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Nonrandom duplication of the chromosome bearing a mutated Ha-*ras-1* allele in mouse skin tumors

(chemical carcinogenesis/oncogenes/mouse chromosome 7/trisomy)

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ABSTRACT **We analyzed the normal/mutated allelic ratio of the Ha-*ras-1* gene in mouse skin squamous cell carcinomas induced by initiation with dimethylbenz[*a*]anthracene and promotion with phorbol 12-myristate 13-acetate. DNA for these studies was obtained from short-term tumor cultures (24–72 hr) to eliminate the contribution of stromal and inflammatory cells to the sample. The allelotypic analysis was performed in 25 squamous cell carcinomas by quantitative radioanalysis of the *Xba* I restriction fragment length polymorphism as detected by BS9, a v-Ha-*ras* probe, and rehybridization of the Southern blots with probes for chromosomes 7 and 8. Approximately 85% of the tumors presented overrepresentation of the mutated allele in the form of 1 normal/2 mutated (12 tumors), 0 normal/3 mutated (4 tumors), 0 normal/2 mutated (3 tumors), and gene amplification (3 tumors). No tumor was found with a 2 normal/1 mutated allelic ratio. These results support our previous cytogenetic studies, indicating that trisomy of chromosome 7 is present in the majority of these tumors and show that nonrandom duplication of the chromosome carrying the mutated Ha-*ras-1* allele appears to be a major mechanism by which the mutated gene is overrepresented.**

Cytogenetic studies performed in our laboratory (1–3) found that numerical chromosomal changes in the form of progressive aneuploidy are characteristic of chemically induced mouse skin papillomas from very early stages. These gross chromosomal abnormalities are temporally related to indicators of malignant progression, such as dysplastic changes, loss of differentiation-associated keratins, and expression of the enzyme γ-glutamyl transpeptidase (2, 3).

We have identified (4) sequential trisomization of chromosomes 6 and 7 as the primary nonrandom cytogenetic events in this model. Trisomy of chromosome 7 (Ts7) was found in severely dysplastic papillomas and in most of squamous cell carcinomas (SCCs) induced by initiation with dimethylbenz[*a*]anthracene (DMBA) and promotion with phorbol 12-myristate 13-acetate (PMA).

In this same model, the initiation event was postulated (5–8) to be a point mutation in one of the alleles of the Ha-*ras-1* gene. This finding plus the fact that the Ha-*ras-1* gene resides on mouse chromosome 7 (9) led us to speculate about a possible link between the dosage of the mutated gene and the generation of Ts7 by nondisjunction. Specifically, we have now carried out a Southern blot analysis to estimate the relative number of mutant vs. normal Ha-*ras-1* alleles. Our results indicate that Ts7 occurs by nonrandom duplication of the chromosome 7 carrying the mutated Ha-*ras-1* allele, providing further evidence for the putative mechanistic role played by this oncogene not only at the initiation stage but also as a determinant factor for skin tumor progression.

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MATERIALS AND METHODS

Animal Treatment and Tumors. Carcinomas were induced on the dorsal skin of SENCAR mice by initiation with DMBA (10 nmol) and promotion with 2 μg of PMA twice weekly, as described (1). Sections of the tumors were characterized histologically and the remainder was enzymatically dispersed at 37°C for 40–50 min, as described (10), without the addition of demecolsine. The dispersed cells were centrifuged, resuspended in low calcium (50 μM $CaCl_2$) Eagle's modified essential medium (MEM), essentially as described (11), but supplemented with 1% fetal bovine serum and plated at 5×10^5–1×10^6 viable cells per 25 cm^2. After 24 hr, cultures were washed to eliminate unattached cells and harvested at 48–72 hr for DNA extraction.

DNA Extraction and Hybridization. High molecular weight DNAs were isolated from the cultures as described (12). DNA (10 μg) was digested with a 10-fold excess of *Xba* I (Boehringer Mannheim) for 12 hr. Digested samples were electrophoresed through 0.8% agarose gels and transferred to nylon filters (Nytran; Schleicher & Schuell) according to the supplier's conditions. Purified insert from the various plasmids was labeled with [α-^{32}P]dCTP (3000 Ci/mmol; 1 Ci = 37 GBq; Amersham) by the random-priming method (Boehringer Mannheim). Blots were hybridized under stringent conditions [50% (vol/vol) formamide/6× SSC at 42°C; 1× SSC = 0.15 M NaCl/0.015 M sodium citrate, pH 7.0] and washed to a final stringency of 0.1× SSC at 65°C for 30 min.

Recombinant DNA Probes. Recombinant DNA probes used in this study were BS9 (13) for the Ha-*ras-1* gene; pCR1, for the mouse androgen-regulated gene D7Rp2 (14) mapped to chromosome 7 (gift of F. Berger, University of South Carolina, Columbia) and pSMA3.1 (gift of P. Stambrook, University of Cincinnati Medical Center, Cincinnati, OH) for the mouse adenine phosphoribosyltransferase (*Aprt*) gene mapped to chromosome 8 (15).

Quantitative Radioanalysis. β-Particle emission counts were measured with an AMBIS radioanalytic imaging system (Scanner Mark II; software version 1.83), based on gas ionization using a 90% argon/10% methane mixture as detector gas/quenching agent. Results were quantitated above an experimental and instrument background averaging 0.06 cpm, using a resolution plate factor of 18.54 (896-aperture plate). Detection time was 1080 min. Scans were normalized to control spleen DNA by assigning a relative value of 1.0 to the corresponding ratios listed in Table 2.

Autoradiography. After the radioanalytic scanning, blots were exposed to Kodak film in the presence of intensifying screens for 48–72 hr at −70°C.

Abbreviations: SCC, squamous cell carcinoma; DMBA, dimethylbenz[*a*]anthracene; PMA, phorbol 12-myristate 13-acetate; RFLP, restriction fragment length polymorphism; Ts7, trisomy of chromosome 7.

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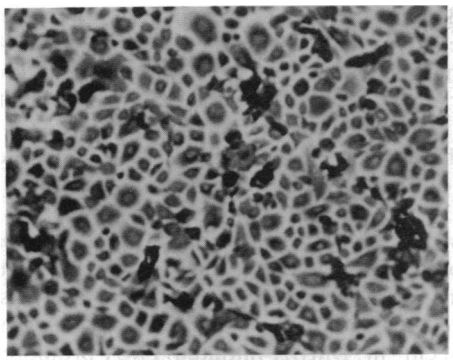

FIG. 1. A 48-hr SCC culture, immunostained with anti-keratin antibody (αK14) by the avidin–biotin peroxidase technique. No counterstain. (×100.)

Immunohistochemistry. Culture microdishes were fixed in 70% ethanol and stained with αK14 anti-keratin antibody (gift of D. Roop, Baylor College of Medicine, Houston) using an avidin–biotin peroxidase kit (VectaStain; Vector Laboratories) as described (3).

RESULTS AND DISCUSSION

To determine the allelic composition of the tumors studied, we quantified the signals derived from the *Xba* I restriction fragment length polymorphism (RFLP) displayed by tumors induced by initiation with DMBA followed by sequential treatment with PMA. This RFLP is generated by a specific $A^{182} \rightarrow T$ transversion at codon 61 in one of the alleles of the Ha-*ras-1* gene (6, 8) that gives rise to a novel restriction site for *Xba* I and is characterized by a 12-kilobase (kb) band corresponding to the normal allele plus two additional bands of 8 kb and 4 kb derived from the mutated allele (8), as detected by hybridization with the v-Ha-*ras* DNA probe BS9 (13). The mutant-specific bands would be expected to show hybridization signals of similar intensity since the BS9 probe contains regions homologous to exons 1 and 2 and part of exon 3 of the c-Ha-*ras* gene, spanning approximately the same length from each side of codon 61.

The presence of stromal and inflammatory cells should not be neglected when performing an allelic analysis of solid tumors. In this respect, the proportion of contaminating nontumoral cells in SCCs can reach values as high as 60% of the total cell subpopulations (C.J.C., unpublished results). Vogelstein *et al.* (16) have stated that loss-of-heterozygozity studies are not reliable when nontumoral cells represent

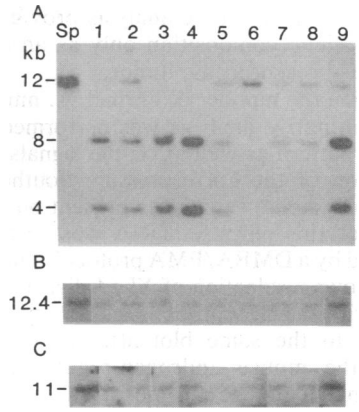

FIG. 2. (*A*) *Xba* I RFLP in chemically induced SCC, as detected by Southern blot analysis with the v-Ha-*ras* BS9 probe. Sp, DNA from spleen. Lanes 1–9 contain DNA extracted from short-term (24–72 hr) cultures of skin carcinomas. (*B* and *C*) Pattern obtained by rehybridization of the Southern blot in *A*. (*B*) Mouse androgen-regulated gene D7Rp2 (pCR1) (14). (*C*) Mouse *Aprt* gene (pSAM3.1) (13). Lane numbers are the same as those assigned in *A* and in Table 2.

>30% of the sample. To overcome this, strategies based on microdissection (16) or cell sorting of aneuploid cells (17) have been developed. We used a short-term (24–72 hr) culture approach with low calcium and 1% fetal bovine serum to inhibit the attachment and proliferation of nonepithelial cells that would otherwise alter the *Xba* I polymorphism pattern by contributing to the 12-kb signal derived from the normal allele. Tumor cultures displayed a typical epithelial morphology and several samples were tested immunohistochemically with monospecific keratin antibodies, confirming that >99% of the attached cells were of epithelial nature (Fig. 1). The presence of normal epithelial cells may be ruled out based on the histopathologic evaluation of every sample that demonstrated that normal epithelial structures were absent or were minimally represented in dissected samples of fully developed carcinomas. Moreover, *in vitro* selection among epithelial cell subpopulations is very unlikely considering the short-term nature of the cultures. In support of this, we observed (18) that the cytogenetic profile of 24- to 72-hr cultures of tumors induced by different initiators and promoters was identical to the *in vivo* picture, presenting the same chromosomal abnormalities.

In addition, an allelic study as depicted in Table 1 should take into account that epithelial neoplasias are dynamic lesions that may contain various cell subpopulations undergoing malignant progression by clonal selection at various

Table 1. Relative signal intensity values and ratios expected for various Ha-*ras-1* allelotypes

Ha-*ras-1* allelotype, normal/ mutated	Relative signal intensity			Ratio			
	12 kb	8 kb	4 kb	Ha-*ras-1*/*Aprt*	Ha-*ras-1* (8 kb + 4 kb)/*Aprt*	D7Rp2/ *Aprt*	Ha-*ras-1*/ D7Rp2
2/0	4	0	0	1	0	1	1
1/1	2	1	1	1	0.5	1	1
0/2	0	2	2	1	1	1	1
1/2*	2	2	2	1.5	1	1.5	1
2/1*	4	1	1	1.5	0.5	1.5	1
0/3*	0	3	3	1.5	1.5	1.5	1

A normal vs. mutated 1/1 Ha-*ras-1* allelotype corresponds to a diploid cell presenting a heterozygous mutation in codon 61 of the c-Ha-*ras* gene. DNA was digested with *Xba* I and probed with BS9. Relative signal intensity values of the normal 12-kb band and the mutation-derived bands of 8 kb and 4 kb would be 2:1:1. Likewise, a lesion with Ts7 would present relative values of 2:2:2, 4:1:1, or 0:3:3 for the allelotypes 1/2, 2/1, or 0/3, respectively. Ratios of normalized signals obtained with probes hybridizing to the same chromosome would be equal to 1, while ratios of signals detected by probes homologous to sequences present on chromosome 7 (Ha-*ras-1* gene and RP2 gene) and on chromosome 8 (*Aprt* gene) would be equal to 1.5 for cells with Ts7.

*Ts7.

rates. Thus, this quantitative analysis provides the basis to estimate the allelic composition only as an average of the various clones present in the tumor.

To determine the number of normal vs. mutated Ha-*ras-1* alleles, a quantitative analysis was performed based on the *Xba* I RFLP pattern as well as on the signals obtained after rehybridization of the corresponding Southern blots with probes homologous to sequences present on different chromosomes. For this purpose, DNA was extracted from 26 SCCs induced by a DMBA/PMA protocol. The Southern blot in Fig. 2*A* shows a selection of *Xba* I-digested tumor DNAs hybridized with the BS9 probe. Fig. 2 *B* and *C* correspond, respectively, to the same blot after rehybridization with probes for the mouse androgen-regulated gene D7Rp2 (pCR1), mapped to mouse chromosome 7 (14), and the mouse *Aprt* gene (pSAM 3.1) (15), mapped to mouse chromosome 8, which, according to our cytogenetic studies (4), was not numerically abnormal in the majority of SCCs analyzed.

As shown in Table 2, the typical *Xba* I polymorphism, as detected by the BS9 probe, was observed in 92% of the tumors studied. Of the 26 tumors analyzed quantitatively, 12 (46%) showed ratios indicative of lesions presenting a predominant cell subpopulation with two copies of the mutated allele and one copy of the normal allele of the Ha-*ras-1* gene. Interestingly, no tumors presented values characteristic of a 2 normal/1 mutated allelic composition, which can be distinguished very clearly from other allelotypes by a 4:1:1 ratio among the *Xba* I RFLP signals (Table 1). In addition, of the 14 tumors that did not exhibit the 1 normal/2 mutated allelic ratio, 10 SCCs (38% of the total) showed partial or complete loss of the normal Ha-*ras-1* allele, as demonstrated by the absence of the 12-kb band. Of these, four tumors (Table 2, samples 1, 3, 15, and 25) presented a 0/3 allelotype, whereas three other tumors (samples 7, 12, and 13) showed partial loss of the normal allele with ratios suggesting the existence of a predominant 0/2 allelotype. Three tumors (samples 4, 9, and 16) showed evidence of gene amplification, as was suggested (8) to occur in DMBA-initiated/PMA-promoted mouse SCCs. As shown in Table 2, tumors 6 and 23 were the only samples negative for the *Xba* I RFLP. This small percentage (≈8%) of cases not presenting an A → T transversion at codon 61 of the Ha-*ras-1* gene is in accordance with findings on tumors induced by a DMBA/PMA protocol (8) and might represent an alternative pathway of tumorigenesis in the mouse skin system. Based on the D7Rp2/*Aprt* signal ratio, the 1/0 allelic composition of tumor 6 does not appear to be due to a monosomy of chromosome 7, which, furthermore, was not found in any of the tumors reported in our cytogenetic analysis (4). Instead, this allelotype suggests the pres-

Table 2. Assignment of Ha-*ras-1* allelotypes in mouse skin tumors

Sample	Pathology	Ha-*ras-1* RFLP, counts			Ratio				Ha'*ras-1* allelotype proposed, normal/mutated
		12 kb	8 kb	4 kb	Ha-*ras*-1/*Aprt*	Ha-*ras*-1 (8 kb + 4 kb)/*Aprt*	Ha-*ras*-1/D7Rp2	D7Rp2/*Aprt*	
Co	Spleen	1708	0	0	1.0	0	1.0	1.0	0/2
1	SCC III	85 (0.2)	404 (1.1)	363 (1.0)	1.6	1.5	1.1	1.4	0/3
2	SCC I	280 (1.0)	387 (1.4)	340 (1.2)	1.5	1.1	1.2	1.3	1/2
3	SCC II	54 (0.1)	709 (1.0)	717 (1.0)	1.4	1.3	1.1	1.3	0/3
4	SCC IV	48 (0)	1520 (1.0)	1692 (1.1)	3.2	3.2	4.3	0.8	GA
5	SCC I	246 (1.0)	332 (1.3)	304 (1.2)	1.6	1.1	1.2	1.3	1/2
6	SCC III	360	0	0	0.6	0	0.5	1.3	1/0
7	SCC I	134 (0.5)	312 (1.2)	260 (1.0)	1.1	0.8	1.1	1.1	0/2*
8	SCC I	290 (1.0)	309 (1.1)	278 (1.0)	1.4	0.9	1.1	1.2	1/2
9	SCC IV	307 (0.3)	1830 (1.7)	1091 (1.0)	4.5	4.0	2.2	2.0	GA
10	SCC I	92 (1.0)	112 (1.2)	108 (1.2)	—	—	—	—	1/2†
11	SCC I	340 (1.0)	437 (1.3)	568 (1.7)	1.4	1.0	1.1	1.3	1/2
12	SCC I	127 (0.4)	359 (1.2)	296 (1.0)	1.1	0.9	1.0	1.2	0/2*
13	SCC II	105 (0.3)	401 (1.1)	358 (1.0)	1.2	1.1	1.0	1.2	0/2*
14	SCC II	224 (1.0)	322 (1.4)	301 (1.3)	1.4	1.0	1.2	1.2	1/2
15	SCC II	102 (0.3)	445 (1.2)	380 (1.0)	1.4	1.2	1.0	1.4	0/3*
16	SCC III	30 (0.1)	580 (1.2)	499 (1.0)	1.7	1.7	2.2	0.8	GA
17	SCC I	395 (1.1)	370 (1.1)	347 (1.0)	1.7	1.1	1.1	1.5	1/2
18	SCC I	345 (1.0)	387 (1.1)	434 (1.3)	1.4	1.0	1.1	1.3	1/2
19	SCC I	325 (1.0)	335 (1.1)	313 (1.0)	1.6	1.0	1.1	1.4	1/2
20	SCC I	331 (1.6)	208 (1.0)	210 (1.0)	1.3	0.7	0.9	1.5	1/2
21	SCC I/PAP‡	230 (2.4)	95 (1.0)	142 (1.5)	1.2	0.6	0.9	1.3	1/1
22	SCC II	138 (1.0)	190 (1.4)	246 (1.8)	1.3	1.0	0.8	1.7	1/2
23	SCC II	581	0	0	1.2	0	0.9	1.3	2/0
24	SCC II	192 (1.0)	217 (1.1)	203 (1.1)	1.6	1.1	1.1	1.5	1/2
25	SCC II	18 (0.1)	215 (1.3)	171 (1.0)	1.7	1.6	1.1	1.4	0/3
26	SCC I/PAP‡	137 (2.1)	79 (1.2)	65 (1.0)	1.0	0.5	1.0	1.0	1/1

Ha-*ras-1* *Xba* I RFLP values represent counts above instrument and experimental background detected in 1080 min (SD < ±15% of the measured net counts, with 95.5% certainty). Values in parentheses represent relative proportions among the Ha-*ras-1* RFLP signals. Counts were normalized to the signal derived from control spleen DNA by assigning a relative value of 1.0 to the corresponding ratios. Ratios were calculated between normalized counts derived from the total (12 kb, 8 kb, 4 kb) and mutant-specific (8 kb, 4 kb) Ha-*ras-1* signals, the *Aprt* 11-kb signal, and the D7Rp2 12.4-kb signal. Histopathology grading was according to ref. 19. SCC I, well differentiated carcinoma; SCC II, moderately differentiated carcinoma; SCC III, poorly differentiated carcinoma; SCC IV, spindle cell carcinoma (20); GA, Ha-*ras-1* gene amplification; Co, control.

*Histopathology analysis revealed a predominant SCC II area and small SCC I areas. The 12-kb band may be derived from minor subpopulations in the lesion presenting a normal allele component.

†Allelotype assignment based solely on the *Xba* I RFLP (counts not significantly different than zero in the *Aprt* and D7Rp2 signals).

‡Histopathology analysis showed SCC I fields as well as large areas graded as papillomas (PAP).

ence of a partial deletion in chromosome 7 containing the Ha-*ras-1* but apparently not the D7Rp2 locus.

It is also clear from the data that the loss of the normal Ha-*ras-1* allele that occurred in some tumors is not the result of hemizygosis at the Ha-*ras-1* locus (Table 2). Instead, the loss of heterozygosity at the Ha-*ras-1* locus may be accounted for by different mechanisms, as schematically represented in Fig. 3. One possibility is recombination between homologous chromosomes after the S phase. A parental cell presenting a composition of 1 normal/2 mutant H-*ras-1* alleles may generate progeny cells with a 0/3 allelotype by mitotic recombination. This mechanism was proposed as one of the possible second events in the development of retinoblastoma according to the two-hit model postulated by Knudson *et al.* (21). Another possibility is an additional mutation at codon 61 of the normal Ha-*ras-1* allele, which has been proposed to be a "hot-spot" for spontaneous mutations based on studies with noninitiated mice (22) and cultured mouse keratinocytes (23). Finally, a cell presenting a 1/2 allelotype could lose the normal allele becoming a 0/2 allelotype and subsequently gain an additional mutated allele by a double nondisjunction mechanism (24). Interestingly, the presence of tumors showing loss of the normal allele with two copies of the mutated counterpart suggests that double nondisjunction may be a likely pathway for tumor progression in this model.

Although the number of cases reported here prevents us from establishing a definite correlation, histopathological evaluation (Table 2) of these tumors revealed a clear association between the degree of tumor malignancy and the overrepresentation of the mutated Ha-*ras-1* allele. As shown in Table 3, most of the SCCs presenting loss of the normal allele were moderately or poorly differentiated tumors, classified as SCC II and SCC III (classification according to ref. 19), whereas the majority of the tumors having at least one copy of the normal allele were well-differentiated SCCs, classified as SCC I (Fig. 4). In this latter group, tumors with

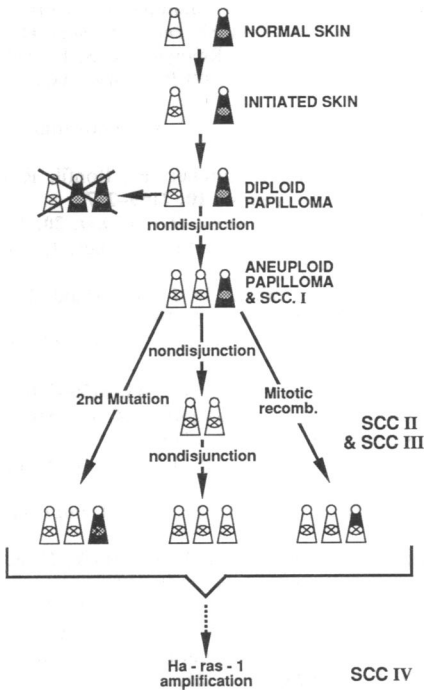

FIG. 3. Schematic representation of the normal/mutated allelic composition of the Ha-*ras-1* gene at various stages of tumor development. SCC I, well differentiated carcinoma; SCC II or SCC III, moderately or poorly differentiated carcinoma, respectively; SCC IV, spindle cell carcinoma, as defined in ref. 19. Open and stippled loci represent the normal parental alleles; locus with cross represents the mutated allele.

Table 3. Ha-*ras-1* allelotype of mouse skin tumors according to histopathology grading

Pathology	Ha-*ras-1* allelotype, normal/mutated				
	1/1	1/2	0/2	0/3	GA
SCC I	2	9	2	0	0
SCC II	0	3	1	3	0
SCC III	0	0	0	1	1
SCC IV	0	0	0	0	2
Total	2	12	3	4	3

Tumors with *Xba* I polymorphism (92% of the total) were used. The numbers of samples under each allelotype and histopathology grade are indicated. Histopathology grading was according to ref. 19. GA, Ha-*ras-1* gene amplification.

a 1/1 allelotype (Table 2, samples 21 and 26) were classified as SCC I and presented large areas graded as papilloma that may be contributing significantly to the overall allelic composition.

Conversely, two of the three tumors showing evidence of gene amplification and loss of the normal Ha-*ras-1* allele (samples 4 and 9) were classified as spindle cell carcinomas (SCC IV), a tumor type regarded as an ultimate step in tumor progression based on its high degree of malignancy and metastatic potential (20), whereas the remaining tumor (sample 16) was very poorly differentiated (SCC III). Table 3 also shows that the proportion of SCC I and SCC II tumors is approximately the same in the 1/2 and 0/2 allelotypes. However, there seems to be a marked shift toward less-differentiated grades when three or more copies of the mutated Ha-*ras-1* allele are present, suggesting that this event might be associated with the acquisition of a more malignant phenotype.

The above histopathological evidence and the fact that almost all SCCs showing *Xba* I polymorphism in this study (22 out of 24), presented a Ha-*ras-1* gene dosage disbalance in the form of 1 normal/2 mutant alleles or loss of the normal allele with an elevated copy number of the mutant Ha-*ras-1*, provides further strong support that activated *ras* oncogenes not only are relevant in the initiation of skin carcinogenesis but also may constitute a necessary step for tumor development and/or progression, probably in a dose-dependent manner. The presence of extra copies of the mutated Ha-*ras-1* gene was previously advanced based on our cytogenetic studies (4) that demonstrated Ts7 in 90% of the tumors

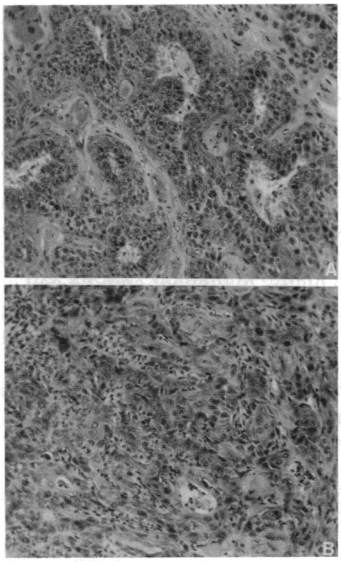

FIG. 4. (*A*) Well differentiated SCC (tumor 19, Table 2). (*B*) Poorly differentiated SCC (tumor 16). (Hematoxylin/eosin; ×55.)

analyzed, suggesting that this numerical chromosomal abnormality may be a major mechanism by which overrepresentation of the mutated Ha-*ras-1* allele occurs in mouse skin tumorigenesis. This study also provides evidence that generation of Ts7 appears to be a major early mechanism by which overrepresentation of the mutated Ha-*ras-1* allele occurs.

Chromosomes carrying an activated oncogene have been shown to be duplicated during tumor progression in other systems. For instance, in chronic myelogenous leukemia, duplication of the Philadelphia chromosome is frequently associated with the terminal acute phase of the disease (25). In our model, it is possible that the disbalance between the activated and the normal Ha-*ras-1* alleles may play a critical mechanistic role. Klein (26, 27) proposed a similar hypothesis with murine T-cell lymphomas postulating that the preferential duplication of the altered chromosome 15 observed in trisomic tumors may be involved in overcoming the influence of a trans-acting suppressive regulator coded by the normal homologue. Trisomic lymphomas having retrovirally rearranged *myc* genes have also shown bias in the duplication of the chromosome 15 carrying the rearranged c-*myc* (28).

A similar chromosome dosage dependency was observed in the suppression of tumorigenicity in hybrids derived from the human carcinoma cell line D98AH2 (29) and the fibrosarcoma cell line HT1080 (30). In the latter, the relative number of chromosome 1 alleles bearing the normal vs. activated N-*ras* gene was suggested to play a critical role in the suppression of tumorigenicity. Specifically, it was speculated that the normal N-*ras* gene may be suppressing the transforming activity of the mutated gene. A similar concept has been advanced with respect to the human and murine p53 gene (31, 32). Spandidos and Wilkie (33) have also proposed that the normal human Ha-*ras-1* gene may exert transformation suppressor activity over the mutated counterpart. However, other authors (34, 35) found no support for this hypothesis, although nonepithelial models were used in those studies. In this context, our findings may be consistent with the hypothesis that the normal Ha-*ras-1* gene may be acting as a suppressor gene whose inactivation, loss, or overbalancing by its mutated counterpart may be a necessary step for the development of the fully malignant phenotype. It is possible that the mutant allele might overbalance the normal allele through a competition mechanism at either the gene or expression levels. On the other hand, it could be postulated that the natural progression of the tumors selects for cells with higher copy numbers of mutated Ha-*ras-1* genes, by virtue of their growth advantage. Consistent with the latter hypothesis, Bizub *et al.* (36) have observed a direct correlation between the content of Ha-*ras-1* p21-altered protein and the degree of morphological transformation and tumor latency of NIH 3T3 cells. Similarly, we observed a constant progression from the well differentiated diploid papillomas with one mutated copy of the Ha-*ras-1* gene to the more aggressive dysplastic papillomas and carcinomas, with Ts7 and with two or three copies of the mutated Ha-*ras-1* allele, and possibly to highly malignant spindle cell carcinomas as a putative ultimate step in neoplastic progression with amplification of the mutated Ha-*ras-1* gene (Fig. 3).

In summary, our results not only stress the importance of activated Ha-*ras-1* genes in epithelial tumor progression but also suggest a role for numerical chromosomal abnormalities in solid tumors.

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