Frequent Activation of c-*kis* as a Transforming Gene in Fibrosarcomas Induced by Methylcholanthrene

Abstract. *The DNA's from two of four methylcholanthrene-induced mouse fibrosarcomas contained transforming genes that were identical in their pattern of restriction endonuclease resistance to inactivation of biologic activity. This transforming gene was identified as the activated homolog of the Kirsten murine sarcoma virus* onc *gene, v-*kis. *The finding that a defined carcinogen reproducibly leads to activation of* kis *as a transforming gene should be of value in elucidating the role of oncogenes in the neoplastic process.*

The development of DNA-mediated gene transfer (transfection) techniques has led to the detection of transforming DNA sequences in various tumor cells. Such DNA's can induce morphological transformation of NIH/3T3 cells (*1*), a continuous mouse line that is contact-inhibited and susceptible to DNA transfection. One approach to identifying genes that may be preferential targets for somatic mutations that could lead to their acquisition of transforming activity is the analysis of animal tumor cells induced by specific carcinogens. Transforming genes have been detected in certain rodent cells transformed in tissue culture by chemical carcinogens (*2–7*). Moreover, the DNA's from several independently derived methylcholanthrene (MCA) transformants have shown an identical pattern of susceptibility to restriction endonuclease cleavage, suggesting that the same transforming gene was activated in each case (*3*).

We sought to detect transforming genes in tumor cells induced in vivo by MCA. In view of studies linking transforming genes of some human malignancies to the *onc* genes of certain transforming retroviruses (*8–11*), we also wanted to determine whether any transforming genes detected could be identified as the activated cellular homolog of a known retroviral *onc* gene.

We used high molecular weight DNA's of tumor cell lines derived from four independent MCA-induced mouse fibrosarcomas for transfection of NIH/3T3 cells. The MCA-induced fibrosarcomas, T-92497 and MCA-1GP12, demonstrated transforming activity (Table 1). A second cycle of transfection was performed with DNA's from the two primary transfectants to confirm that the foci observed were induced by exogenous DNA. Each DNA preparation retained transforming activity for NIH/3T3 cells (Table 1). As an independent confirmation that the transformants had taken up exogenous DNA, the plasmid pBR322 was used as a marker for cotransfection. Multiple copies of pBR322 sequences were present in DNA's isolated from representative transformants induced by T-92497 and MCA-1GP12 tumor cell DNA's (lanes 2 and 3 in Fig. 1).

We next analyzed the ability of various restriction endonucleases to inhibit the transforming activity of the T-92497 transforming gene. The biologic activity of this gene was completely inactivated by Eco RI, Hind III, Pst I, Sac I, and Bgl II, while Bam HI had no effect (Table 2). A similar pattern of inactivation was observed with the transforming gene of MCA-1GP12 cells (data not shown).

To identify the transforming genes, we subjected primary and secondary transfectants to Southern blotting analysis using several *onc* genes as molecular probes. Since the transforming genes were of mouse origin, their detection would require the presence of restriction enzyme sites that differed from those of the respective normal homologs already present in NIH/3T3 mouse cell DNA. Because Bam HI did not inactivate the transforming activity of T-92497 DNA, we digested some first-cycle T-92497 transformant DNA's with Bam HI before subjecting them to a second cycle of transfection. By this strategy, Bam HI sites originally flanking the transforming gene would be lost, thus altering the pattern of Bam HI cleavage of the gene in second-cycle transformants. The Kirsten-MSV *onc* gene, v-*kis*, detected a new high molecular weight DNA fragment in Bam HI–cleaved DNA of a second-cycle transformant of the T-92497 tumor (lane 6 in Fig. 1). These results demonstrated that exogenous *kis* sequences were associated with the transformed phenotype of the transfectants. None of a number of other retroviral *onc* genes detected any additional bands in comparison with those present in the DNA of NIH/3T3 cells (data not shown).

When high molecular weight DNA's from first- or second-cycle transfectants of the T-92497 tumor were cleaved with Hind III, an additional *kis*-related fragment of approximately 9.2 kilobase pairs (kbp) was also observed (lanes 9 and 10 in Fig. 1). Since these restriction fragments were not present in Hind III–cleaved DNA of the original tumor, we presume that the altered cleavage pattern resulted from the process of transfection and integration. An extra *kis*-related band was also observed after Hind III cleavage of all primary and secondary transfectants induced with high molecular weight DNA of the MCA-1GP12 tumor (data not shown).

To compare the structure of c-*kis* in the original tumor cells with that present in DNA from control mice, the respective DNA's were cleaved with various enzymes, including Bam HI, Hind III, and Eco RI. We detected no differences in cleavage patterns with v-*kis* as a molecular probe (data not shown), suggesting that activation of c-*kis* in the MCA tumors was not associated with any gross rearrangement of this gene. In some cases, the intensity of *kis*-related fragments in DNA's from transfectants was substantially increased over that present in the NIH/3T3 or MCA tumor cell DNA's analyzed (Fig. 1), suggesting the possibility of c-*kis* amplification.

When the original MCA tumor cells or transfectants were analyzed for *kis*-related transcripts, polyadenylated RNA's of 5.2 and 2.1 kilobases were observed. The sizes of these transcripts were similar to those detected in untreated NIH/3T3

Table 1. Transforming activity of DNA's of methylcholanthrene-induced mouse tumors. Efficiency of transfection is given as the ratio of the number of foci to the number of recipient cultures. High molecular weight DNA (~ 30 μg) was used to transfect 1.5×10^5 NIH/3T3 cells by calcium phosphate precipitation (*22*) as described (*23, 24*). Cells were maintained in culture with twice-weekly changes of Dulbecco's Modified Eagle's Medium supplemented with 5 percent calf serum. Focus formation was scored at 14 to 21 days.

Donor DNA	Mouse strain	Efficiency of transfection	
		Primary	Secondary
	Control cells		
NIH/3T3	NIH Swiss	0/20	
BALB-MSV NIH/3T3	NIH Swiss	8/4	
	MCA-induced tumor cells		
T-92497	C57BL/6	5/20	51/14
MCA-1GP12	NIH Swiss	6/4	17/4
T-94736	NIH Swiss	0/20	
MCA-1GP17	C57BL/6	0/4	

cells, a further indication that there was no gross alteration in the activated *kis*-related gene (data not shown). The amount of *kis*-related transcripts in the transfectants tested appeared to be greater than that in the MCA tumor cells, a finding also consistent with *kis* DNA amplification in the transfectants.

In the present studies, two of four MCA fibrosarcomas analyzed were shown to contain transforming genes capable of serial cycles of transfection. Moreover, we observed a common pattern of restriction endonuclease inactivation of the biologic activity of these genes. Finally, it was possible to demonstrate that the transforming sequences transmitted were specifically hybridized by the retroviral *onc* gene, v-*kis*. These findings establish that c-*kis* is activated as a transforming gene at high frequency in such tumors.

A large number of transforming genes derived from tumors of many different somatic cell types are related to the *onc* genes of just a few retroviruses: Kirsten-, BALB-, and Harvey-MSV (*8–11*). These findings suggest that the number of cellular genes that can demonstrate transforming activity in the NIH/3T3 transfection assay may be limited. It is possible that the transfection assay selects for these oncogenes, although NIH/3T3 cells are comparably sensitive to transfection by a number of other retroviral *onc* genes. The *kis* gene and related *onc* genes of Balb- and Harvey-MSV are capable of inducing a variety of tumors (*12–15*). In tissue culture these *onc* genes transform fibroblasts (*16–18*), as well as cells of hematopoietic origin (*15, 19*), and can even alter the growth and differentiation of epithelial cells (*20, 21*). Thus, the diverse cell types in which these genes have been detected as transforming genes may reflect the pleiotropic nature of their transforming actions for cells at many stages of differentiation.

Shilo and Weinberg (*3*) demonstrated a dominant transforming gene associated with mouse cells transformed by MCA in tissue culture. The pattern of restriction endonuclease inactivation of this transforming gene (*3*) was similar to that observed in our present studies, suggesting that c-*kis* may be activated as a transforming gene in the MCA transformants analyzed by Shilo and Weinberg as well. Comparison of the transforming and normal alleles of c-*kis* combined with knowledge of the mode of action of MCA should provide detailed information concerning the genetic alterations that lead to the frequent activation of this gene in MCA tumors. Moreover, identification of this transforming gene should be helpful in establishing whether it is activated at a specific stage in the neoplastic process.

Table 2. Restriction enzyme inactivation of the T-92497 transforming gene. High molecular weight DNA (~ 30 μg) or digested DNA of 4-8-1, a T-92497 first-cycle transfectant, was applied to 1.5×10^5 NIH/3T3 cells. High molecular weight DNA was incubated with the indicated restriction endonuclease according to the conditions recommended by the manufacturer. Cells were maintained in culture, and foci were scored as described in legend to Table 1.

Restriction endonuclease	Ratio of foci to recipient cultures
Untreated	16/8
Bam HI	27/8
Pst I	0/8
Hind III	0/8
Eco RI	0/8
Bgl II	0/8
Sac I	0/8

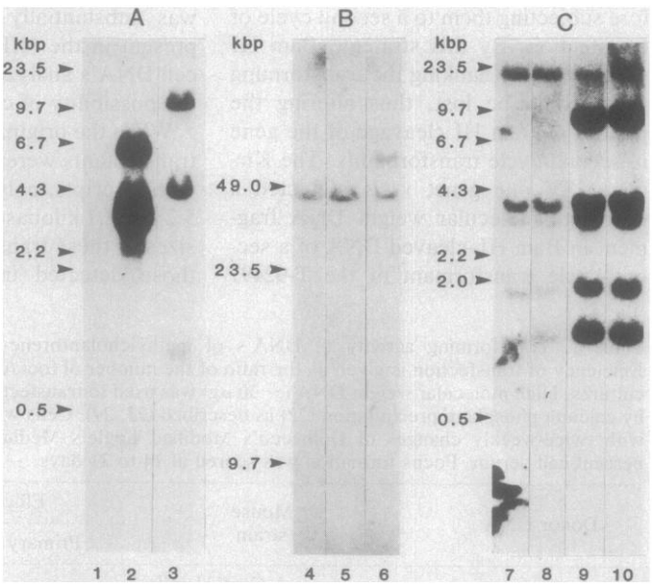

Fig. 1. Detection of exogenous DNA sequences in T-92497–derived first- and second-cycle transfectants. Southern blot analysis of digested cellular DNA's from transfectants were probed with v-*kis* and pBR322 specific probes. Each DNA (20 μg) was digested with (A and C) Hind III or (B) Bam HI, fractionated by electrophoresis through an agarose gel, and transferred to nitrocellulose paper. The filters were incubated with (A) nick-translated ^{32}P-labeled pBR322 DNA (2.5×10^6 cpm/ml) or (B and C) Pvu II to Pvu II v-*kis* DNA (*25*) specific probes. The DNA's analyzed were from the following cell lines: (lanes 1, 4, and 7) NIH/3T3; (lane 2) 13-1-1, a T-92497–derived second-cycle transfectant; (lane 3) 34-1-1, an MCA-1GP12–derived first-cycle transfectant; (lane 5) 30-3-1, a T-92497–derived second-cycle transfectant; (lane 6) 30-2-1, a cell line derived from a focus obtained by transfecting 4-8-1 DNA digested with Bam HI; (lane 8) T-92497; (lane 9) 4-8-1, a T-92497–derived first-cycle transfectant; and (lane 10) 8-2-2, a T-92497–derived second-cycle transfectant. DNA's shown in lanes 4, 5, and 6 were run for 60 hours at 30 V in a 0.5 percent agarose gel. Other DNA's were run for 16 hours at 30 V in a 0.7 percent agarose gel.

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