yes	AS-2M-3T, AS-2M-3M
	4	0	0	6.0	10.5	19.0	Yes	AS-2M-4T, AS-2M-4M
Human placenta	5	0	0	0	0	0	—	
	6	0	0	0	0	0	—	—
None	7	0	0	0	0	0	—	—
	8	0	0	0	0	0	—	—

*Each group represents a pool of G418-resistant colonies and each line a nude mouse injected with 5×10^6 cells from two plates.

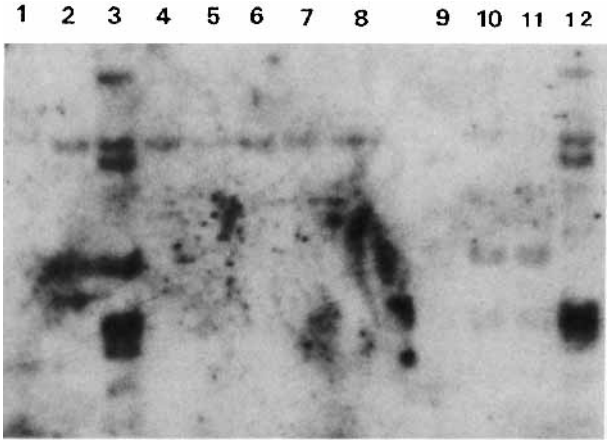

Fig. 4. Presence of human *alu* sequences in secondary tumors and metastases induced in nude mice by injection of NIH 3T3 cells transfected with DNAs from AS-3T tumor as AS-2M metastasis. Various DNAs were analyzed for human repeat sequences as described in Materials and Methods. **Lane 1:** NIH 3T3. **Lane 2:** AS-3T-1T.**Lane 3:** AS-3T-2T. **Lanes 4–8:** Individual lung metastases of AS-3T-1M. **Lane 9:** AS-2M-3T. **Lanes 10, 11:** Individual lung colonies of AS-2M-3M. **Lane 12:** AS-2M-4T.

tumors and metastases with an Ha-*ras* probe revealed that all of them contained the unique, highly amplified 9.4-kb *Bam*HI fragment similar to those found in AS-3T tumor and AS-2M metastasis (Fig. 5).

DISCUSSION

Until recently, DNA transfection studies aimed at identifying oncogenes were limited to using the NIH 3T3 "focus assay." Very often the genes from tumors that had focus-forming activity were found to be members of the *ras* family of oncogenes. In an attempt to detect new transforming genes, Blair et al [9] and Fasano et al [10] developed an NIH 3T3 "tumorigenicity assay," in which transfected NIH 3T3 cells are injected into athymic nude mice. Using this assay, we simultaneously transferred tumorigenic and metastatic phenotypes by transfection of NIH 3T3 cells with genomic DNA from a human squamous cell carcinoma (AS). Both the tumors and metastases induced in nude mice by injection of transfected cells contained the human Ha-*ras* oncogene.

We were surprised to find that s.c. injection of NIH 3T3 cells cotransfected with DNAs from AS tumor and pSV2-*neo* not only induced tumors at the site of injection but also metastasized spontaneously to the lungs in 100% of mice injected. The tumorigenic and metastatic phenotypes again cotransferred in a second round of DNA transfection. Southern blot hybridization with *ras* probes revealed that a majority of the tumors and metastases induced by AS DNA contained highly amplified Ha-*ras* oncogene. Interestingly, DNA from AS tumor also contained high copy numbers of the Ha-*ras* gene compared with that present in human placental DNA. Therefore, we conclude that further amplification of the Ha-*ras* gene in tumors and metastases induced by AS DNA might have occurred during or after gene transfer. Such

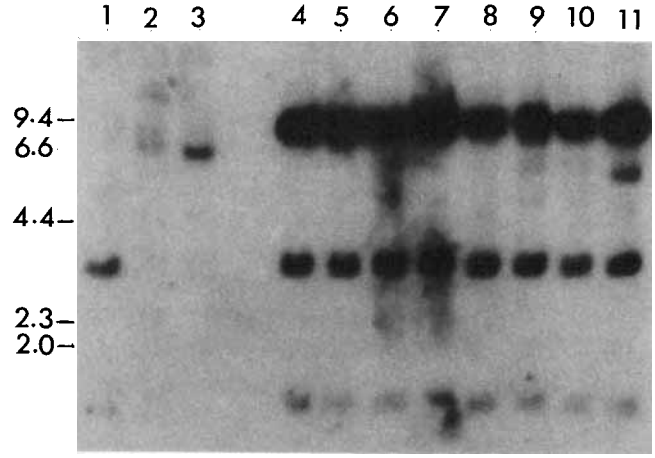

Fig. 5. Presence of Ha-*ras* sequences in secondary tumors and metastases induced in nude mice by injection of NIH 3T3 cells transfected with DNAs from AS-3T tumor and AS-2M metastasis. DNAs from indicated tumors and metastases were analyzed for Ha-*ras* sequences as described in Materials and Methods. **Lane 1:** NIH 3T3. **Lane 2:** Human placenta. **Lane 3:** AS tumor. **Lane 4:** AS-3T-1T. **Lanes 5, 6:** Individual lung colonies of AS-3T-1M. **Lane 7:** AS-3T-2T. **Lane 8:** AS-2M-3T. **Lanes 9, 10:** Individual lung colonies of AS-2M-3M. **Lane 11:** AS-2M-4T. Numbers on the left are *Hind*III-digested lambda phage DNA molecular weight markers.

amplification and rearrangement of genes are known to occur during DNA transfection [10,37,38].

DNAs from most tumors and metastases induced by primary and secondary transfectants contained amplified copies of the Ha-*ras* gene. In addition, DNAs from a majority of primary tumors and metastases and all secondary tumors and metastases induced by AS tumor DNA contained a unique 9.4-kb *Bam*HI fragment of the amplified Ha-*ras* gene. This suggests that this amplified 9.4-kb *Bam*HI fragment of the Ha-*ras* gene was responsible for induction of both tumorigenic and metastatic phenotypes in NIH 3T3 cells transfected with DNA from AS tumor. However, we do not know at present whether the amplified Ha-*ras* gene in AS tumor and in tumors and metastases induced by AS DNA contains structural mutations. Previous studies have shown that introduction of activated c-Ha-*ras*-1 gene into NIH 3T3 [39–43] and early-passage rat fibroblast [42,44,45] cells results in induction of both tumorigenic and metastatic phenotypes. In contrast, NIH 3T3 cells transformed by elevated levels of the Ha-*ras* proto-oncogene were tumorigenic but not metastatic [42]. Based on these and our studies, we can speculate that the Ha-*ras* gene in AS DNA and in tumors and metastases induced by AS DNA may, in addition to being amplified, contain structural alterations.

Other strategies for studying the molecular basis of metastasis have included introduction of either cloned activated *ras* gene [42,46,47] or genomic DNA from metastatic cells [40] into tumorigenic but nonmetastatic cells, and assessment of the ability of transfected cells to metastasize in syngeneic immunocompetent hosts. Using this approach, Vousden et al [46] and Collard et al [47] demonstrated that activated human c-Ha-*ras*-1 oncogene conferred metastatic potential when introduced into nonmetastatic but tumorigenic cells by DNA-mediated gene transfer. In contrast, Muschel et al [42] found that transfection of tumorigenic, but nonmetastatic murine C127 cells with activated c-Ha-*ras* gene did not confer metastatic potential, even though the C127 transformants expressed increased levels of the Ha-*ras* protein. On the other hand, Bernstein and Weinberg [40] reported that the metastatic phenotype induced by transfection of *ras*-transformed, nonmetastatic tumor cells with genomic DNA from a human metastatic tumor was associated with an unidentified gene. In another study, Gallick et al [48] found that human metastatic colon tumors expressed decreased levels of $p21^{ras}$ protein compared with the levels expressed in primary tumors. Thus, these contradictory findings imply that both *ras* and non-*ras* genes may be involved in the regulation of the metastatic phenotype. Although our data strongly suggest that the amplified Ha-*ras* oncogene is associated with the metastatic phenotype in NIH 3T3 cells transfected with AS tumor DNA, the involvement of other non-*ras* genes, if any, in the regulation of the metastatic phenotype remains to be determined.

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