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Overexpression and Amplification of the c-*myc* Gene in Mouse Tumors Induced by Chemicals and Radiations

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We examined expression of the c-*myc* gene by the dot blot hybridization of total cellular RNA from mouse primary tumors induced by chemicals and radiations. Expression of the c-*myc* gene was found to be elevated in 69 cases among 177 independently induced tumors of 12 different types. DNA from tumors overexpressing the *myc* gene was analyzed by Southern blotting. No case of rearrangement was detected. However, amplification of the c-*myc* gene was found in 7 cases of primary sarcomas. These included 4 cases out of 24 methylcholanthrene-induced sarcomas and 3 cases out of 7 α-tocopherol-induced sarcomas. We also analyzed 8 cases of sarcomas induced by radiations, but could not find changes in the gene structure of the c-*myc* gene. Thus, our data indicate tumor type specificity and agent specificity of c-*myc* gene amplification.

Key words: *myc* amplification — Mouse sarcoma — Methylcholanthrene — Vitamin E — Radiation

Oncogenic transformation of cells involves a variety of changes in cellular genes, particularly oncogenes. Many cellular oncogenes have been conserved throughout the evolution of mammals, vertebrates and even invertebrates. The *ras* gene family serves as a good example of evolutional conservation of oncogenes.[1] Cellular oncogenes are thought to participate in cellular proliferation, differentiation and development. Some oncogenes are expressed in limited tissues and cells at particular states of differentiation, while others are expressed in a wide range of cell types. The c-*myc* gene, a member of the *myc* gene family, belongs to the latter class of oncogenes and is expressed in almost all types of cells with a high potential for proliferation.

Activated forms of the c-*myc* gene are found in a variety of tumors. The mode of activation involves retrovirus insertion,[2-4] rearrangements[5-9] and amplification.[10-12] Retrovirus insertion and rearrangements of the c-*myc* gene are frequently observed among leukemias and lymphomas. The amplified form of the c-*myc* gene is found in a wider range of tumors.

Changes in the c-*myc* gene have been well studied in human tumors by many investigators. The number of tumors studied in some cases was large enough so that the frequency of the c-*myc* gene activation could be estimated.[13] We attempted to survey the c-*myc* gene activation in mouse tumors induced by a variety of agents. This type of study, if done on a sufficient number of tumors, can answer the following two important questions. 1) What is the tumor type specificity of the c-*myc* gene activation? 2) What is the agent specificity of the *myc* gene activation? The results of our study are presented in this communication.

MATERIALS AND METHODS

Mice C57BL/6N×C3H/He F_1 (BCF_1) mice and Balb/c mice were purchased from Charles River Japan, Inc., Atsugi, Kanagawa Pref. Balb/c *nu/nu* mice were kindly supplied by Dr. A. Matsuzawa, Institute of Medical Science, University of Tokyo. NFS mice and C3H/He× C57BL/6N F_1 (CBF_1) mice were bred in the animal facility of our institute.

Tumor induction Seven- to 8-week-old mice were treated with a variety of chemicals and radiations. Chemicals used and their modes of application were as follows. Methylcholanthrene (MCA) of 0.1 mg, 0.8 mg or 1 mg was dissolved in 0.1 ml of olive oil and injected subcutaneously into mice. Sarcoma induction by α-tocopherol (TP) was done using a procedure established by others.[14] Briefly, 20 mg of α-tocopherol was dissolved in 0.1 ml of soybean oil and injected weekly into mice subcutaneously until a tumor developed at the site of injection. Diethylene glycol (DG) was added at 0.5% in drinking water. Nitrosoethylurea (NEU) dissolved in 10% ethanol was administered orally through polyethylene tubing at 4 mg per mouse first, and a further 1 mg per mouse was given 6 days after the first treatment. Diethylenenitrosamine (DEN) was added at 0.01% to drinking water. Mice were fed basal diet containing 0.05% phenobarbital (PB).

Tumors were also induced by a variety of radiations (Rad). Irradiations of mice with ^{252}Cf neutrons and ^{60}Co gamma rays were carried out at room temperature using the irradiation units at our institute. Tritiated water (HTO) was injected into mice intraperitoneally, and

mice thus treated were kept at the tritium research unit of our institute.[15]

Mice were killed when the development of tumors was evident. Tumors were weighed, and part of the tumor tissues was fixed in 10% formaldehyde and processed for histological examinations. Another part was processed to isolate RNA and DNA.

Purification of DNA and RNA Tumor tissues were quickly frozen by immersing them in liquid nitrogen and then ground to fine powder in a mortar. For the isolation of DNA, ground tissues were processed as described previously.[16] Briefly, tissues were lysed in a buffer containing 1% sodium dodecyl sulfate, 0.1 *M* NaCl, 5 m*M* EDTA, 20 m*M* Tris-HCl (pH 8.0) and 100 μg/ml RNaseA. After incubation at 37°C for 1 h, proteinase K was added to 100 μg/ml and the lysate was further incubated. DNA was recovered by ethanol precipitation after phenol-chloroform extraction of the lysate.

For the purification of RNA. the ground tissues were lysed in 4 *M* guanidine thiocyanate, 25 m*M* sodium citrate, 0.5% sodium N-lauroyl-sarcosine and 0.1 *M* 2-mercaptoethanol. The lysate was layered onto 5.7 *M* CsCl and total cellular RNA was isolated through sedimentation.[17]

DNA RNA hybridization and densitometric analysis Cellular DNA was digested with appropriate restriction enzymes and processed for Southern blotting hybridization.[18] Total cellular RNA was denatured in 10% formaldehyde and applied to nitrocellulose filters for dot blot hybridization.[19] ^{32}P-labeled probes were made by the nick-translation procedure.[20] The intensity of autoradiograms was quantified by densitometric scanning.

Plasmids The recombinant plasmid, pSVmycKp10, which carries the 9 kb *Kpn*I fragment of the mouse c-*myc* gene was a generous gift of Dr. K. A. Marcu. The recombinant plasmid pα-2 carries the 2 kb fragment of the mouse α-globin gene[21] and was kindly provided by Dr. K. Simotohno. The plasmid pmβG carries the 3.5 kb *Eco*RI-*Xba*I fragment of the mouse β-globin gene and was supplied by Dr. M. Obinata. The plasmid p15 carries the mouse ribosome RNA gene[22] and was kindly given to us by Dr. R. Kominami.

RESULTS

Survey of *myc* gene overexpression in mouse tumors Total cellular RNA's from 177 independently induced primary tumors of 12 different types were analyzed. RNA was extracted from mouse fibroblasts at a semi-confluent stage of growth and was used as a control. Duplicate filters were made and each was probed with the c-*myc* gene and ribosomal RNA gene. The autoradiograms were scanned by a densitometer. The intensity of the c-*myc*-probed dot blot on X-ray film was normalized for the amount of RNA assayed by using the ribosomal RNA. Tumors were judged as overexpressing the *myc* gene when the autoradiogram of the dot was more than twice as intense as the control. Table I summarizes the number of cases of *myc* overexpression for each tumor type. The level of c-*myc* gene expression was elevated in a variety of tumors. Out of 177 tumors, 62 (35%) were expressing the c-*myc* gene at significantly higher levels.

When each tumor type was examined, there existed variations in the frequency of the overexpression. Statistical analysis by use of the χ^2 test indicated that the frequency was significantly lower among hepatomas and thyroid tumors. On the contrary, the frequency of the

Table I. Frequency of c-*myc* Gene Overexpression in Mouse Tumors

Tumor	Inducer[a]	Case no.	Overexpressed case	%
Sarcoma	MCA, TP, Rad	39	18	46
Ovarian tumor	Rad	28	7	25
Hepatoma	TP, DEN, DG, PB, Rad	28	1	3.5
Mammary tumor	NEU, Rad	12	4	33
Lung tumor	TP, Rad	4	0	0
Thyroid tumor	Rad	14	0	0
Lymphosarcoma	Rad	4	3	75
Pituitary tumor	Rad	4	2	50
Stomach tumor	TP	3	0	0
Adrenal tumor	DG	2	2	100
Thymoma	NEU, Rad	31	20	65
Non-thymic lymphoma	Rad	8	5	63
Total		177	62	35

a) Abbreviations of inducers are given in the "Materials and Methods" section of the text.

overexpression was high in thymomas. The number of cases was too small to evaluate the significance for lung tumors, lymphosarcomas, pituitary tumors, stomach tumors and adrenal tumors.

It is our experience that tumor tissue fragments transferred *in vitro* shed numerous macrophages and granulocytes during the initial few days of culture. This suggested that tumors *in vivo* carried cells participating in inflamation and immunological reactions. Thus, the total cellular RNA isolated from the primary tumors is usually contaminated with that of macrophages and granulocytes. Therefore, the significance of the c-*myc* overexpression might be questionable for some of the tumor samples.

Amplification of the *myc* gene in mouse sarcomas DNA's of 62 tumors overexpressing the c-*myc* gene were analyzed by Southern blotting. DNA was restricted with *Eco*RI and the filters were hybridized with the ^{32}P-labeled 9 kb *Kpn*I fragment of the mouse c-*myc* gene which carries all the exons and introns together with the 5′ and the 3′ flanking regions of the gene. The *Eco*RI digest of mouse DNA yielded a 30 kb band when probed with the c-*myc* gene. As an internal marker, the mouse β-globin gene was used as a probe which gave a 7.8 kb band. We noticed that cases with the amplified c-*myc* gene were always sarcomas (data not shown).

We then examined 39 cases of independently induced sarcomas for possible changes in the c-*myc* gene. The

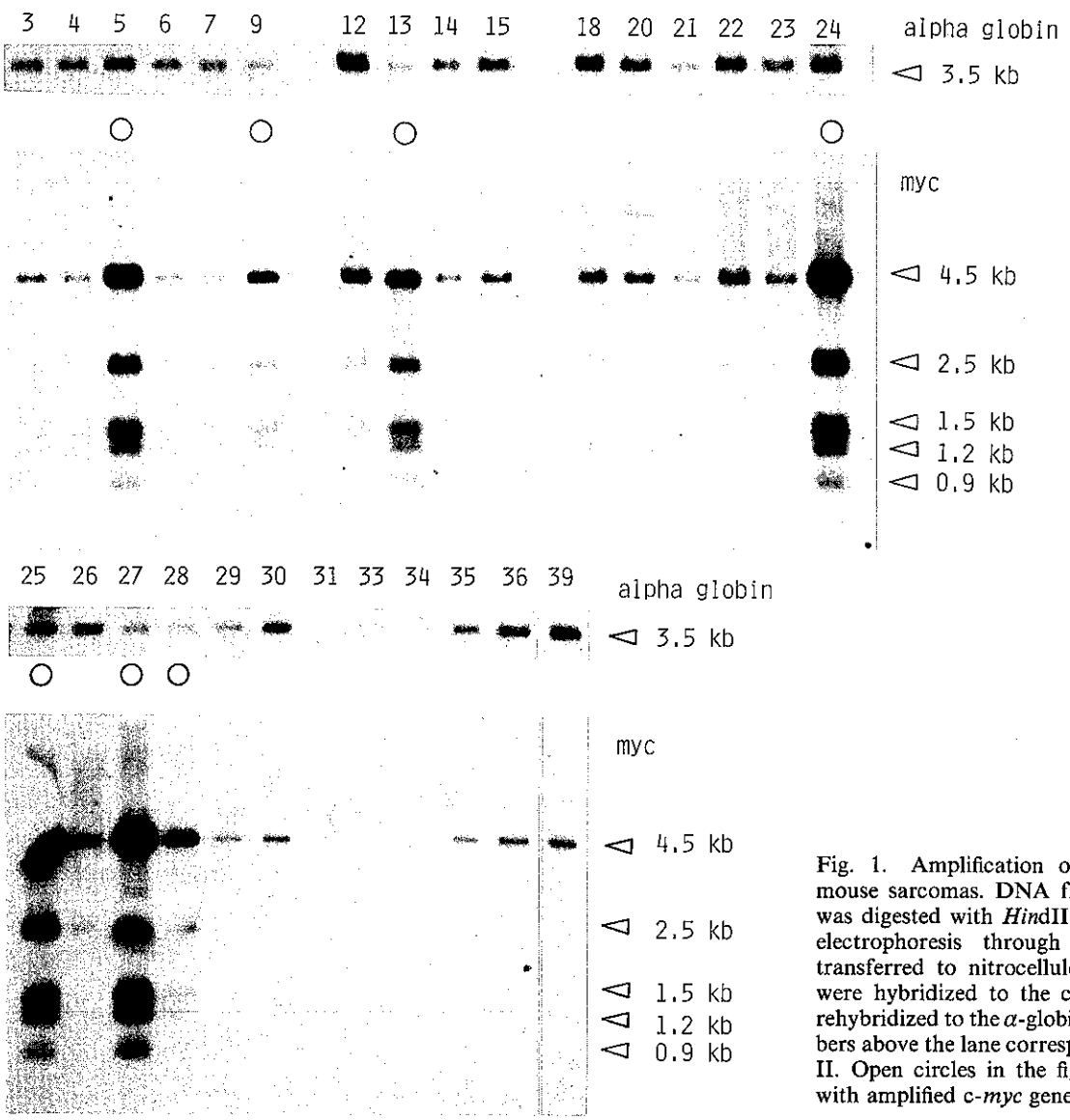

Fig. 1. Amplification of the c-*myc* gene in mouse sarcomas. DNA from sarcomas (5 μg) was digested with *Hin*dIII, size-fractionated by electrophoresis through 0.7% agarose and transferred to nitrocellulose filters. The filters were hybridized to the c-*myc* probe and then rehybridized to the α-globin probe. Sample numbers above the lane correspond to those of Table II. Open circles in the figure indicate samples with amplified c-*myc* gene.

Table II. Overexpression and Amplification of the c-*myc* Gene in Sarcomas

Sample no.	Tumor no. (inducer, dose)	Latency (months)	Overexpression	Amplification
1	NFS 1618 (MCA, 0.1 mg)	3	++	–
2	1619 (MCA, 1.0 mg)	3	–	–
3	1624 (MCA, 0.1 mg)	3	+	–
4	1634 (MCA, 0.1 mg)	5	–	–
5	1635 (MCA, 0.1 mg)	5	+	×5
6	1714 (MCA, 1.0 mg)	2.5	–	–
7	Balb/c *nu/nu* 566 (MCA, 0.1 mg)	3	–	–
8	576 (MCA, 0.1 mg)	3	–	–
9	582 (MCA, 1.0 mg)	3.5	+	×5
10	583 (MCA, 0.1 mg)	3.5	–	–
11	594 (MCA, 0.1 mg)	4	–	–
12	601 (MCA, 1.0 mg)	4.5	–	–
13	709 (MCA, 0.1 mg)	5	++	×10
14	Balb/c335 (MCA, 1.0 mg)	3.5	–	–
15	336 (MCA, 1.0 mg)	3.5	–	–
16	344 (MCA, 0.1 mg)	5	–	–
17	350 (MCA, 0.1 mg)	6	–	–
18	$CBF_1$6296 (MCA, 0.8 mg)	3.5	+	–
19	6304 (MCA, 0.8 mg)	3.5	–	–
20	6312 (MCA, 0.8 mg)	4.5	+	–
21	6328 (MCA, 0.8 mg)	5	+	–
22	6329 (MCA, 0.8 mg)	5	+	–
23	6330 (MCA, 0.8 mg)	5	+	–
24	6334 (MCA, 0.8 mg)	5.5	++	×5
	subtotal, MCA-induced		11/24	4/24
25	NFS 1757 (α-tocopherol)	8	+++	×30
26	$BCF_1$3067 (α-tocopherol)	7	–	–
27	3224 (α-tocopherol)	10	++	×30
28	3225 (α-tocopherol)	10	+	×10
29	3226 (α-tocopherol)	10	–	–
30	3249 (α-tocopherol)	10	–	–
31	4063 (α-tocopherol)	10	–	–
	subtotal, α-tocopherol-induced		3/7	3/7
32	$BCF_1$2309 (HTO, 7.5 mCi)	15	–	–
33	2940 (HTO, 20 mCi)	14	–	–
34	3104 (neutron, 271 rad)	12	+	–
35	3114 (γ-ray, 271 rad)	12	–	–
36	3116 (γ-ray, 271 rad)	17	–	–
37	3400 (γ-ray, 271 rad)	17	++	–
38	3414 (γ-ray, 271 rad)	17	++	–
39	3992 (γ-ray, 271 rad)	14	+	–
	subtotal, radiation-induced		4/8	0/8

amplified c-*myc* gene was detected in 7 cases of the sarcomas. When restricted with *Eco*RI, the mouse c-*myc* gene is cut into a fragment larger than 30 kb in length. The resolution of the Southern blot analysis using 0.7% agarose is rather poor for DNA of such size. Although the amplified c-*myc* gene in sarcomas seemed to be similar in mobility to the normal c-*myc* gene, there is still a possibility that the amplified gene might have suffered small rearrangements.

DNA from sarcomas was restricted with *Hin*dIII, which cleaves the mouse c-*myc* gene internally at multiple sites. When compared with the c-*myc* gene from

normal tissues, the amplified c-*myc* gene had the same pattern of restriction fragments (Fig.1). This indicated that the amplification was not accompanied with rearrangements.

The level of the amplification was determined by densitometric scanning of the X-ray films. The intensity of the 4.5 kb *Hin*dIII fragment of the c-*myc* gene was normalized for the amount of DNA applied as judged by that of the 3.5 kb *Hin*dIII fragment of the α-globin gene. Relative levels of the c-*myc* gene amplification thus determined are listed in Table II. Amplification was found among sarcomas induced by MCA and by α-tocopherol. The frequency of the amplification of the c-*myc* gene was especially high in α-tocopherol-induced sarcomas (3 out of 7 cases, 43%). It is interesting to note that none of the radiation-induced sarcomas had amplification of the c-*myc* gene (0 out of 8 cases, 0%).

Effect of mouse strains and the latency of sarcomagenesis on the amplification Sarcomas with the amplified c-*myc* gene were found in NFS, Balb/c *nu/nu*, CBF_1 and BCF_1 strains of mice. We could not detect the amplification in 3 sarcomas of Balb/c strain. Although the sample size of the tumors for each strain is too small to evaluate the strain dependency, it seems likely that there is not much strain dependency of c-*myc* gene amplification.

The latency of sarcomas induced by various agents is listed in Table II. MCA induced sarcomas at the latency of 2.5 to 6 months. Administration of α-tocopherol induced sarcomas within 7 to 10 months. Sarcoma induction by various radiations, such as HTO, neutron and gamma-ray, required the longest latency (from 12 to 17 months) among the agents tested in the present study. As has been shown in the previous section, only MCA and α-tocopherol yielded sarcomas with the amplified c-*myc* gene.

Levels of overexpression and amplification of the c-*myc* gene in sarcomas and other tumors The level of the c-*myc* gene overexpression in sarcomas was studied in detail. Autoradiograms of the c-*myc*-probed and the ribosome RNA gene-probed dot blots were scanned by a densitometer (Fig. 2). The level of c-*myc* gene expression was normalized for the amount of RNA per dot based on the result with the ribosomal RNA gene. When the level of expression was more than twice, four times and six times as high as that of the control sample, it was scored as +, ++ and +++, respectively. The results are summarized in Table II. Sarcomas with the amplified c-*myc* gene usually expressed a high level of the gene. However, the reverse was not the case. Some sarcomas overexpressed the c-*myc* gene without amplification. For example, NFS 1618 sarcoma (sample number 1) expressed the c-*myc* gene at the level of ++ but the c-*myc* gene was not amplified in this tumor. Overexpression without amplification was more common among tumors

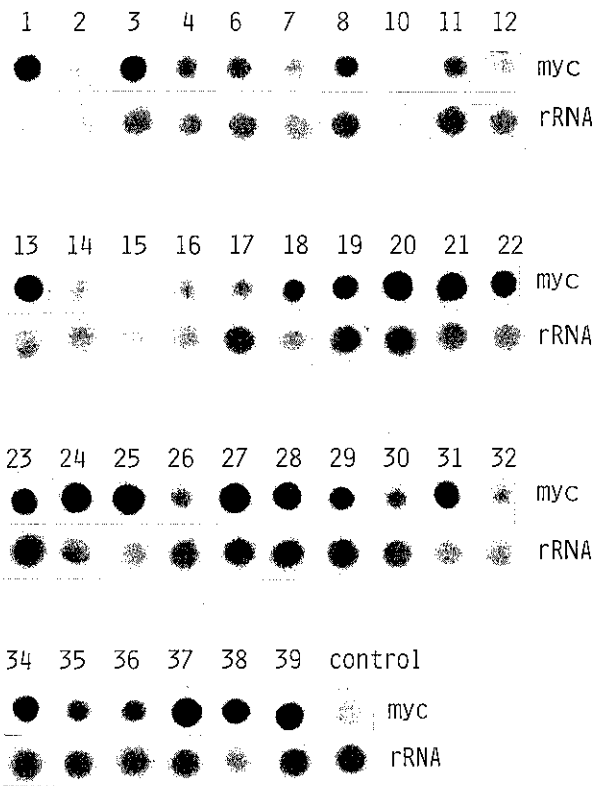

Fig. 2. Expression of the c-*myc* gene in mouse sarcomas. Total cellular RNA (2 μg) from sarcomas was spotted onto a nitrocellulose filter. The filter was hybridized to the c-*myc* probe and then rehybridized to the ribosomal RNA gene. Sample numbers above the spot correspond to those of Table II. RNA from mouse fibroblasts was used as a control. Upper rows are those hybridized to the c-*myc* probe and the lower rows to the ribosomal RNA.

other than sarcomas. As has been shown in Table I, and in the previous sections, we could not detect changes in the gene in tumors overexpressing the c-*myc* gene except for sarcomas. Among 148 tumors other than sarcomas, 44 cases had overexpression of the c-*myc* gene. None of these 44 cases showed amplification of the c-*myc* gene.

DISCUSSION

In our present study, we examined overexpression of the c-*myc* gene in 177 independently induced primary mouse tumors. Overexpression of the c-*myc* gene was observed in a variety of tumor types, except hepatomas and thyroid tumors, where the frequencies were 3.5% and 0% respectively. The c-*myc* gene is expressed at high levels in cells involved in immune responses.[23] Because of invasions of macrophages and other types of cells which are involved in immune responses and inflamatory reac-

tions, c-*myc* expression detected in some tumor tissues may well be due to contamination with transcripts from these cells. This may result in overestimation of the frequency for many types of tumors in which invasion of lymphocytes is common. Therefore, the low frequency of the c-*myc* overexpression in hepatomas and thyroid tumors seems rather significant.

Overexpression of the c-*myc* gene was common among tumors other than hepatomas and thyroid tumors. However, amplification or rearrangement of the gene was not found in these tumors except sarcomas. Even for thymomas, in which the c-*myc* gene was overexpressed at high frequency, there was no detectable change in the gene (data not shown).

Activation of the c-*myc* gene was noted in a variety of human and mouse tumors. Activation by translocations and rearrangement of the c-*myc* gene was found among tumors of hematopoietic origin, such as mouse plasmacytomas and human Burkitt lymphomas.[5, 7–9] Amplification of the c-*myc* gene was, on the contrary, found in solid tumors as well as in leukemias.[10, 12, 13, 24, 25]

In our present study, the amplified c-*myc* gene was found only in sarcomas. The frequency of sarcomas overexpressing the c-*myc* gene was similar among sarcomas induced by various agents; the frequencies for MCA-induced, α-tocopherol-induced and radiation-induced sarcomas were 46%, 43% and 50%, respectively. However, the frequency of amplification differed considerably between sarcomas induced by these agents; the frequencies were 17%, 43% and 0% for sarcomas induced by MCA, α-tocopherol and radiations, respectively. Thus, there seems to be some inducer specificity for the amplification of the c-*myc* gene.

It is interesting to note that the c-*myc* gene was frequently amplified in α-tocopherol-induced sarcomas. Amplification of DNA sequences was proposed to result from overreplication of DNA, which is usually induced by DNA damage.[26] It is hard to conceive how α-tocopherol dissolved in soybean oil damages DNA. Thus the mechanism of sarcoma induction by α-tocopherol itself is a great puzzle,[14] and the mechanism of acquisition of the amplified c-*myc* gene during the progression of the these tumors is another question to be answered in the future.

We could not detect the amplification of the c-*myc* gene in 8 cases of radiation-induced sarcomas. The difference in the frequency of amplification between radiation-induced and α-tocopherol-induced sarcomas is significant as judged by the χ^2 test. However, it is not significantly different from the frequency in MCA-induced sarcomas. MCA-induced sarcomas had the shortest latency while radiation-induced sarcomas had the longest among the three agents. Thus, there seems to be no correlation between the latency and the frequency of amplification. It has been reported that chemically induced and radiation-induced transformation of C3H/10T1/2 cells was associated with enhanced expression of the c-*myc* gene without amplification.[27] On the other hand, skin carcinomas induced by ionizing radiations in rats frequently carry the amplified c-*myc* gene.[28] There is no simple explanation for the lack of amplification of the c-*myc* gene in radiation-induced sarcomas in the present study or for the high frequency of amplification in rat carcinoma.

Expression of the c-*myc* gene was frequently elevated in a variety of tumors studied in the present investigation. In most cases, c-*myc* overexpression was not associated with changes in the gene. Thus, there seem to exist at least two mechanisms for the overexpression: an increase in the transcripts through amplification of the gene and through a high rate of transcription accompanied with a low rate of mRNA degradation. Both of these mechanisms were shown to operate in a variety of tumor cells.[29] Frequent overexpression of the c-*myc* gene without amplification in radiation-induced sarcomas provides another example of transcriptional activation of the gene.

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