Genetic Regulatory Mechanisms and Carcinogenesis

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DURING the past few decades, experimental oncology has described both the multiplicity and the vast spectrum of agents which can, under the appropriate conditions, induce tumor formation and the heterogeneous biological and biochemical alterations which occur in the resultant tumors. In very general terms, carcinogenesis can be described as a series of changes that occur in a tissue when it is exposed to the appropriate stimulus and result in the phenotypic traits which characterize a tumor.

In a given experiment, both the pattern of phenotypic changes occurring during carcinogenesis and the life history of the resultant tumor are unique. Although this is generally true, and indeed each tumor seen clinically has unique characteristics, nevertheless a convergence of biological and enzymatic characteristics occurs during carcinogenesis. Thus, tumors arising from different tissues tend to lose their specialized characteristics and become more similar to one another than to the tissues from which they originated. Based on enzyme analyses of a number of tumors, Greenstein formulated the "convergence" hypothesis of cancer in which he concluded that "tumors tend to converge enzymatically to a common type of tissue" (*1*).

A number of different agents or conditions may effectuate the carcinogenic transformation—the DNA and RNA viruses; radiation of different types; chemical agents including literally thousands of different types and structures; films of metals, plastic, or glass; hormonal stress of the appropriate severity; and, finally, normal cells become malignant when they are simply propagated in tissue culture in vitro.

The multiplicity and heterogeneity of causal agents clearly indicate that the initial cellular sites of action of the carcinogen in each case may be quite different. However, the convergence of biological and enzymatic character during carcinogenesis suggests that these agents are affecting common cellular mechanisms. This is suggested by other data as well; thus, carcinogens may act either synergistically or antagonistically. A good example of this is the well-known promoting or inhibiting activities of certain hormones on chemical carcinogenesis (*2*, *3*). Another interesting example is the synergism between X-radiation and the carcinogen urethan in experimental leukemogenesis (*4*).

Although the diversity of carcinogenic agents implies that there is no single, common, initial

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site of action for all carcinogens, the ability of these agents to act synergistically or antagonistically suggests that their carcinogenic action may be the result of their impingement on a common cellular system which is being altered during carcinogenesis.

Phenotypic Changes

Figure 1 shows generally the kinds of phenotypic deviations from the normal which are seen in tumors. Thus, there is a loss of growth control, a loss of contact inhibition (*5*), greater invasiveness and metastasis, a loss of specialized function, a deletion (*6*) or appearance (*7*) of specific antigens, an alteration in the enzyme profile primarily involving the loss of enzyme activity (*1*), and finally a loss of responsiveness to environmental stimuli as seen by a decreased enzyme inducibility (*8*).

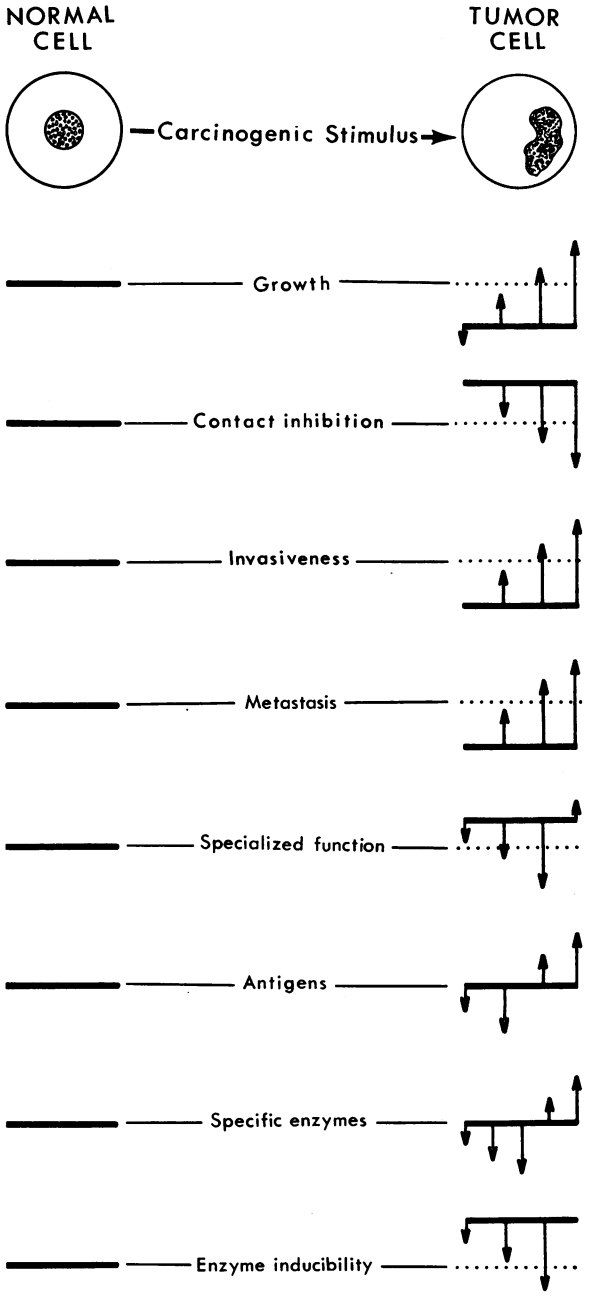
The degree of change of a given characteristic from the normal varies. Furthermore, in some cases a given character may remain either similar to that of the normal tissue or may deviate only minimally, and in other cases the deviation may be gross. The deviation from the normal may be present in a relatively stable and biologically unrecognizable state, as is the case of the initiated skin tumor cell (*9*), or be recognizable as a benign tumor.

On the other hand, the phenotypic deviation may not be stable, but rather progress from the normal either slowly or rapidly and thus reflect the relative malignancy of the tumor (*10*). In addition, a relatively stable condition may be modified to the progressive state as in the case of two-stage tumorigenesis (*9*). Here the carcinogen-induced first stage represents an alteration from the normal state, which remains stable and unrecognized until it is modified to the tumor state by subsequent treatment with croton oil, a noncarcinogenic irritant.

Since the phenotypic alterations observed in the tumor are inherited by successive generations of its cells, it is necessary to relate the observed changes in the tumor either to the ultimate and transmissable repository of biological information, the gene, or to the system which converts genetic information into specific phenotypic character, that is, the gene-action system (*11*).

In other words, the phenotypic changes observed in carcinogenesis must be related either to the chemical structure of the gene itself or to changes in the system that transcribes information from the gene into particular messages, which are then translated into specific enzyme proteins. These end products of the gene-action

Figure 1. Phenotypic changes observed in tumor tissue



system, the proteins, when ordered into the appropriate cellular architecture, impart specific phenotypic characters to the cell. The early alterations in gene-action systems induced by carcinogens may involve alterations either in gene structure or in gene activity.

Changes in the structure of the gene can occur either by mutation (by the deletion or alteration of a nucleotide in DNA) or, in the case of viral carcinogenesis, by incorporation of the viral genome into the host DNA. The second possibility is that gene structure remains intact but that there is an alteration in gene expression, that is, in the activity of specific genes. Thus, genes that are normally inactive may be activated and those normally active may be repressed. Current concepts suggest that embryonic differentiation involves a progressive alteration in gene expression.

Therefore, cells of widely varied phenotype develop from a common ancestor and have no detectable differences in either their DNA content (*12*), in the ratio of nucleotides in their DNA (*13*), the hybridizing properties of the DNA (*14*), or in their chromosome morphology. It has been estimated that a relatively small proportion of a cell's DNA is active as an information template. Hence, the diversity of phenotype among different tissues may be the result of differing loci of activity on identical DNA molecules.

Although the early events of carcinogenesis may be alterations in either gene structure or in gene expression, it seems likely that the subsequent events must involve a large number of progressive alterations in the expression of different genes. The reason for this conclusion is that a single mutation or even several mutations, if taken as isolated events, would delete or alter a single enzyme or several enzymes. Furthermore, even in the case of viral carcinogenesis, it has been estimated that there are no more than 5–10 genes present in the viral genome. This is based on the finding of a molecular weight of 3×10^6 for polyoma virus DNA (*15*). These genes would therefore impart perhaps 5–10 new characteristics to the infected cell.

The large phenotypic differences between normal cells and tumor cells cannot be explained by such a relatively small number of changes, and they must involve a large number of events subsequent to the initial changes. In other words, the initial changes involving alterations in either structure or activity of a few genes must be subsequently multiplied into variations in the activities of a large number of genes. This progression would result in inactivation of a number of genes that are normally active and vice versa, thereby producing a pattern of gene expression which would eventually characterize the tumor cell. It seems, therefore, that carcinogenesis is a process in which there must be progressive alterations in the nature of macromolecular information transferred from the gene into specific phenotypic character.

Nature of Biological Code

What is known about the biochemical nature of this information transfer system? In respect to the nature of the biological code (*16*), we are in a rather enlightened position. First, it is now well documented that DNA is the genetic material and it is DNA that is transmitted from one generation to the next and which carries within its structure the information for succeeding generations.

We also know a great deal about the structure and composition of DNA. It is a double-stranded molecule consisting of two complementary strands. The strands are composed of four subunits. In the complementary strands, adenine is paired with thymine and guanine is paired with cytosine.

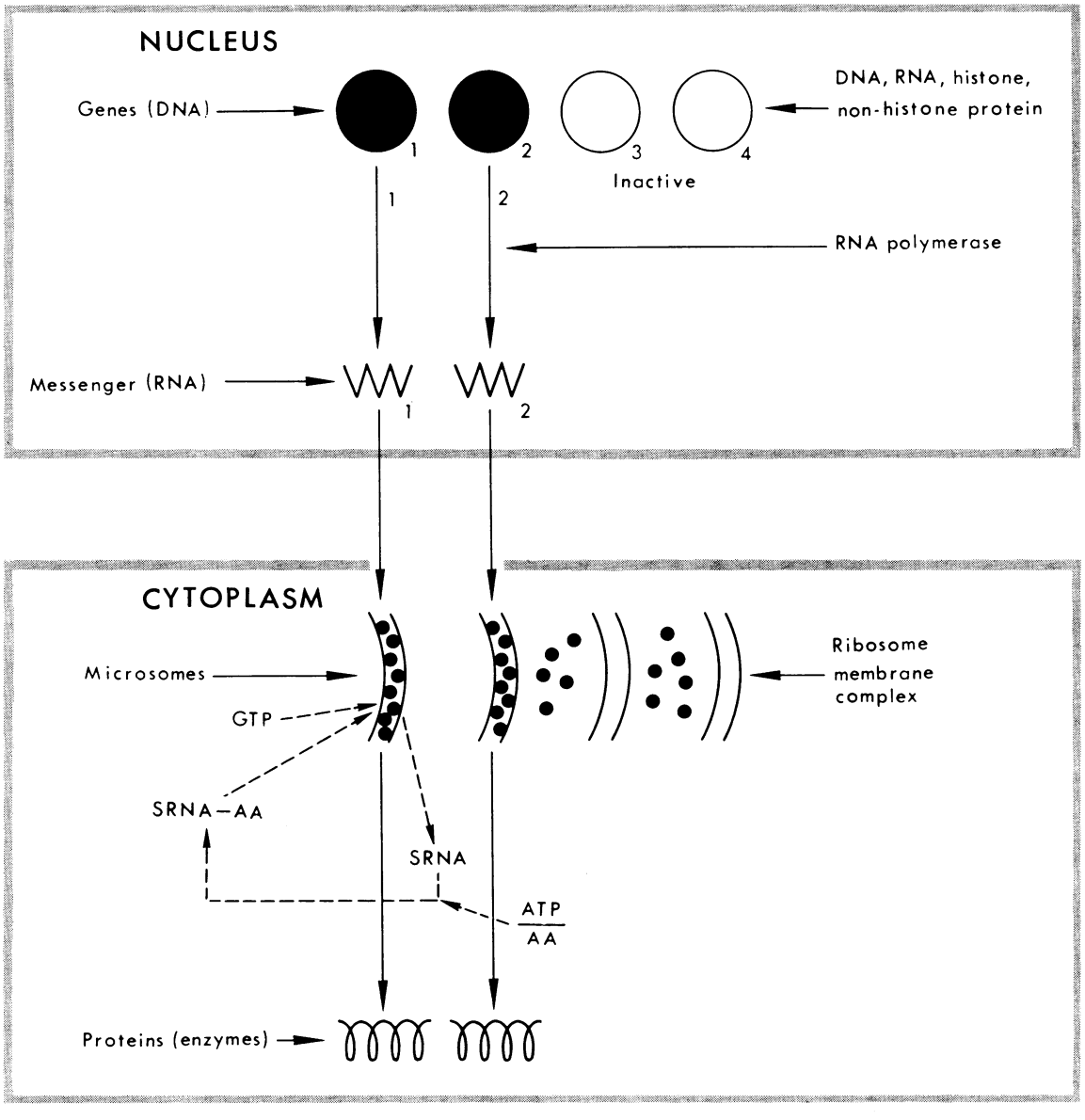
Yanofsky and co-workers have shown that a strict colinearity exists between the sequence of bases in DNA, the genetic material, and the sequence of amino acids in the product protein (*17*). Other workers have shown that the sequence of nucleotides in DNA specifies the sequence of amino acids through the formation of an intermediate molecule, messenger RNA, which relays the message from DNA to the protein synthesizing site, the ribosomes. The RNA represents a complementary reading of the DNA. We know a great deal about the nature of the code in messenger RNA. Thus, the message coding for a single amino acid consists of a sequence of three nucleotides in messenger RNA. The recent studies of Nirenberg and coworkers (*18*) are very close to demonstrating

the specificities of each of the possible 64 codon triplets. Consequently, we are approaching a knowledge of the exact nature of the biological code.

Gene-Action System

Although we have almost a complete understanding of the nature of the genetic code, by comparison we know extremely little about the processes regulating the transcription of DNA into messenger RNA or those processes regulating the translation of the message into protein. We do know, however, that certain genes may affect the activity of other genes and also that this regulation of one gene by another is sensitive to various molecules in the environment of the cell. These molecules affecting gene activity may be of either endogenous origin, such as certain metabolites, substrates, or hormones, or

Figure 2. Gene-action system



NOTE: GTP, guanosine triphosphate; ATP, adenosine triphosphate; AA, amino acid; SRNA, soluble ribonucleic acid (transfer RNA).

may be of exogenous origin; for example, certain carcinogens or drugs. In this way genetic activity becomes responsive to the environmental changes to which the cell is exposed.

A simple formulation of the gene-action system is shown in figure 2. First is the question of the nature of the mechanism regulating the activity of genes. As shown, certain genes, 1 and 2, are active in the synthesis of messenger RNA's while other genes, 3 and 4, are inactive. We do not know the nature of this regulation, but we do know that when DNA is transcribed into RNA the reaction is catalyzed by an enzyme, RNA polymerase. A useful inhibitor which blocks this reaction is actinomycin-D (*19*).

After the messenger RNA is made in the nucleus, it is transferred to the cytoplasm and more specifically to the ribosome-membrane complex where it is translated into a specific polypeptide chain or protein. Although a fair amount is known about the mechanism of protein synthesis (*16*), very little is known about the mechanisms regulating this process. For example, we know little concerning those factors governing the relative stability of the messenger RNA or the number of times each message is read.

Effects of MC on gene activity. One indicator of possible change in the gene-action system is enzyme induction, that is, the appearance of increased levels of specific enzymes. An example of this phenomenon is the stimulatory effect of methylcholanthrene (MC), a carcinogenic polycyclic hydrocarbon on the level of a number of microsomal drug metabolizing enzymes (*20*). One of the microsomal enzymes induced to high levels within 24 hours after MC is administered is benzpyrene hydroxylase, an enzyme which hydroxylates aromatic rings (*21*).

In each of the tissues shown in figure 3, MC caused a rapid increase in enzyme activity. Actinomycin-D, an inhibitor of DNA-dependent RNA synthesis, and puromycin, an inhibitor of protein synthesis, either prevented or diminished the increase in enzyme activity in all of the tissues studied. These results suggested that the increased activity was due to new enzyme synthesis. If this were so, changes might occur in the protein synthesizing system.

In order to investigate this possibility we turned to in vitro studies on the effect of methylcholanthrene on microsomal protein synthesis of rat liver. We found that in vivo MC treatment of rats increased the incorporation of C^{14}-

Figure 3. The effect of actinomycin-D and puromycin on the MC stimulation of benzpyrene hydroxylase activity of rat tissue

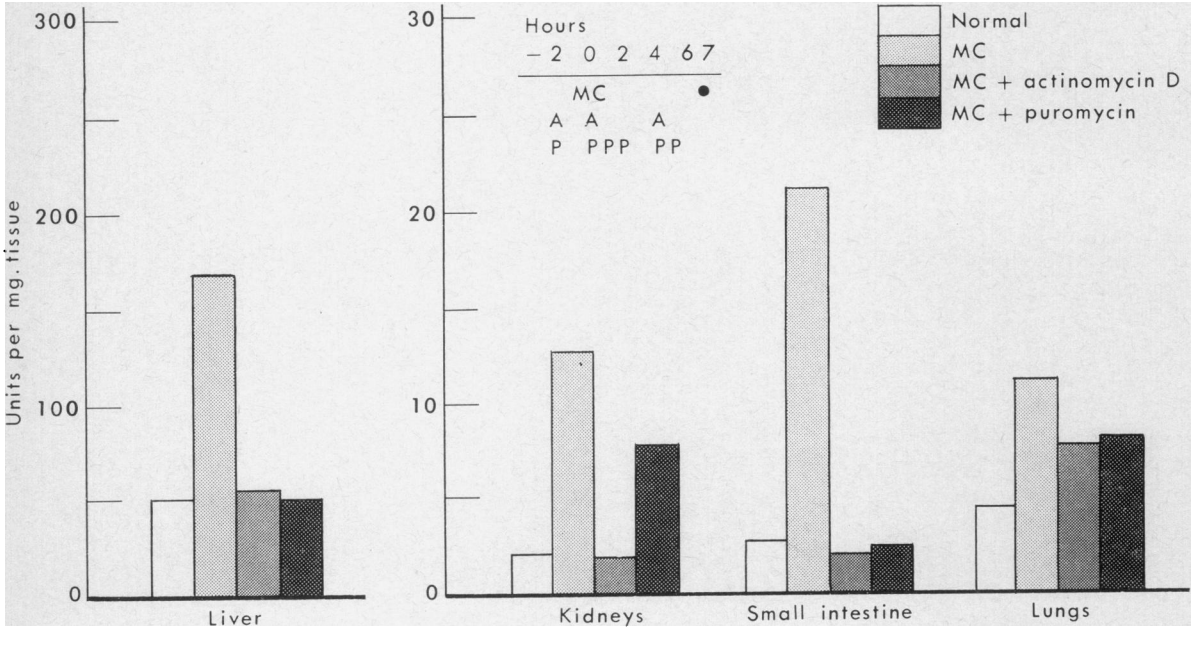
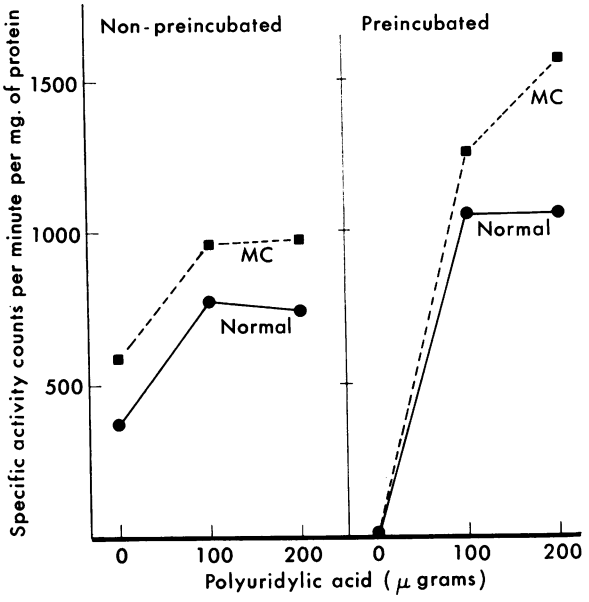


Figure 4. Effect of polyuridylic acid on l phenylalanine-C^{14} incorporation in microsomes from normal and MC-treated rats



leucine in vitro by microsomes prepared from their livers. One milligram of MC injected 18 hours before sacrificing increased in vitro incorporation by 40 percent. The maximum incorporation in both the normal and MC microsomes was dependent on those co-factors known to be required in protein synthesis (*22*).

Microsomes can be said to consist of two operationally separate components. One is the ribosome-endoplasmic reticulum complex, and the other is the messenger RNA which is attached to this complex and which represents the informational template that directs the incorporation of amino acids in a specific sequence. It was of interest to know which of these components was affected by MC. We found that MC affected both the level of microsomal messenger RNA and the ribosome-reticulum complex to which the messenger RNA is attached (*17*).

Figure 4 shows that in the nonpreincubated microsomes the MC microsomes are more active than the normal ones when no exogenous messenger is added. Also, both MC and normal microsomes are fully saturated with 100 μg of the synthetic messenger, polyuridylic acid. After preincubation, 100 μg of polyuridylic acid saturates the normal microsomes, but fails to saturate the MC microsomes. This suggests that more sites were made available for the added polyuridylic acid in the MC microsomes during preincubation, and the increase in the number of sites available suggests that more messenger RNA was removed from the MC microsomes. The results suggest that MC microsomes contain an increased level of messenger RNA.

The effect of polyuridylic acid, the synthetic messenger RNA, on phenylalanine incorporation in the preincubated microsomes is also shown in figure 4. These microsomes are completely dependent on polyuridylic acid for activity. The microsomes from MC-treated rats are more active than are normal microsomes in the presence of identical amounts of messenger RNA. Thus, MC treatment also increases the microsomal sensitivity to added exogenous messenger RNA.

Actinomycin-D, an inhibitor of DNA-dependent RNA synthesis, prevents the increased enzyme activity as well as the increase in protein synthesizing activity (*22*). This result suggested that these effects may be due to an activation of specific genes. If this were so, there might be alterations in DNA-directed messenger RNA synthesis in the nucleus. We then directed our attention to the effect of MC on nuclear RNA metabolism and specifically on the production of messenger RNA.

Early experiments with highly purified nuclei (*23*) showed that MC has no effect on the

Table 1. Nuclear RNA content [1] in livers of normal rats and rats treated with methylcholanthrene

Experiment No.	Normal rats	MC-treated rats	Percent difference
		4 hours	
1	0. 20	0. 20	0
2	. 20	. 20	0
		16 hours	
1	. 20	0. 23	+15
2	. 20	. 23	+15
3	. 20	. 30	+50
4	. 24	. 33	+38
5	. 22	. 25	+14
6	. 25	. 32	+28

[1] Ratio of RNA protein to DNA protein.

level of DNA per gram of rat liver. However, 16 hours after MC treatment, the amount of nuclear RNA is increased (table 1). Although at 4 hours no differences were detected, at 16 hours after MC treatment, 15–50 percent more RNA per DNA was observed in the nuclei of rat livers.

The incorporation into RNA of a radioactive precursor, orotic acid-C^{14}, is stimulated to a marked extent by MC pretreatment (fig. 5). The greatest stimulation was observed at 4 hours after the MC was administered. Thus, there is an increase in the rate of RNA synthesis and an increase in the amount of RNA present.

When RNA was isolated from the nuclei of MC-treated rats and control rats and tested for its ability to stimulate or to direct amino acid incorporation in an *Escherichia coli* protein synthesizing system, we found that, per microgram of RNA added, the RNA from MC-treated rats had considerably greater messenger activity than did the RNA from similarly isolated normal nuclei. As shown in table 2, the RNA from MC nuclei had 29–57 percent more stimulatory activity than did identical amounts of normal RNA. Considering both the MC-induced increase in the amount of nuclear RNA and the increase in its specific messenger RNA activity, MC more than doubles the messenger RNA activity of the liver nuclei. More recently we have found that MC increases rat liver RNA polymerase activity.

Figure 5. The effect of methylcholanthrene on orotic acid-C^{14} into rat liver nuclear RNA

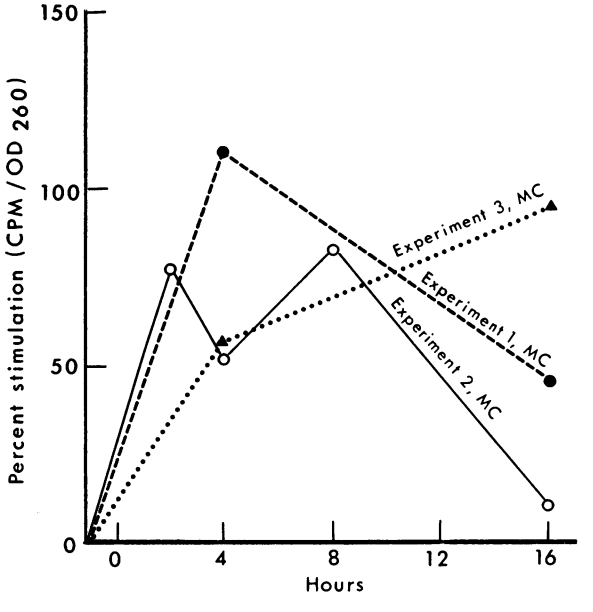


Table 2. Stimulatory activity on C^{14} phenylalanine incorporation [1] into protein by nuclear RNA from normal and MC-treated rats

Experiment No.	Nuclear RNA (μg)	Normal rats	MC-treated rats (16 hours)	Percent difference
1	8	11.3	16.2	+43
2	11	14.8	23.2	+57
3	25	12.3	15.9	+29

[1] Micromicromoles of phenylalanine incorporated per 100 μg of nuclear RNA.

The following list summarizes the effects of MC on the gene-action system:

Microsomes

Increase of:
- Specific enzymes
- Amino acid incorporation:
  - More messenger RNA
  - More sensitive to added messenger RNA
- Effects prevented by puromycin and actinomycin-D

Nucleus

Increase of:
- Orotic acid-C^{14} to RNA
- RNA per DNA ratio
- Messenger RNA content
- RNA polymerase activity

Initial alteration. Some of the results presented thus far suggest that MC has an effect on gene activity; that is, it affects the DNA-dependent production of RNA. We do not know that the primary interaction of MC is with the gene, but we do know that soon after MC is administered there is an alteration of gene activity. However, other compounds which are noncarcinogenic, such as hormones and some drugs, also induce changes in the gene-action system. Furthermore, carcinogens may alter gene activity in tissues which are resistant to malignant transformations as well as in those tissues which are susceptible. It may be that in tissues not undergoing malignant transformation the alterations in gene expression revert back to the normal state as in a target

tissue's response to a hormone. In tissues susceptible to the carcinogen's action, these changes may be irreversible.

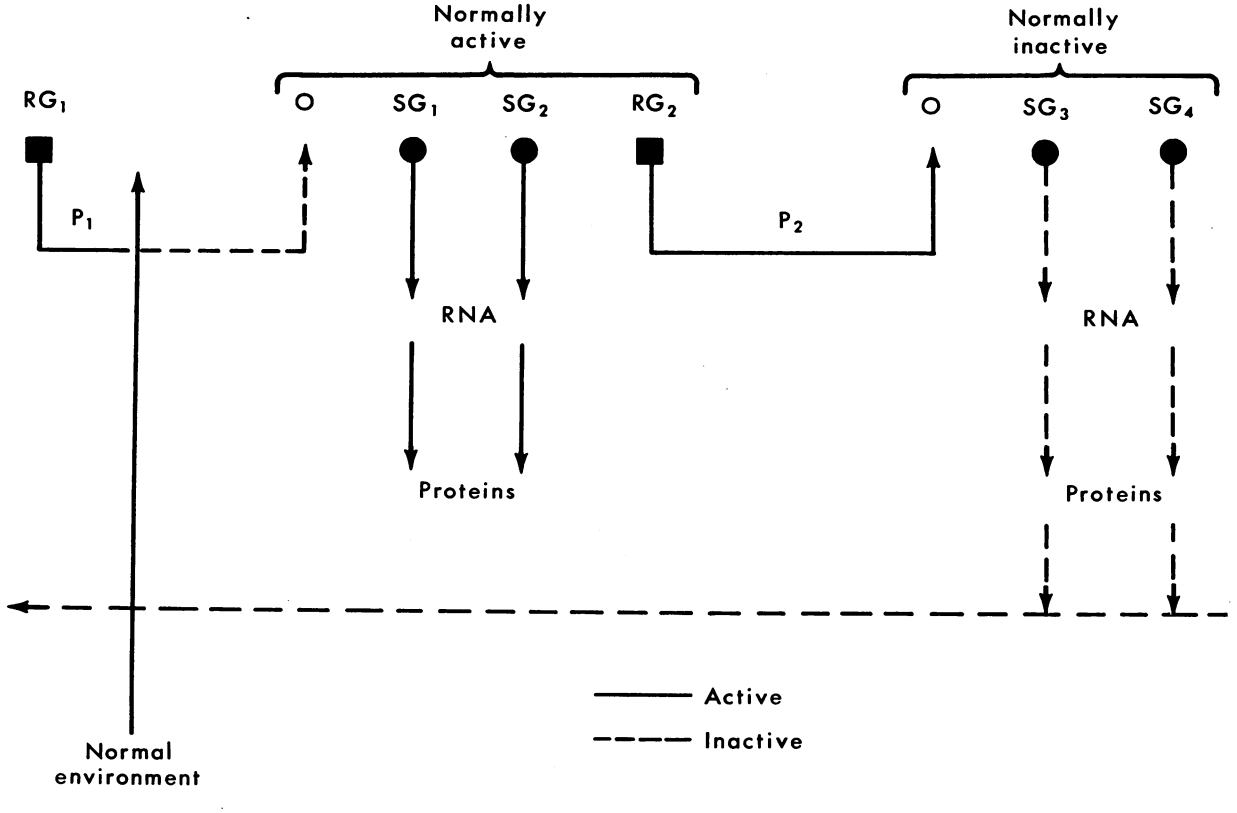
Under several experimental conditions, a single small dose of MC can initiate the tumorigenic process. More than 95 percent of the carcinogen disappears from mouse skin within 24 hours (*24*). Thus, the early carcinogen-induced changes in the gene-action system may represent the initiation stage of carcinogenesis. In certain cases, this initial alteration may progress to the eventual tumor without continuous application of carcinogen or promoter. In other cases, the alteration in the pattern of messenger RNA synthesis may require further progression by the imposition of either more carcinogen, the proper hormonal conditions, or a so-called promoting agent.

The progression in the case of carcinogenesis may be an inexorable one, since the cell with the new pattern of messenger RNA synthesis may have the advantage over its normal neighbors if it is somewhat more active in cell division. A shift in the expression of specific genes would then result in a shift in the synthesis of specific enzymes, and a consequence of this would be an altered metabolism and an alteration in the environmental milieu of the nucleus.

Interlocking Gene-Action Systems

In order for the initial alterations to be progressive and the progression to take place without continuous carcinogen application, the regulation of activity of different sets of genes must be interlocked in some manner. Thus, the turning on of one set of genes would concomitantly switch off another set. A model of an interlocking gene-action system is shown in figure 6. The model is based on the Monod-Jacob repressor model (*25*) with the added suggestion of Waddington that there is more than one regulator gene for each structural gene (*26*). This type of "cascade regulation" (*27*) focuses attention on the potential interlocking nature of the gene-action system. Thus, an active operon is controlled by regulator gene, RG_1, and contains a second regulator gene,

Figure 6. Interlocking gene-action system



RG_2, which produces a molecule, P_2, which is repressing a second set of genes. In this way sets of genes can be interlocked so that when one set is active in RNA and protein synthesis, the other is inactive.

Although the model shown is based on repression, any number of models with interlocking gene-action systems can be proposed if one accepts two premises: (*a*) that certain genes affect the activity of other genes, and (*b*) that environmental conditions can alter this regulation. The first premise has been well established in micro-organisms by both classic genetic studies and the more recent studies in enzyme induction (*25*). In higher organisms, the first premise is suggested by some studies of Halvorson (*28*) in yeast and McClintock (*29*) in maize. The second premise, that environmental conditions can alter this regulation, is supported by a number of studies on mammalian enzyme induction and hormone action (*30*).

With an interlocking system, the phenotype of a cell can be permanently altered by a transitory exposure to a specific type of environmental agent. This can occur with no change in genotype. Beale (*31*) has shown that the transitory exposure of paramecium to a specific temperature will induce the formation of a specific antigen. The production of this antigen will continue for more than 50 generations after the original organism's single experience with the new temperature. The facts of carcinogenesis are similar. The phenotype of a cell is altered by its transitory exposure to a carcinogen. If a carcinogen-induced alteration in the expression of gene information is indeed the nature of the initiation of tumorigenesis, then a block of gene expression during the time in which the tissue is exposed to the carcinogen might be expected to inhibit the carcinogenic process.

Inhibition of Tumorigenesis

One experimental approach to the testing of this hypothesis is with the use of specific metabolic inhibitors. One inhibitor of gene activity is actinomycin-D. We used the two-stage system of skin carcinogenesis of Berenblum and Shubik (*32*). In this study we were joined by Dr. Michael Klein (*24*). A single small dose of carcinogen, 7,12-dimethylbenz(a)anthracene, applied topically to the skin initiates the tumorigenic process. Subsequent weekly application of croton oil, a promoting agent which is essentially noncarcinogenic, causes tumors to appear after a period of 13–20 weeks.

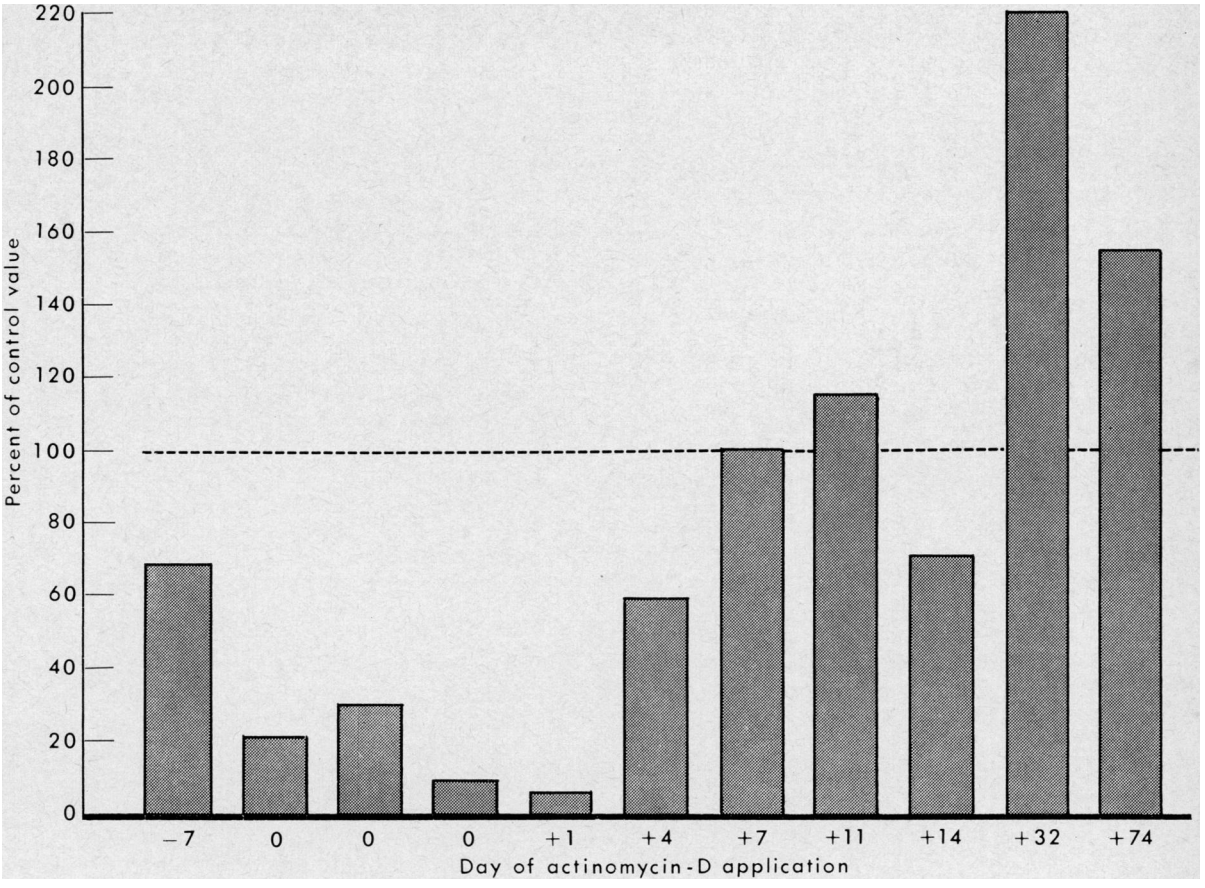
Figure 7 shows the effect of actinomycin-D on initiation when it was given at varying times relative to the time the carcinogen was given. Mice treated with actinomycin-D on day "zero," the day the carcinogen was applied, developed 5–33 percent as many tumors as the controls. Actinomycin-D given 1 day after the carcinogen was as effective an inhibitor as it was on day "zero." When actinomycin-D was given 7 days prior to the carcinogen, there was only a slight inhibition, probably due to the presence of residual actinomycin-D at the time the carcinogen was applied. When actinomycin-D was given at day 4, inhibition was considerably less than that observed at day "zero"; when it was given at day 7, essentially no inhibition occurred.

These experiments clearly demonstrated that actinomycin-D inhibits the initiation stage of skin tumorigenesis. We concluded from this that the initiation of skin tumorigenesis requires the simultaneous activity of DNA. This activity may be either in DNA synthesis or in RNA synthesis. Furthermore, our evidence delineates the time it takes for initiation of skin tumorigenesis. At 0 and 1 day, there is almost complete inhibition by actinomycin-D. At 4 days, there is very little; and at 7 days, there is none. Thus, the initiation stage of tumorigenesis sensitive to actinomycin-D is essentially completed in a few days.

Carcinogenesis and Gene Action

The work of many investigators has demonstrated an alteration in the enzyme profile during carcinogenesis and in the resulting tumor. Further, by electrophoretic analysis, Sorof and co-workers (*33*, *34*) showed marked changes in the distribution of soluble proteins during the carcinogenic process. Since enzymes are the end products of gene-action systems, the altered enzyme profile of the tumor cell must reflect an altered message flow. Kidson and Kirby have offered experimental support for this conclusion by showing that the pattern of messenger

Figure 7. Effect of time of application of actinomycin-D on tumorigenesis induced by DMBA and croton oil on mice



NOTE: Three different experiments were conducted on the three "zero" days.

RNA synthesis during carcinogenesis (*35*) and in the resulting tumor (*36*) is different from the pattern of messenger RNA synthesis in normal liver.

More recently Sporn and co-workers (*37*, *38*) found that the liver carcinogens 3'methyl-dimethyl aminoazobenzene and aflatoxin B–1 lower the RNA content of liver nuclei. These findings are consistent with recent results obtained in our laboratory showing that the liver carcinogens mentioned markedly suppress DNA-directed RNA synthesis. Here, the carcinogenic action may be related to the suppression of DNA activity as a template for RNA synthesis.

McCarthy and Hoyer (*14*) also showed that the RNA from mouse tumor cells grown in culture hybridizes with DNA differently than the RNA obtained from normal mouse tissues. These data suport the hypothesis that carcinogenesis involves alterations in the flow of genetic information. Other experimental results support this concept. For example, Abelev (*39*) has shown that liver tumor cells contain an antigen which is identical to an antigen present in embryonic tissue but not in normal adult liver. This suggests that genetic information which was once expressed in the embryo and subsequently repressed during differentiation is again being expressed in the liver tumor.

We also know that hormones greatly modulate carcinogenesis, in some cases promoting the process and in other cases inhibiting the process. Recent studies on the mechanism of hormone action suggest that hormones affect gene activity (*30*) and thus, if they are acting on the same system as the carcinogen, one would expect them to act as modulators of carcinogenesis.

The results of experimental studies of carcinogenesis lead to the conclusion that the neoplastic transformation involves numerous progressive changes in the activities of specific genes. One of the primary problems of carcinogenesis, therefore, is to determine how the primary interactions of carcinogens with various components of the cell lead to alterations in the gene-action system. An inherently related task is to determine which gene or sets of genes need to be modified in order to initiate those progressive changes which are termed carcinogenesis.

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Scholarship and Loan Funds for Fiscal 1967

Scholarship and loan funds for fiscal year 1967 have been made available to schools of medicine, dentistry, optometry, osteopathy, podiatry, and pharmacy through the Public Health Service. Each of the 227 schools receiving scholarship funds and the 196 receiving loan funds will be responsible for administering these grants, including selecting the students and the amount awarded to each.

The Health Professions Scholarship Program will provide up to $2,500 per academic year for full-time first-year students from low-income families. Increased enrollment in health professions schools is the basic objective of the program.

Under the Health Professions Student Loan Program, full-time students can borrow up to $2,500 per academic year. They must repay the schools over a 10-year period beginning 3 years after completion or termination of study. Interest accrues on the loan only during repayment period.

The scholarship program funds total $3,807,800. Schools of medicine will receive $1,733,200; dentistry, $787,400; optometry, $145,800; osteopathy, $97,800; podiatry, $51,000; and pharmacy, $992,600. Of a total $12,716,583 in loan funds, which represents 50 percent of the authorized appropriation for fiscal year 1966, schools of medicine will receive $7,369,659; dentistry, $3,113,779; optometry, $427,985; osteopathy, $442,902; podiatry $79,361, and pharmacy, $1,282,897. The final allocation of funds for the loan program for fiscal year 1966 will be made after congressional action on the appropriation bill for the Department of Health, Education, and Welfare.