Jpn. J. Cancer Res. **81**, 333–339, April 1990

Alterations of Mouse Proto-oncogenes in Sarcomas Induced after Transplantation of Human Tumors in Athymic Nude Mice

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During serial subcutaneous transplantation of several types of human tumors into nude mice, the local development of malignant mouse-specific sarcomas has been observed. Although the frequency of sarcoma induction is low, this phenomenon is very important because the mouse-specific sarcomas completely replaced the human tumors during serial transplantation. The DNA of five independently induced mouse-specific sarcomas was transfected into NIH/3T3 cells in order to detect oncogenes associated with mouse-specific sarcoma induction. Two of these DNAs were found to carry activated mouse c-N-*ras* and c-Ki-*ras* genes. The sequence analysis of the molecularly cloned mouse c-N-*ras* oncogene showed a single nucleotide transition from G to A at the 12th codon. This results in substitution of aspartic acid for glycine at this position. The mouse c-*myc* gene was also found to be amplified in a sarcoma. In these mouse sarcoma DNAs, human *Alu* sequences were not detected. These data strongly suggest that the mouse-specific sarcomas were not induced by the transfer of human transforming sequences but by the alterations of mouse proto-oncogenes.

Key words: Oncogene — *ras* gene — c-*myc* gene — Nude mouse — Transplantation

The ability of athymic nude mouse to maintain human tumor xenografts has been utilized extensively by many laboratories for cancer research. However, the induction of mouse-specific sarcomas has been reported in nude mice after transplantation of human tumors.[1,2] In our laboratory, five mouse-specific sarcomas appeared and replaced the respective human tumors, out of 187 human tumor xenografts, during serial transplantation in nude mice. It is important to elucidate the mechanism of sarcoma induction not only to prevent new sarcoma induction during the transplantation of human tumors but also to clarify the effect of human tumor cells on the surrounding stromal cells. In previous reports,[1,3] it has been suggested that human transforming sequences may have been transferred from human tumor cells to the adjacent mouse cells. We tried to detect transforming genes by transfecting mouse-specific sarcoma DNA into NIH/3T3 cells. Many human and animal transforming genes have been detected in a wide variety of human and animal tumor cells.[4,5] The majority of cellular transforming genes identified in this manner are related to the *ras* gene family.[6-9] The activation of *ras* oncogenes in human and animal tumors is most commonly due to point mutations at position 12 or 61 in the *ras* coding sequences.[10-12]

MATERIALS AND METHODS

Transfection assays DNA transfection analysis was carried out according to the calcium phosphate precipitation method as described previously.[13,14] NIH/3T3 cells, plated 24 h earlier at 1.5×10^5 cells per 10-cm Petri dish, were transfected with 30 μg of high-molecular-weight DNA. Cells were maintained in culture with twice-weekly changes of Dulbecco's modified Eagle's medium supplemented with 5% calf serum. The foci of transformed cells were scored at 14–21 days.

DNA blot analysis High-molecular-weight DNA was digested with appropriate restriction endonucleases under the conditions suggested by the manufacturers. The digested DNAs were electrophoresed in 1% agarose gels, which were blotted onto nitrocellulose filters as described before.[15]

Molecular cloning The λgtWES.λB strain of λ phage was propagated in *Escherichia coli* strain BNN45.[16] Phage DNA and cellular DNA were digested with *Eco*RI and purified independently by preparative sucrose gradient centrifugation. Purified phage arms and cellular DNA fragments were ligated at 1:1 molar ratio by T4 DNA ligase and packaged *in vitro* into phage particles.

Positive plaques were identified by *in situ* hybridization of phage plaques.[16)]

Nucleotide sequencing DNA was sequenced directly from a plasmid.[17)] Single-stranded DNA was obtained from the plasmid by an alkaline treatment and annealed with specific synthetic oligomers. Then, nucleotide sequences were determined by the dideoxy chain termination method.[18)]

RESULTS

Transforming activities of mouse-specific sarcoma DNA Table I lists the five mouse-specific tumors used in this study. All the tumors were accidentally found to be fibrosarcomas on histological examination after serial subcutaneous transplantation of human tumors into athymic nude mice. All the tumors showed a mouse-specific karyotype on karyological analysis. To examine whether human DNA sequences were present in the mouse-specific tumor cells, the DNAs of the five mouse tumors were subjected to Southern blot analysis using a probe specific for human *Alu* family repetitive sequences (0.3 kbp *Bam*HI fragment of pBLUR8).[19)] None of the mouse tumor DNAs showed the presence of the *Alu* sequences (Fig. 1, lanes 3–7), whereas the human placenta DNA demonstrated numerous bands containing the *Alu* sequences (Fig. 1, lane 1). As a positive control, Fig. 1, lane 2 shows that the *Alu* probe could detect the human sequences in the 84-3 hepatoma DNA-derived mouse tertiary transformants.[20)] The mouse tumor DNAs did not show any hybridizing band with total human DNA as a probe, either (data not shown). These results clearly show that these tumors were not human tumors but mouse tumors. The mouse-specific tumors were given designations such as M(LM1-JCK), where

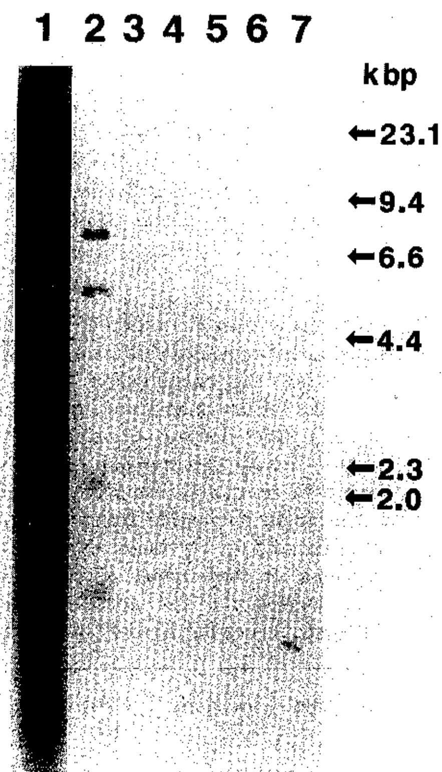

Fig. 1. Detection of human DNA sequences in mouse-specific tumor DNAs. The DNAs, 20 μg except in lane 1 (5 μg), were digested with *Eco*RI and then analyzed by Southern hybridization for the presence of human repeat sequences using the nick-translated *Alu* sequence as a probe. DNAs were obtained from human placenta (lane 1), the 84-3 hepatoma DNA-derived mouse tertiary transformants (lane 2), M(LM1-JCK) (lane 3), M(RB1) (lane 4), M(NB-5-JCK) (lane 5), M(Hpb-T-JCK) (lane 6) and M(CRA-1-JCK) (lane 7) tumors. Coelectrophoresed DNA fragments of *Hin*dIII-digested λcI857 DNA served as standards. kbp, kilobase pairs.

Table I. Transforming Activity of DNAs Isolated from Mouse-specific Sarcomas and Their NIH/3T3 Transfectants

Mouse-specific sarcomas	Original human tumors		Transfection efficiency (no. foci/no. recipient cultures)	
			Primary	2nd cycle
M(LM1-JCK)	T cell lymphoma		1/24	72/8
M(RB1)	Retinoblastoma	Exp. 1	2/8	78/16
		Exp. 2	2/7	
		Exp. 3	4/7	
M(NB-5-JCK)	Neuroblastoma		0/32	
M(Hpb-T-JCK)	Hepatoblastoma		0/16	
M(CRA-1-JCK)	Craniopharyngioma		0/16	

The DNAs prepared from mouse-specific sarcomas as well as first-cycle NIH/3T3 transfectants were used to transfect NIH/3T3 cells as described in "Materials and Methods."

the designation of the original human tumors is given in parenthesis.

When high-molecular-weight DNA of the five mouse tumors was subjected to transfection analysis using NIH/3T3 cells, we observed focus formation in two tumors, M(LM1-JCK) and M(RB1) (Table I). In the case of M(RB1), 8 foci in total appeared in the three independent experiments. Both foci-derived transfectant DNAs also transformed NIH/3T3 cells at the second cycle (Table I).

Identification of two transforming genes To determine if there was a relationship between the two transforming genes and any known oncogenes, we subjected the restriction enzyme-digested transfectant DNAs to molecular hybridization with the Ha-*ras* (0.5 kbp *Eco*RI fragment of pBS9), Ki-*ras* (0.9 kbp *Hin*cII fragment of v-K-*ras*) and N-*ras* (0.9 kbp *Pvu*II fragment of human N-*ras*), and *raf* (1.3 kbp *Bst*EII-*Xba*I fragment of p-v-*raf*) and *erb*B2/*neu* (1.6 kbp *Eco*RI fragment of pCER 237) genes as probes. If the two transforming genes were mouse oncogenes, it would be difficult to identify the transforming genes in mouse NIH/3T3 cells, because the mouse transforming genes would give the same sized hybridizing bands as mouse proto-oncogenes. We therefore used the second cycle transfectant DNAs and suitable restriction enzymes for hybridization to detect subtle rearrangements of the transforming genes, which might occur during the transfection process.

When the M(LM1-JCK) transfectant DNAs were analyzed using the N-*ras* specific probe, two transfectant DNAs exhibited additional hybridizing bands not observed in the NIH/3T3 cells (Fig. 2, left panel, lanes 5 and 6). The first cycle and other second cycle transfectant DNAs showed a band that was of the same size but amplified compared with the band of NIH/3T3 cells (Fig. 2, left panel, lanes 3 and 4), implying that there were extra c-N-*ras* genes other than the endogenous c-N-*ras* genome in the transfectants. When the same filter was rehybridized with the Ki-*ras* specific probe, the mouse

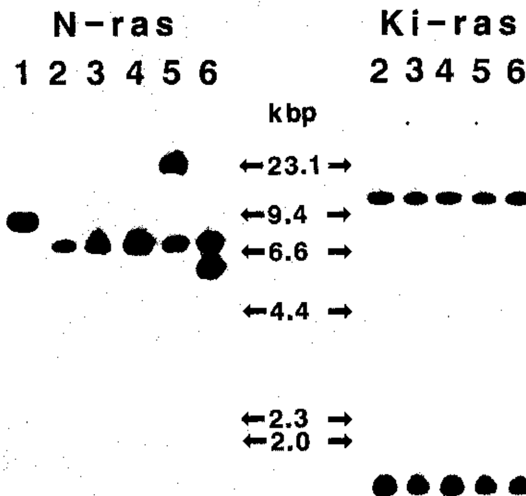

Fig. 2. Identification of the M(LM1-JCK) transforming gene. The DNAs (10 μg) were digested with *Eco*RI and then analyzed by Southern hybridization. The filter was hybridized with the human c-N-*ras* first exon-specific *Pvu*II 0.9 kbp fragment (left panel) and the v-Ki-*ras* *Hin*cII 0.9 kbp fragment[21] (right panel). The DNAs analyzed were from the following cells: (lane 1) normal human skin fibroblasts, M413; (lane 2) NIH/3T3; (lane 3) an M(LM1-JCK)-derived first cycle transfectant; (lanes 4–6) M(LM1-JCK)-derived second cycle transfectants. Size markers in kbp appear at the center.

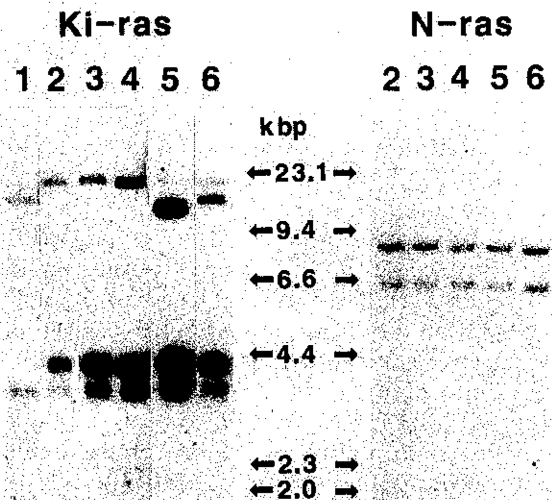

Fig. 3. Identification of the M(RB1) transforming gene. The DNAs (10 μg) were digested with *Hin*dIII and then analyzed by Southern hybridization. The filter was hybridized with the v-Ki-*ras* (left panel) and c-N-*ras* (right panel) specific probes. The DNAs analyzed were from the following cells: (lane 1) human M413; (lane 2) NIH/3T3; (lane 3) an M(RB1)-derived first cycle transfectant; (lanes 4–6) M(RB1)-derived second cycle transfectants. Size markers in kbp appear at the center.

tumor DNAs showed three bands that were of the same size and same intensity as the bands of NIH/3T3 cells (Fig. 2, right panel). Thus, all the transfectants invariably contain the extra mouse c-N-*ras* genes in addition to the endogenous c-N-*ras* genome, suggesting that the transforming gene in the M(LM1-JCK) tumor cells is an activated mouse c-N-*ras* gene.

Several *Hin*dIII-digested M(RB1) transfectant DNAs with the Ki-*ras* specific probe showed extra hybridizing bands not seen in the NIH/3T3 cells (Fig. 3, left panel, lanes 5 and 6). The first cycle and other second cycle transfectant DNAs exhibited amplified bands (Fig. 3, left panel, lanes 3 and 4). When the same filter was rehybridized with the N-*ras* specific probe, the tumor DNAs showed two bands that were indistinguishable from the bands of NIH/3T3 cells (Fig. 3, right panel). *Eco*RI-digested M(RB1) transfectant DNAs demonstrated three bands that were of the same size but also amplified compared with the bands of NIH/3T3 cells with the Ki-*ras* specific probe (data not shown). These data indicate that the M(RB1) transforming gene is an activated mouse c-Ki-*ras* gene.

Molecular cloning and characterization of the M(LM1-JCK) oncogene The restriction map of the mouse c-N-*ras* gene is presented in Fig. 4A.[22] To characterize the c-N-*ras* oncogene associated with the M(LM1-JCK) tumor, we cloned its sequence from a second cycle transfectant, 56-1-6. *Eco*RI-digested DNA of this transfectant showed an endogenous 7.4 kbp band and a new 6.1 kbp band with the human c-N-*ras* first exon specific probe (Fig. 2, left panel, lane 6). The 56-1-6 DNA was digested completely with *Eco*RI, and the fragments were separated by sucrose density gradient centrifugation. Fractions that contained the 6.1 kbp fragment were ligated to phage λgtWES. λB purified arms and packaged *in vitro* into phage particles. From 200,000 plaques screened, three positive clones were obtained using the c-N-*ras* first exon specific probe. Following plaque purification, the clone DNAs were subjected to agarose gel electrophoresis after *Eco*RI cleavage. Each insert showed the expected size of 6.1 kbp. One representative insert was subcloned into plasmid pAT153 and the subclone was designated as pLM1.

Restriction enzyme analysis was carried out to compare the normal c-N-*ras* and pLM1 sequences, using *Bam*HI, *Hae*II, *Pvu*II and *Sac*I. Fig. 4A shows that the restriction map of the pLM1 sequences closely corresponded with the mouse c-N-*ras* gene. Divergence was noted beyond the second *Hae*II site at the 5′ end of the c-N-*ras* gene. Presumably this was due to rearrangement introduced at the end of the DNA during the transfection process.

The great majority of mutations identified in activated human and animal *ras* genes involve the 12th and 61st codons, with the majority of activated N-*ras* genes showing mutations at codon 61.[11] We performed nucleotide sequence analysis on the first and second exons of the pLM1 sequence, in comparison with the corresponding exons of the mouse c-N-*ras* proto-oncogene.[22] The pLM1 single-stranded DNA was annealed with four synthetic 20mers to sequence codons 12 and 61 from both strands. Analysis of the first exon revealed the presence of a single base change of G to A within the 12th codon of the coding sequence (Fig. 4B). This results in a change from glycine to aspartic acid at this position in the protein. No differences were observed in the corresponding sequences of the second exon containing codon 61 (Fig. 4B). These results suggest that a point mutation in codon 12 is

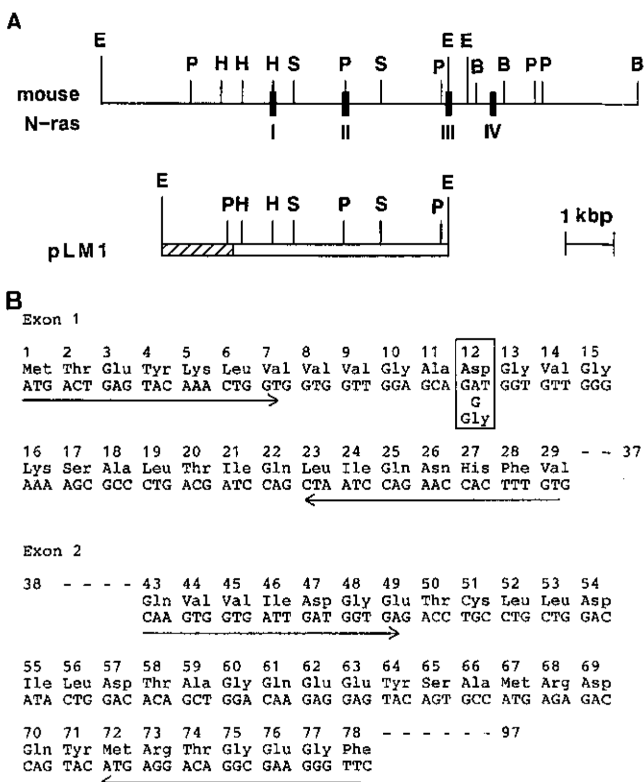

Fig. 4. A. Restriction endonuclease maps of the mouse c-N-*ras* gene (top) and the cloned c-N-*ras* gene; hatched block, sequences unrelated to the mouse c-N-*ras* gene. B, *Bam*HI; E, *Eco*RI; H, *Hae*II; P, *Pvu*II; S, *Sac*I. B. Nucleotide sequence and predicted amino acid sequence of the first and second exons of pLM1. Arrows indicate the four 20mers used as specific primers. They are ATGACTGAGTACAAACTGGT, ACAAAGTGGTTCTGGATTAG, CAAGTGGTGATTGATGGTGA, and AACCCTTCGCCTGTCCTCAT. Sequences are derived from Guerrero *et al.*[22] A single base change (G to A) and the consequent amino acid change of glycine to aspartic acid are boxed.

responsible for the activation of the M(LM1-JCK) oncogene.

Amplification of a c-*myc* gene To examine the possible involvement of other oncogenes in mouse sarcoma induction, the mouse sarcoma DNAs were hybridized with 16 oncogene probes; *abl* (0.7 kbp *Bgl*II fragment of v-*abl*), *erbB*1 (2.4 kbp *Cla*I fragment of pE7), *fgr* (1.7 kbp *Bam*HI fragment of pv-*fgr*1700), *fos* (1.3 kbp *Pst*I fragment of p*fos*-1), *fps/fes* (0.4 kbp *Bam*HI fragment of pBR-FO4), *mos* (2.5 kbp *Eco*RI fragment of human c-*mos*), *myb* (2.6 kbp *Eco*RI fragment of human c-*myb*), c-*myc* (0.8 kbp *Cla*I-*Eco*RI fragment of human c-*myc*), N-*myc* (2.0 kbp *Eco*RI fragment of pN-*myc*), Ha-*ras*, Ki-*ras*, N-*ras*, *rel* (1.0 kbp *Eco*RI fragment of p-v-*rel*), *ros*, (2.2 kbp *Hin*dIII-*Eco*RI fragment of pROS-BH3.2), *sis* (1.3 kbp *Pst*I fragment of p-v-*sis*) and *yes* (1.7 kbp *Sal*I fragment of pYS2). No oncogene probes showed rearrangement of proto-oncogenes in the mouse sarcoma DNAs. None of the probes other than the c-*myc* gene showed amplification of proto-oncogenes in these tumors. Among five mouse sarcomas, the M(RB1) tumor DNA showed a higher intensity of c-*myc*-related DNA fragment (Fig. 5). The amplified c-*myc*-related bands exhibited the same size as the mouse c-*myc* proto-oncogene (Fig. 5) but not as the human c-*myc* proto-oncogene[23] with three restriction endonucleases, *Eco*RI, *Hin*dIII and *Bam*HI. These data indicate that the mouse c-*myc* gene is amplified. The degree of amplification was about 20-fold. As a control, these DNAs were hybridized with c-*mos* probe (Fig. 5). The intensity of the c-*mos* bands in the M(RB1) tumor was essentially the same as in the control mouse spleen DNA.

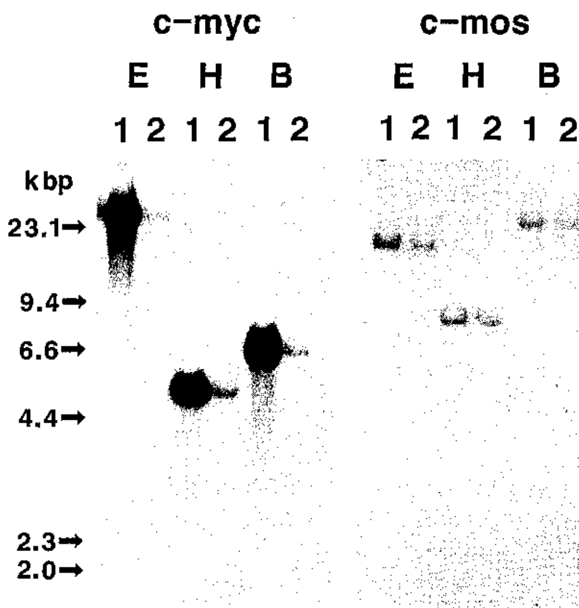

Fig. 5. Southern blot analysis of mouse sarcoma and normal cellular DNAs. The DNAs (10 μg) were separately digested with (E) *Eco*RI, (H) *Hin*dIII or (B) *Bam*HI. The probes used were the 0.8 kbp *Cla*I-*Eco*RI fragment containing almost the entire sequence of the human c-*myc* exon 3 and the 2.75 kbp *Eco*RI fragment containing the entire human c-*mos* gene. The DNAs were obtained from the M(RB-1) sarcoma (lane 1) and a nude mouse spleen (lane 2). Size markers in kbp appear on the left.

DISCUSSION

It has been reported that mouse-specific sarcomas replaced human tumor xenografts during serial transplantation in nude mice.[1, 2] The occurrence of spontaneous sarcomas in BALB/c nude mice is very rare. The sarcomas appeared at the sites of the transplanted human tumors. Therefore, the mouse-specific sarcomas must have been induced by a certain factor or stimulus derived from the human tumors directly or indirectly. There are at least three possible mechanisms for the sarcoma induction. Firstly, a transforming virus may have been transferred from the human tumor cells to the surrounding mouse stromal cells. Alternatively, a transforming virus may have been induced within the mouse cells adjacent to the human tumor xenografts. Secondly, transforming sequences may have been transferred from the human tumor cells to the adjacent mouse cells. Thirdly, one of the mouse proto-oncogenes may have been activated by a certain factor or stimulus derived from the human tumor cells.

We infected mouse embryo fibroblasts with mouse sarcoma extracts. However, we could not detect any morphological change of the cells (unpublished data). Extensive attempts to demonstrate retrovirus activity in rat or human tumors have been unsuccessful in the case of rat sarcomas induced in rats treated with anti-thymocyte sera after transplantation of human tumor cells.[3] These data imply that transforming viruses are not involved in sarcoma induction.

Are human sequences related to the mouse-specific sarcoma induction? The five mouse sarcoma DNAs did not show any hybridizing bands with the *Alu* sequence or total human DNA as probes, indicating that there were no detectable human repeated sequences in the mouse tumors. We also hybridized the mouse sarcoma DNAs with 16 oncogene probes as described above. No additional hybridizing bands were detected with any of the probes other than the respective mouse proto-oncogene-related bands.

The M(LM1-JCK) and M(RB1) tumor DNAs transformed NIH/3T3 cells. Several second cycle transfectant DNAs exhibited extra hybridizing bands with the N-*ras* and Ki-*ras* specific probes, respectively. Since all the first

cycle transfectant DNAs showed the same-sized bands as the NIH/3T3 cells, it is most likely that these extra bands were derived from the mouse genes during the transfection process. If these bands had originated from the human genomes, the M(LM1-JCK) and M(RB1) tumor DNAs should have demonstrated *Alu*-related bands because both the human c-N-*ras* and c-Ki-*ras* 2 genes contain *Alu* sequences in their introns.[8, 24] The restriction map of the cloned c-N-*ras* fragment from the M(LM1-JCK) transfectant also showed that the extra c-N-*ras*-related band was derived from the mouse c-N-*ras* gene. Therefore, the M(LM1-JCK) and M(RB1) transforming genes are the activated mouse c-N-*ras* and c-Ki-*ras* genes, respectively. These results indicate that human transforming sequences are not responsible for at least the maintenance of the transformed state of the mouse sarcoma cells.

Gupta *et al.*[25] reported that establishment of cell lines *in vitro* from a human lung cancer xenograft in nude mice resulted in transformed mouse cell lines. Human DNA sequences were present in their cell lines, indicating that spontaneous transfection of human tumor DNA into host cells had occurred. Malignant transformation of mouse cells occurred in culture in their case,[25] while it occurred in mice in our cases. The difference might be closely related to whether human DNA is directly involved in the transformation process or not.

Transforming activity was detected in two DNAs from the five mouse sarcomas. Both were found to be activated mouse *ras* genes. The M(LM1-JCK) c-N-*ras* oncogene was shown to contain a point mutation at the 12th codon. The mechanism of activation of the M(RB1) c-K-*ras* oncogene is now under investigation. At least in these two mouse sarcomas, the activation of the *ras* proto-oncogenes would have been induced by a factor or stimulus derived from the human tumor xenografts and would play an important role in the induction of sarcomas. The factor might be a growth factor, such as a transforming growth factor.[26] It remains to be determined what kind of factor is involved in the activation of the *ras* proto-oncogenes. It has been reported that many chemical carcinogens activate *ras* proto-oncogenes in mice and rats.[12, 27, 28] However, our findings show that the activation of *ras* proto-oncogenes can be induced or enhanced by a certain biological factor.

Activated *ras* oncogenes and other uncharacterized oncogenes have been detected in spontaneously occurring mouse liver tumors.[29] Thus, nude mice fibroblasts with activated *ras* genes could have been present before the transplantation of the human tumors. Then, growth factors and/or other agents from the human tumors could have promoted the previously "initiated" mouse cells. This possibility cannot be ruled out at present.

In general, malignant neoplasms are thought to be formed through a multistep process. Land *et al.* have reported that the tumorigenic conversion of rodent primary embryo fibroblasts requires the cooperation of the *ras* and *myc* oncogenes.[30] The coexistence of the activated c-N-*ras* gene and the amplified c-*myc* gene has also been reported in the HL-60 human promyelocytic leukemia cell line.[31] The M(RB-1) DNA was found to contain an elevated level of the mouse c-*myc* gene. Thus, the amplified mouse c-*myc* gene, together with the mouse transforming c-Ki-*ras* gene, might be involved in the M(RB-1) sarcoma induction. Further investigations are necessary to clarify the mechanism of mouse sarcoma induction, especially in the cases of the three sarcomas for which altered proto-oncogenes were not detected.

ACKNOWLEDGMENTS

We thank Drs. Nobuo Tsuchida, Tadashi Yamamoto, Angel Pellicer, Marianne Oskarsson and the Japanese Cancer Research Resources Bank for the oncogenes, Dr. Kyoichi Okubo for the M(RB1) tumor, Dr. Tadayuki Takeda and Dr. Masayuki Yamamoto for synthetic oligomers, Dr. Hiroo Hoshino for his encouragement, Yasushi Sato for his technical assistance, and Ms. S. Fujii and S. Yoshida for preparing the manuscript. This work was supported in part by Grants-in-Aid for Cancer Research and for Special Project Research on Cancer Bioscience (61210008) from the Ministry of Education, Science and Culture of Japan.

(Received November 24, 1989/Accepted February 14, 1990)

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