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20. Swimming strains were examined by high-intensity dark-field light microscopy in the laboratory of R. M. Macnab (Yale University) with techniques described by Macnab [*J. Clin. Microbiol.* 4, 258 (1976)].
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24. Supported by NSF grant OCE-8214889. We thank R. M. Macnab for his assistance in dark-field light microscopy and S. Tamm and P. Greenberg for many useful suggestions. This is Woods Hole Oceanographic Institution contribution No. 5945. We dedicate this report to Norbert Pfennig on the occasion of his 60th birthday.

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Partial Inversion of the Initiation-Promotion Sequence of Multistage Tumorigenesis in the Skin of NMRI Mice

Abstract. *Alterations in NMRI mouse skin induced by the phorbol ester 12-O-tetradecanoylphorbol-13-acetate in "stage I of tumor promotion" are slowly reversible, and this reversibility has a half-time of 10 to 12 weeks. The tumor response observed in the course of an initiation-promotion experiment in vivo is independent of whether stage I of promotion occurs before or after initiation. Since the time interval between treatment with the promoter, and subsequent initiation can be extended up to at least 6 weeks, an enhancement of initiation because of promoter-induced cellular DNA synthesis seems to be unlikely. This result may be inconsistent with the two-stage model of tumor promotion because it indicates that in skin the existence of initiated cells is not required for the induction of cellular alterations that are essential for the stage of skin tumorigenesis called stage I of promotion.*

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The terms initiation and promotion were introduced to distinguish between sequential stages of tumor induction observed under strictly controlled experimental conditions (*1*). Initiation is brought about by treatment of a tissue with a carcinogenic agent at a dose that is insufficient to cause tumor development by itself. Tumors develop in such initiated tissues after long-term application of a promoting agent which, under the conditions of the experiment, does not exhibit tumorigenic potency in non-initiated tissues (*1*).

The temporal sequence of initiation and promotion has been thought to be of critical importance—that is, the experiment could not be done in reverse (*1, 2*). Boutwell (*3*) proposed that in mouse skin the process of promotion itself consists of two different stages. Recently, this concept has gained considerable support by the introduction of "incomplete" tumor promoters. When these substances were used in multistage tumorigenesis experiments in mouse skin, stage I of promotion was brought about by a single treatment with a so-called complete promoter such as the phorbol ester 12-*O*-tetradecanoylphorbol-13-acetate (TPA), whereas stage II was brought about by long-term treatment of the animals with an incomplete promoter such as 12-retinoylphorbol-13-acetate (RPA) (*4*) or the diterpene ester mezerein (*5*). Whereas promotion had generally been thought to be a fully reversible process, stage I of promotion in the skin of NMRI mice was characterized by persistent effects (*6*). We now report experiments showing that stage I of promotion is slowly reversible and can occur before initiation.

The proof of persistent effects in stage I of promotion was a major prerequisite for the experiments to be described. Therefore, initiation was carried out on the skin of female albino mice (strain NMRI) by a single topical application of 7,12-dimethylbenz[*a*]anthracene (DMBA) in a subthreshold dose. Stage I of promotion was carried out by two topical applications of TPA (or RPA as control) 1 week after initiation. This treatment was followed after different time intervals by repeated applications of the incomplete skin tumor promoter RPA twice weekly for at least 18 weeks (stage II of promotion). As shown in Fig. 1, the effect induced by TPA (stage I) disappeared gradually with a half-time of 10 to 12 weeks. If in stage I, TPA was replaced by RPA, only a slight increase of tumor incidence was observed when the time interval between stage I and stage II was increased. This result indicates that in NMRI mouse skin, RPA-induced stage I promotion became more prominent with time but was still not comparable with the strong effect of TPA within the time interval of our experiment. The persistence of the TPA effect in stage I suggested the possibility of applying TPA a number of weeks before initiation. To permit a complete regression of the short-term TPA effects such as hyperplasia and inflammation, we allowed 2 to 6 weeks to elapse before DMBA was applied. One week after initiation with DMBA, RPA treatment was started.

The tumor rate and tumor yield achieved by this partially inverted approach were almost the same as those obtained with the usual initiation-promotion sequence, whether initiation was carried out by the topical or the intragastral route (Fig. 2). When the animals were treated with RPA instead of with TPA 6 weeks before DMBA application,

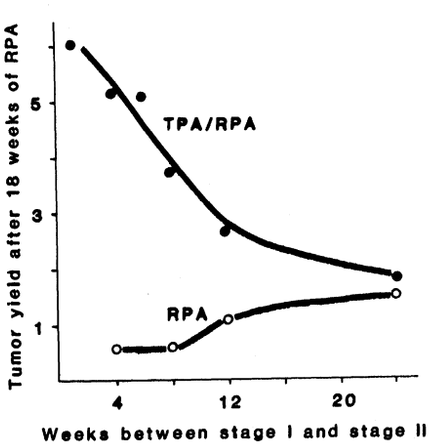

Fig. 1. Two-stage tumor promotion in NMRI-mouse skin—the effect on tumor incidence of increasing the time between stage I and stage II. Female NMRI mice (7 to 8 weeks old; 20 per group) were treated topically with 100 nmol of DMBA dissolved in 100 μl of acetone. Stage I of promotion was carried out with two topical applications of TPA (●) or RPA (○) (20 nmol per 100 μl of acetone) on day 8 and 11 after initiation. For stage II of promotion, long-term RPA treatment (10 nmol per 100 μl of acetone, twice a week) was started at 3, 6, 12, and 24 weeks after stage I treatment (abscissa) and continued for 18 additional weeks. At the end of the experiment, 90 percent or more of the animals were alive. All experiments were repeated twice with similar results. The tumor response is expressed as tumor yield (number of papillomas divided by the number of survivors) after 18 weeks of RPA treatment in stage II.

fewer tumors developed (Table 1). Very little tumor development was observed when DMBA application or long-term RPA treatment or both were omitted. These results show that the persistent changes involved in what has been called stage I of promotion may occur after initiation or before initiation, without a change in the final outcome of the multistage tumorigenesis experiment in NMRI mouse skin.

Initiation in mouse skin has been repeatedly found to be enhanced by a single pretreatment with a promoter, provided the time interval between the two treatments did not exceed 24 hours (*7–11*). These results led to the assumption that initiation is especially effective in cells that are undergoing DNA synthesis. Such an explanation for our observations is not probable since the interval between pretreatment with the promoter and initiation could be extended to at least 6 weeks, whereas the effects of TPA-induced epidermal hyperproliferation and irritation had disappeared after 2 weeks (*12*). Moreover, in contrast to the studies cited above, in our experiments the stage of promotion carried out after initiation was brought about by RPA, a noncarcinogenic hyperplasia-inducing agent, which has been shown to be a very poor promoter in initiated NMRI mouse skin in the absence of TPA-pretreatment. Other experiments demonstrating an inversion of the initiation-promotion sequence may be considered less conclusive since they included either long-term pretreatment with the promoter (*13*), or initiation was performed by repeated applications of an aromatic hydrocarbon in a rather high dose instead of a subthreshold dose (*14, 15*).

Although the noninvertibility of the initiation-promotion sequence has been thought to be one of the essentials of the multistage concept of tumorigenesis (*2*), we do not believe that our results disprove this concept, since they do not contradict the central idea, that—at least in animal experiments—tumorigenesis proceeds in several qualitatively different stages.

On the basis of experiments showing that croton oil and the nonpromoting irritant turpentine have a synergistic effect on promotion, Boutwell (*3*) concluded that the development of visible skin tumors from initiated cells requires more than one step. He suggested that there are at least two steps of promotion: (i) conversion of initiated cells into dormant tumor cells, and (ii) propagation, involving activation and clonal expansion of the dormant tumor cells. The concept of dormant tumor cells has been challenged, for example, by Hennings and Yuspa (*16*), who postulated that an initiated cell is identical to a papilloma cell—that is, promotion can be explained solely by a clonal expansion of initiated cells. Since neither initiated cells nor dormant tumor cells can be unequivocally identified and distinguished from one another by experimental means (the only way to verify the existence of such cells is by promotion!), both concepts must be regarded as more or less equivalent working hypotheses based on operational definitions. We prefer Boutwell's concept, mainly because the introduction of incomplete skin tumor promoters such as mezerein (*5*) and RPA (*4*) has allowed us and others (*4, 5*) to carry out conversion and propagation (stage I and stage II of promotion) in clearly separated steps. Our results on the inversion of the sequence of DMBA and TPA treatment indicate that in NMRI mouse skin the presence of initiated cells is not required for conversion. Within the framework of Boutwell's concept this would mean that for the final outcome of the tumorigenesis experiment it is unimportant whether an epidermal cell is initiated first and then converted into a dormant tumor cell

Table 1. Inversion of the sequence of initiation and stage I of promotion in NMRI mouse skin. Groups of 20 female NMRI mice each (age 7 weeks) were treated as described in Fig. 2 except that two applications of TPA (20 nmol), RPA (20 nmol), or acetone (0.1 ml) were made 6 weeks before topical DMBA treatment. Long-term RPA treatment was started 2 weeks after administration of DMBA. Tumor development is shown as rate (percentage of tumor bearing animals) and yield (number of papillomas per animal) after 15 weeks of RPA treatment.

Stage I	Weeks between stage I and initiation	Initiation	Stage II	Tumor development	
				Rate	Yield
TPA	6	DMBA	RPA	95	6.3
RPA	6	DMBA	RPA	30	1.1
Acetone	6	DMBA	RPA	15	0.2

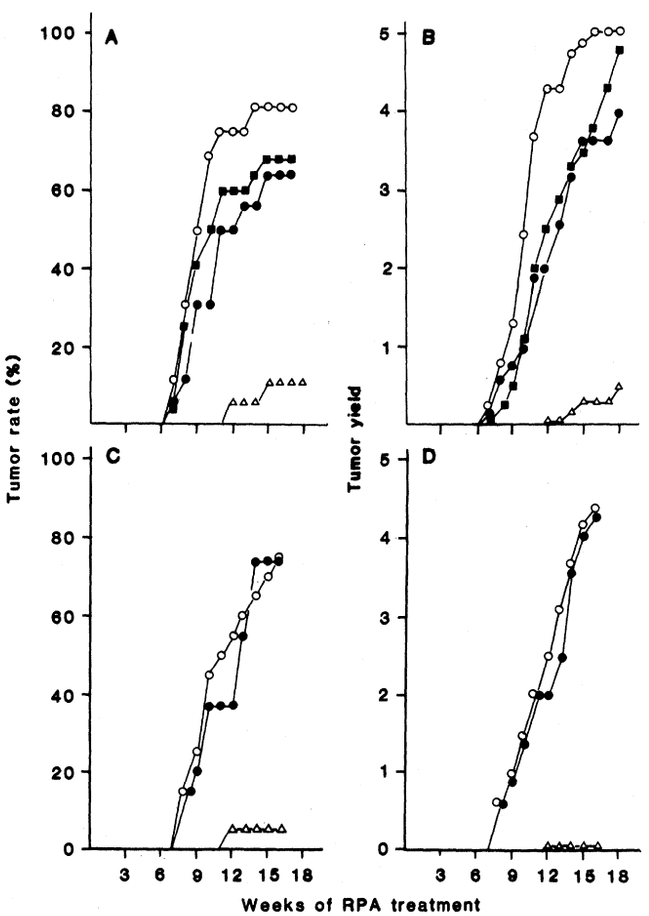

Fig. 2. Inversion of the sequence of initiation and stage I of tumor promotion in NMRI mouse skin. (A and B) Two applications of TPA (20 nmol per 100 μl of acetone each) were made in a 3-day interval, 2 (■) or 6 (●) weeks before topical application of 100 nmol of DMBA. (C and D) Initiation was induced by intragastral instead of by topical application of DMBA (50 μg per gram of body weight), and stage I of promotion was carried out by a single TPA application 3 weeks before initiation (●). In both experiments, stage II of promotion was carried out by repeated RPA application (see Fig. 1) starting 2 weeks after administration of DMBA. In control experiments stage I of promotion was carried out after initiation. For this purpose animals were treated with two applications of TPA (○) or acetone (△) 1 week after topical (A and B) or intragastral (C and D) DMBA application. After another week, RPA treatment (stage II) was started. Tumor development is shown as rate (percentage of tumor-bearing animals) and yield (number of papillomas per animal), depending on the time of RPA treatment in stage II of promotion (abscissa). For other details see Fig. 1.

or the tissue is converted to a state in which treatment with a carcinogen immediately results in the formation of dormant tumor cells.

The inversion experiment thus contradicts the assumption that conversion is due to a selection and clonal expansion of neoplastic cells or to transition of initiated into dormant tumor cells. It may also be incompatible with our former interpretation that conversion is due to an expression of the neoplastic phenotype (*17*), unless TPA pretreatment creates conditions in skin that facilitate a spontaneous phenotypic expression after initation.

We would like, therefore, to modify Boutwell's concept in such a way that the process of conversion is considered as a discrete element of multistage tumorigenesis in mouse skin rather than as a component of promotion. The term "promotion" should actually be restricted to those events that must occur after carcinogen treatment and that have been called "stage II of promotion." We agree with Hennings and Yuspa (*16*) that these events may critically involve a selection and clonal expansion of tumor cells. The term "stage I of promotion" should be avoided in the future and replaced by the term "conversion," which may be operationally defined as a conversion of the tissue into a state of (increased?) promotability. This would mean that the distinction between incomplete and complete skin tumor promoters would be obsolete. Both RPA and TPA are promoters of comparable potency, but for reasons still unknown TPA is a much stronger converting agent than RPA (or mezerein). Such a reevaluation of the nomenclature is more than a matter of semantics, since it may help to overcome the discrepancy that (with few exceptions) no differences between the biological effects of RPA (or mezerein) and TPA could be found in systems other than the skin of the living mouse (*17*). Since the other systems are mainly in vitro models, the necessity of conversion might be restricted to the in vivo situation.

A mechanistic interpretation of our results is hampered by the fact that little is known about the cellular and molecular events occurring in the conversion stage. Events thought to be essential for phorbol ester–induced tumor promotion, such as an interaction with protein kinase C (*17*) and generation of superoxide anion radicals (*18*), appear not to be of critical importance for conversion. Whereas promotion is fully reversible when the intervals between individual RPA treatments are extended up to 2 weeks, conversion leads to much more persistent effects. Originally we had assumed that conversion—at least in NMRI mouse skin—is almost irreversible (*6*). We have now shown that it disappears slowly, with a half-time of approximately 10 weeks. This may be taken as an indication that long-lasting effects on epidermal cell kinetics or metabolism are involved in conversion. However, slow reversibility makes it unlikely that conversion is due to the same molecular mechanism as initiation, which is generally assumed to be irreversible. It is conceivable that—provided initiation is a rare mutagenic event—the effect leading to conversion must occur with a high degree of probability, since otherwise an inversion of the experimental sequence would not be possible.

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A Model for the Tertiary Structure of p21, the Product of the *ras* Oncogene

Abstract. *A model was developed for the structure of p21, the protein with a molecular weight of 21,000 that is produced by the* ras *genes. This model predicts that p21 consists of a central core of* β*-sheet structure, connected by loops and* α *helices. Four of these loops comprise the guanine nucleotide binding site. The phosphoryl binding region is made up of amino acid sequences from 10 to 16 and from 57 to 63 of p21. The latter sequence may contain a site for magnesium binding. Amino acids defining guanine specificity are Asn-116 and Asp-119, and sequences around amino acid 145 may contribute to guanine binding. The model makes it possible to visualize how oncogenic mutations of p21 affect interaction with guanine nucleotides.*

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Proteins with a molecular weight of 21,000 (p21) are produced by a family of proto-oncogenes referred to as *ras* genes. There are at least three members of the *ras* gene family in the human genome, designated H-*ras*, K-*ras*, and N-*ras* (*1*). Closely related proteins are made by the yeast *Saccharomyces*, the fruit fly *Drosophila*, and the slime mould *Dictyostelium* (*2*). Several reports have pointed to sequence homology between p21 proteins and various nucleotide-binding enzymes and regulatory proteins, such as the β-subunit of bovine mitochondrial adenosinetriphosphatase (*3*), bacterial elongation factors EF-Tu and EF-G, α-tubulin, adenylate kinase and other nucleotide-binding enzymes (*4*), and signal-transducing G proteins (*5*). Of this group, the homology between p21 and EF-Tu is particularly interesting, since EF-Tu has been extensively studied at the structural level. The primary sequence of p21 has been aligned with EF-Tu so that 42 percent of p21 amino acids have identical or conservative equivalents in EF-Tu (*6*). Further-