Nontumorigenic squamous cell carcinoma line converted to tumorigenicity with methyl methanesulfonate without activation of *HRAS* or *MYC*

(tumor cells/carcinogenesis/nude mice/RNA and DNA blot-hybridization analyses)

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ABSTRACT   Plasticity of human tumor populations could account for the reason why many tumorigenic human cell lines lose this feature when grown in culture. Methyl methanesulfonate (MMS) was used to convert premalignant squamous cell carcinoma (SCC) cell line SCC-83-01-82 to a malignant phenotype. The MMS-treated SCC-83-01-82 cells (MMS-SCC-83-01-82) produced progressively growing tumors in 5 of 11 splenectomized BALB/c nude mice within 3–5 months. A cell line, designated SCC-83-01-82 CA, was established *in vitro* from one of the mouse tumors and was repassaged successively. This SCC-83-01-82 CA cell line was aggressively tumorigenic. A tumor ≥ 2.0 cm in size was present within a month, as opposed to the 3–5 months required for the tumors produced by the MMS-SCC-83-01-82 cells. Examination of frozen cross sections by *in situ* hybridization revealed that focal areas of the tumor produced by the MMS-SCC-83-01-82 cells expressed *MYC* and *HRAS* mRNA. However, by the third passage *in vivo*, the levels of expression of the corresponding genes in the mouse tumors were undetectable. Blot-hybridization analysis of the RNA from the MMS-SCC-83-01-82 cells and the subsequently derived tumors and cells did not indicate any consistent overexpression of *MYC*, *HRAS*, or *KRAS*. Restriction fragment length polymorphism analysis of both *MYC* and *HRAS* genes revealed neither rearrangement nor amplification of *MYC* nor point mutation in the 11th or 12th codon of *HRAS*. The data suggest that alterations in *MYC* and *HRAS* were not directly involved in either the initial transformation or MMS-induced tumorigenic conversion of the SCC-83-01-82 cell line. Persistence of tumorigenicity after reisolation of the MMS-converted premalignant SCC-83-01-82 cells did not disappear immediately following the treatment with MMS.

An acceptable system for evaluating malignant potential is to use a surrogate host such as a nude mouse to measure tumorigenicity (1, 2). Normal human cells are more resistant than rodent cells to tumorigenic conversion by chemical carcinogens and oncogenes. Even though tumors from human cells have been obtained in nude mice by injection of chemically transformed human fibroblasts (3, 4), this approach has not always been successful (5). Transfection of primary or early-passage human diploid fibroblasts with oncogenes has resulted in anchorage-independent colony formation (6–8) and foci formation (7, 8), but tumorigenicity has not been observed. Yokum *et al.* (9) have reported that transfection of human bronchial epithelial cells with v-Ha-*ras* (viral Harvey *ras* sarcoma oncogene) produced transformed cells that became tumorigenic after 100–120 population doublings.

Unlike the primary or early-passage human cells, cell lines with infinite life span have been more frequently transformed to tumorigenic phenotypes by oncogenes or chemicals. For example, an immortalized human skin fibroblast cell line has been converted to a malignant phenotype by transfection with the human homologue, *HRAS*, of the v-Ha-*ras* oncogene (10). The tumorigenic conversion of an immortalized human bronchial epithelial cell line has been achieved by infecting v-Ha-*ras*-containing retrovirus (11) or by transfecting with a plasmid containing the v-Ki-*ras* region from Kirsten murine sarcoma virus (12). Boukamp *et al.* have reported that malignant transformation of keratinocyte cell lines by the v-Ha-*ras* oncogene (13). *N*-Methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) treatment of nontumorigenic osteosarcoma cell line (14) and T24 human bladder carcinoma cell line (15) has resulted in tumorigenic conversion of these cells.

The basis by which human cells acquire tumorigenic potential is not understood. Oncogenes have been implicated in malignant transformation, and the basic mechanisms of oncogene activation have been associated with neoplasia (16–18). These mechanisms include point mutations, gene rearrangement, amplification and/or overexpression, and mutations in tumor suppressor genes. Conversion of nontumorigenic cell lines to malignant cells by chemical agents may involve one or more of these basic mechanisms.

We have established three cell lines with differing tumorigenic potentials from human carcinomas. One of these cell lines, SCC-83-01-82, is a nontumorigenic squamous cell carcinoma (SCC) cell line. In an effort to study the mechanism of the tumorigenic conversion of the continuous cell line versus the primary cells, we used methyl methanesulfonate (MMS) for the treatment of these cells (1). MMS is not classified as a potent human carcinogen. Yet, this compound converts these SCC cells to a tumorigenic phenotype with a relatively high frequency. In this paper, we present an examination of the malignant transformation of SCC-83-01-82 by MMS.

MATERIALS AND METHODS

Cell Culture. To establish *in vitro* cell cultures of SCC-83-01-82 or other cell lines from progressively growing tumors produced in different nude mice, tumors ≥ 2.0 cm in size were minced into ≈1 × 1 mm sections digested with 0.5% collagenase in growth medium supplemented with an addi-

Abbreviations: MNNG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine; MMS, methyl methanesulfonate; SCC, squamous cell carcinoma; *MYC*, avian myelocytomatosis viral (v-*myc*) oncogene homologue; *KRAS*, Kirsten *ras* sarcoma viral (v-ki-*ras*) oncogene homologue; *HRAS*, Harvey *ras* sarcoma viral (v-Ha-*ras*) oncogene homologue.
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tional 5% (vol/vol) fetal bovine serum at 37°C in a 4% CO_2-enriched air atmosphere for 4 hr (1, 19, 20).

Anchorage-Independent Growth. Cells were evaluated for their ability to produce colonies in soft agar as described by Milo et al. (21). Colonies containing 50 cells $\geq$ 60 μm in diameter were counted after 2 weeks.

MMS Treatment. Two million SCC-83-01-82 cells were seeded into several 100-mm diameter Petri dishes in 10 ml of growth medium. When these cells grew to a monolayer, MMS was added at 50 μg/ml, and the mixture was incubated for 24 hr at 37°C in a 4% CO_2-enriched air atmosphere (1).

Tumorigenicity Assay. All lines were assayed for tumorigenicity. Three- to 4-week-old gnotobiotic nude mice were splenectomized and then injected subcutaneously into the subscapular area with ca. 1 $\times$ 10^7 cells suspended in minimal essential medium (2).

In Situ **Hybridization.** DNA probes for *HRAS*, *MYC*, and the keratin gene were used to examine 8-μm frozen cross sections for the expression of these genes at the RNA level (1). The DNA probes were labeled by nick-translation (30) with biotinylated dUTP and were hybridized with the sections overnight in hybridization solution with 50% (vol/vol) formamide at 37°C (22). The hybridization pattern was detected with horseradish peroxidase-conjugated avidin. The cells expressing the different messages were identified with diaminobenzidine·HCl and H_2O_2, counterstained with hematoxylin, and examined under $\times$40 magnification (2).

RNA (Northern) Blot-Hybridization Analysis. Total cellular RNA was isolated by the guanidinium isothiocyanate/CsCl method (23) and then electrophoresed on a 1.2% agarose denaturing gel by the method of Maniatis et al. (24). The gel was stained with ethidium bromide and visualized for equivalent loading of RNA among lanes by comparison of the intensities of the 28S and 18S RNA bands.

The transfer, prehybridization, hybridization, and wash steps were performed according to the instructions of the supplier of the nylon membranes (Schleicher & Schuell). The membranes were then exposed overnight to Kodak XAR-5 film at -70°C with an intensifying screen.

The DNA probes were radioactively labeled with [α-^{32}P]dCTP (specific activity = 3000 Ci/mmol, ICN; 1 Ci = 37 GBq) by the random-primer method (Pharmacia) according to the manufacturer's instructions. pSVc-myc-1 was obtained from American Type Culture Collection, and the *KRAS* probe was purchased from Oncor, Gaithersburg, MD.

Southern Blot Analysis. High molecular weight genomic DNA was isolated from various SCC cell lines and from SCC tumors produced in nude mice as described by Maniatis et al. (24). Restriction digests of genomic DNA were carried out according to the instructions of the supplier of the restriction enzymes (Bethesda Research Laboratories). Southern transfers were also performed as described by Maniatis et al. (24). The BA85 nitrocellulose filters (Schleicher & Schuell) were baked for 2 hr at 80°C under vacuum and then prehybridized,

hybridized, and washed as described by Fu and Marzluf (25). The filters were exposed to Kodak XAR-5 film at -70°C for 48 hr with an intensifying screen.

The DNA probes were radioactively labeled with [α-^{32}P]dCTP (specific activity = 800 Ci/mmol; NEN) by nick-translation (30). The *HRAS* and *MYC* probes were purchased from Oncor, Gaithersburg, MD.

RESULTS

Malignant Conversion of a Nontumorigenic SCC Cell Line. One SCC cell line, SCC-83-01-82, failed to produce any tumors (0/8) within 3–4 months after subcutaneous injection of 1 $\times$ 10^7 cells into nude "gnotobiotic" (BALB/c nu/nu) mice (Table 1). These cells grew rapidly, and they did express anchorage-independent growth as an indicator of a transformed phenotype.

The nontumorigenic SCC-83-01-82 cells were treated with MMS at 50 μg/ml for 24 hr and allowed to recover until 80–90% confluent monolayers were obtained (3–4 wk). After subcutaneous injection of 1 $\times$ 10^7 MMS-SCC-83-01-82 cells into nude mice, 5 of the 11 mice developed progressively growing tumors after a 3- to 5-month latent period. Histological examination showed the tumors to be undifferentiated adenocarcinomas (Table 2). The original patient tumor was characterized as a poorly differentiated invasive SCC. A cell line designated SCC-83-01-82 CA was established from one of the excised tumors and was found to be even more aggressive than the parental MMS-SCC-83-01-82 cell line, as it produced tumors in 100% (7/7) of the injected nude mice with a latency of 5–7 days. Another cell line, SCC-83-01-82 CA$_1$, was established from one of the SCC-83-01-82 CA tumors and was also found to be highly tumorigenic. Karyological examination of the SCC-83-01-82 CA cell line indicated that it contained only human chromosomes (1). Furthermore, the SCC-83-01-82 CA$_2$ cell line, established from one of the SCC-83-01-82 CA$_1$-derived tumors, also exhibited an aggressive malignant behavior. The tumorigenic characteristics of these different SCC cell lines are presented in Table 1.

MYC and RAS Gene Expression in SCC Cell Lines and Tumors. Northern blot analysis was used to determine whether conversion of the nontumorigenic SCC-83-01-82 cell line to tumorigenicity after MMS treatment involved altered expression of *MYC*, *HRAS*, or *KRAS*. No consistent change in the levels of mRNA could be detected in SCC-83-01-82 cells after MMS treatment for any of these three genes (Fig. 1; data for HRAS mRNA are not shown). The level of MYC and KRAS mRNAs also was inconsistent among the individual cell lines established from the various nude mice tumors (Fig. 1 *A* and *B*). When the level of *MYC* mRNA in the MMS-SCC-83-01-82 cells was compared with the level in mouse tumors derived from these cells, no *MYC* expression was detected in the tumors (Fig. 1C).

Table 1. Characterization of the malignant potential of the various SCC populations

Cell line	No. of mice producing tumor/no. of mice injected	Latent period before tumor initiation	Time between tumor initiation and excision	Tumor size at excision, cm
SCC-83-01-82	0/8	—	—	—
MMS-SCC-83-01-82	5/11	3–4 months	$\approx$6 months	$\approx$2.0
SCC-83-01-82 CA*	7/7	5–7 days	20–25 days	1.5–2.0
SCC-83-01-82-CA$_1$†	4/4	5–7 days	20–25 days	1.0–1.5
SCC-83-01-82-CA$_2$‡	2/2	10–15 days	$\approx$1 month	$\approx$2.0

*Cell line established from a tumor, T_1, produced by MMS-SCC-83-01-82.
†Cell line established from a tumor, T_2, produced by SCC-83-01-82 CA.
‡Cell line established from a tumor, T_3, produced by SCC-83-01-82 CA$_1$.

Table 2.   *In situ* hybridization and histopathology data of the tumors derived from MMS-treated SCC-83-01-82 cells

Tumor	Producer cell line	In situ hybridization detection			Histopathology interpretation of the tumor
		HRAS	MYC	Keratin	
T_1	MMS-SCC-83-01-82	+	+	+,−	Undifferentiated adenocarcinoma
T_2	SCC-83-01-82 CA	−	+	−	Poorly-to-moderately differentiated SCC
T_3	SCC-83-01-82 CA_1	−	−	−	Poorly-to-moderately differentiated SCC

Moreover, this malignant conversion does not appear to be due to infection of the original SCC-83-01-82 cells by an endogenous murine retrovirus in the nude mice host, as Northern blot analysis with the *fms* probe pSM3 did not detect retroviral expression in any of the tumorigenic SCC cell lines (data not shown). A recent report by Walker *et al.* (26) describes the acquisition of mouse retroviral sequences by human cell lines passaged through nude mice and demonstrates that the detection of an 8.5- to 9.5-kilobase (kb) RNA transcript in these cell lines by the pSM3 probe is due to sequence homology between the *pol* regions of the Susan McDonough strain of feline sarcoma virus contained in pSM3 and of AKR murine leukemia virus (AKV). Our results demonstrate that these tumors did not acquire murine retrovirus sequences.

The expression of *MYC* and *HRAS* in the various nude mice tumors was also analyzed by *in situ* hybridization (Table 2). Certain subpopulations of cells in a representative tumor derived from the MMS-treated human SCC-83-01-82 cell line demonstrated increased MYC and HRAS mRNA levels. These subpopulations contained both keratin mRNA and keratin proteins as detected by *in situ* hybridization and immunohistochemistry. However, the levels of HRAS and keratin mRNA decreased to undetectable amounts by *in situ* hybridization in the successively generated tumors. Detectable levels of MYC mRNA were still present in subpopulations of cells in a representative tumor derived from the SCC-83-01-82 CA cell line, but the MYC mRNA level began to decrease in tumors derived from SCC-83-01-82-CA_1 and became undetectable in a SCC-83-01-82 CA_2 cell line derived from a tumor.

MYC and HRAS Genotype in SCC Cell Lines and Tumors. DNA from normal human fibroblasts, SCC-83-01-82, MMS-SCC-83-01-82, SCC-83-01-82 CA, and SCC-83-01-82 CA_1 cells and from cells derived from a MMS-SCC-83-01-82 tumor in mice were digested with *Msp* I and *Hpa* II and subjected to Southern blot hybridization with a *HRAS* probe. A 355-base-pair (bp) fragment alone was detected in all DNA samples we analyzed (Fig. 2), indicating the presence of only the normal *HRAS* gene in all of these cell lines and tumors. A 411-bp fragment, indicative of a codon 12 mutation, was not observed.

To determine whether *MYC* gene rearrangement occurred in the original SCC-83-01-82 cell line or in the MMS-derived cells and tumors, Southern blot analysis was used to detect restriction fragment length polymorphisms indicative of such rearrangement. But the *MYC* probe detected only germ-line *Eco*RI (13.0 kb), *Hind*III (11.5 kb), *Pvu* II (16.5, 2.7, and 1.3
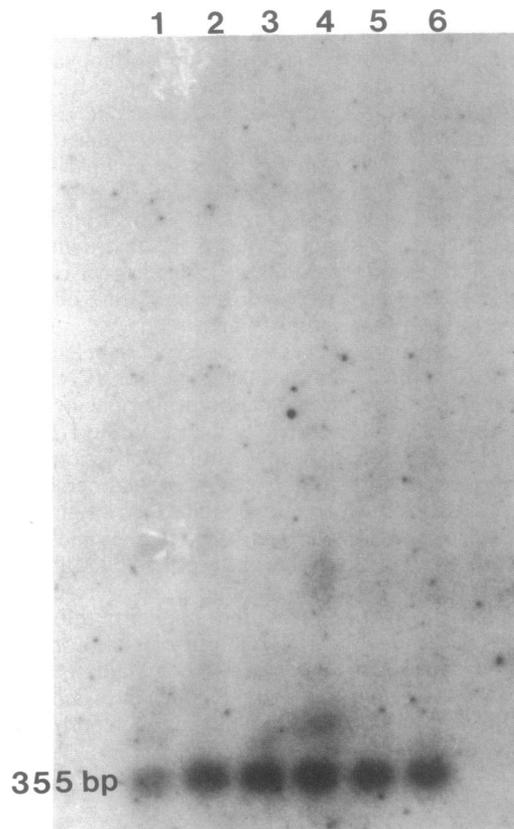
FIG. 2.   Southern blot analysis was used to examine the presence of a point mutation in codon 12 of *HRAS*. DNA (10 μg) from the various samples were digested with *Msp* I and *Hpa* II, electrophoresed on a 1.8% agarose gel, transferred to a nitrocellulose filter, and hybridized with a human *HRAS* probe. Lanes: 1, SCC-83-01-82 cell line; 2, MMS-SCC-83-01-82 cells; 3, tumor T_1 from mouse produced by MMS-SCC-83-01-82; 4, SCC-83-01-82 CA cell line derived from T_1; 5, tumor T_2 from mouse produced by SCC-83-01-82 CA; and 6, normal human fibroblasts. Fragment size was determined by comparison with the 1-kb DNA ladder (Bethesda Research Laboratories). Note that a 411-bp fragment, indicative of a codon 12 mutation, was not observed.
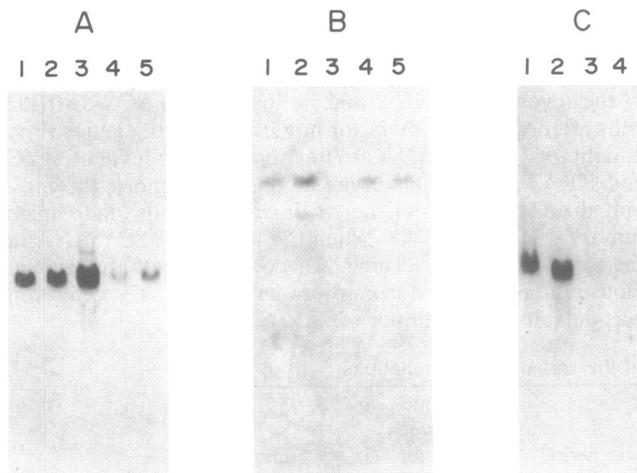

FIG. 1.   Characterization of the MYC and KRAS mRNA levels in MMS-treated SCC cells. Total cellular RNA (20 μg) was loaded into each lane. (*A*) *MYC* hybridization against RNA isolated from untreated SCC-83-01-82 cells (lane 1), MMS-SCC-83-01-82 cells (lane 2), SCC-83-01-82 CA cells from tumor T_1 (lane 3), SCC-83-01-82-CA_1 cells from tumor T_2 (lane 4), and SCC-83-01-82 CA_2 cells from tumor T_3 (lane 5). (*B*) Same filter as in *A* except for KRAS mRNA. (*C*) *MYC* hybridization against RNA isolated from untreated SCC-83-01-82 cells (lane 1), MMS-SCC-83-01-82 cells (lane 2), and two independent tumors produced by MMS-SCC-83-01-82 cells (lanes 3 and 4).

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kb), *Sst* I (14.5, 3.1, and 1.6 kb), and *Xba* I (7.1 and 6.9 kb) fragments (Fig. 3). Comparison of the *MYC* copy number detected in the *Eco*RI digests of the above DNA samples to that in normal human fibroblast DNA did not indicate any *MYC* amplification (Fig. 4).

DISCUSSION

The tumorigenicity of immortal cell lines isolated from human malignancies cannot be consistently predicted. Cell lines have been isolated that have been initially tumorigenic but after prolonged propagation in cell culture have lost this tumorigenic capacity (15). It has been shown that the capacity for tumorigenesis could be acquired in such cell lines after exposure to a carcinogen such as MNNG (14, 15). The mechanism for these conversions was not the same in the different cell lines. In the present study, we have exposed a nontumorigenic SCC cell line to a very weak carcinogen, MMS, and converted the cell line to tumorigenicity.

The carcinogenic potential of MMS and MNNG is not equal. MMNG is an excellent carcinogen in human cells and can convert normal cells to transformed cells that exhibit anchorage-independent growth and cellular invasiveness (3). MMS, while a methylating agent, is a very weak human carcinogen and has not been shown to be able to transform either normal human fibroblasts or normal human keratinocytes (unpublished data). The effects of MNNG in the tumorigenic conversion of immortalized cell lines vary, dependent on the cell line. In a nontumorigenic human osteosarcoma, the effects of MNNG have been associated with a gene rearrangement and onset of expression of the *MET* oncogene (27). The tumorigenic conversion of T24 human bladder carcinoma cells by MNNG was not associated with new expression of *MET* or alteration in the levels of expression of the mutated *HRAS*, which the cells already contained (15). Thus, there does not appear to be a consistent molecular mechanism associated with the activation of nontumorigenic cells by MNNG. In this study, we have examined the molecular changes associated with the tumorigenic conversion of SCC cells by MMS.

The nontumorigenic SCC cells exhibited an altered growth pattern with an extended life-span and pleomorphic morphology. They also retained the capacity for growth in soft agar.

After treatment with MMS, the SCC cells did not demonstrate significant changes in the level of MYC, HRAS, or KRAS mRNAs. Furthermore, neither amplification or obvious rearrangement in specific regions of the *MYC* gene nor a mutation in the 12th codon of *HRAS* associated with transformation was present. However, this does not exclude a mutation in codon 61 of *HRAS* or mutations in either site of *KRAS* or *NRAS*. These MMS-exposed cells acquired the capacity for tumorigenesis and produced progressively growing tumors in nude mice hosts. Analysis of these tumors by *in situ* hybridization revealed increased *HRAS* and *MYC* expression in discrete subpopulations of tumor cells. However, this expression did not persist in subsequent cell lines or tumors.

Thus, there is the possiblity of a transient genetic response of oncogenes (28). Even though these subsequent cell lines do not appear to have alterations in the *MYC* and *HRAS* genes, they are capable of a much more rapid development of tumors, which also do not contain such alterations. The generation of these various cell lines resulted in a further demonstration of the plasticity of the tumorigenic potential of cell lines derived from human malignancies.

Previously we have described the presence of different subpopulations in tumors that have different phenotypic characteristics and different biologic potentials (1). The presence of cell populations that may remain plastic in their commitment to tumorigenesis may be directly related to the methods of identification. In other studies converting nontumorigenic cells to tumorigenicity, the *in vitro–in vivo* transition appears to be a critical point (14, 15). The loss of
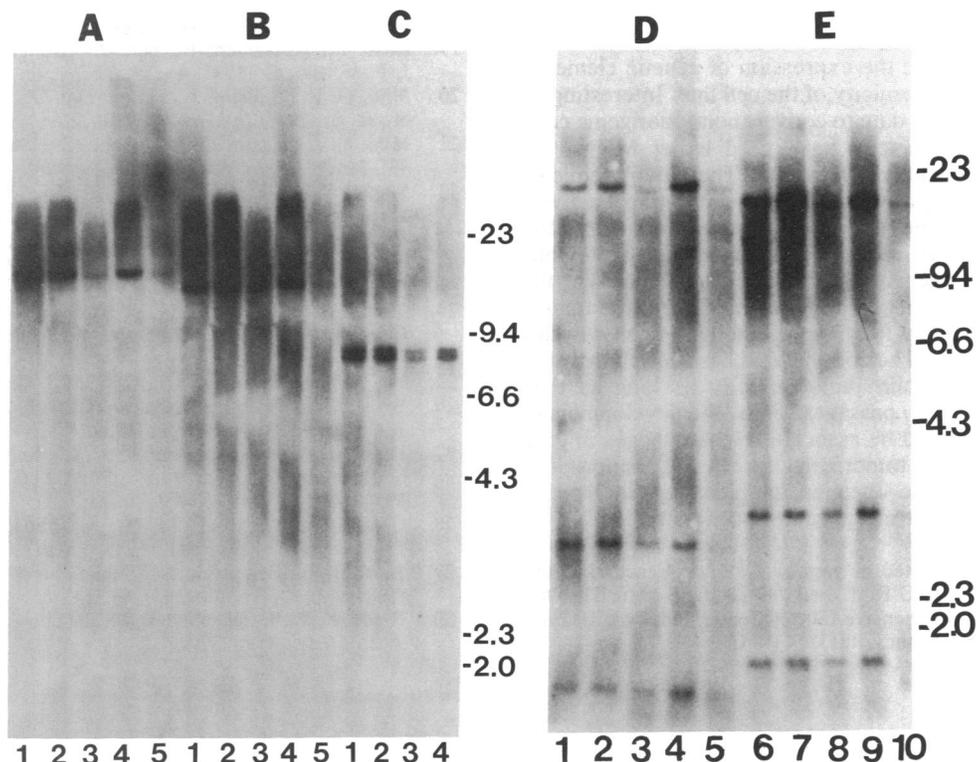

Fig. 3. Screening of DNA for *MYC* rearrangement. DNA preparations (10 μg) were digested with the restriction enzymes *Eco*RI (*A*), *Hind*III (*B*), *Xba* I (*C*), *Pvu* II (*D*), or *Sst* I (*E*), electrophoresed on a 0.8% agarose gel, and hybridized with a human *MYC* probe. Lanes are described in Fig. 2. *Hind*III-digested phage λ DNA size markers (in kb) appear on the right (Bethesda Research Laboratories). Lanes 6–10 in *E* correspond to lanes 1–5 in Fig. 2.
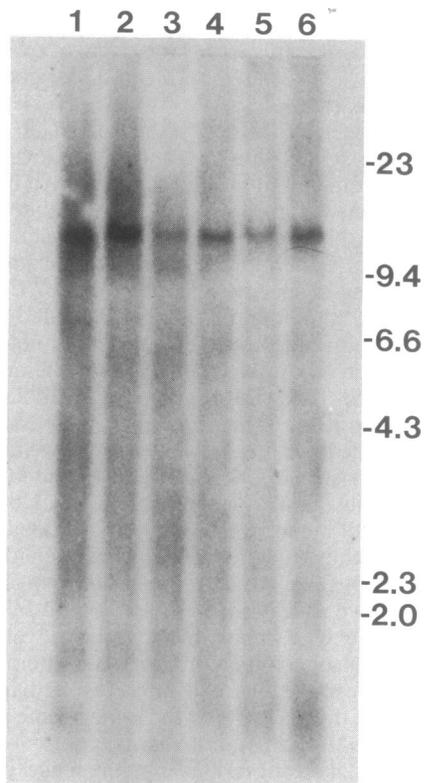
FIG. 4. Screening of the DNA from the various sources for possible *MYC* amplification. Lanes are as described in Fig. 2. DNA preparations were digested with *Eco*RI and hybridized with the human *MYC* probe. *Hin*dIII-digested phage λ DNA size markers (in kb) appear on the right.

the *in vivo* environment results in an alteration in the cells that does not affect immortalization but changes the biologic potential of the cells *in vivo*. The transition to the *in vitro* situation might increase the expression of genetic elements that reverse the tumorigenicity of the cell line. Interestingly, the studies described to date to convert nontumorigenic cells in culture to tumorigenicity have used either MNNG or MMS, which are both alkylating agents. These two chemicals both add methyl groups to DNA, and the mutational activity of these chemicals is associated with the development of the DNA adducts (29). If in the present case and in previous studies a suppressor gene was methylated and mutated by the effects of chemical exposure and thus inhibited, the tumorigenic capacity could be fully expressed by the cells. In this manner, the cycle could be continued as has been shown, and the cells would maintain tumorigenicity as long as they continue *in vivo–in vivo* passages. Thus, the development of *in vitro* cell lines would be associated with changes in gene expression that affect tumorigenicity but not immortalization. The effect of the chemical mutagen would then be needed for the cells to reacquire tumorigenicity.

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