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Larry Paul Yotti

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James W. Kaufman
Major professor

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THE ELIMINATION OF METABOLIC COOPERATION IN CHINESE HAMSTER CELLS BY TUMOR PROMOTERS

By

Larry Paul Yotti

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ABSTRACT

THE ELIMINATION OF METABOLIC COOPERATION
IN CHINESE HAMSTER CELLS BY TUMOR PROMOTERS

By

Larry Paul Yotti

The two stage theory of carcinogenesis, originally postulated almost 40 years ago, envisions cancer as consisting of two distinct and separate stages: initiation and promotion. Initiation consists of administration of a subcarcinogenic dose of a chemical or physical agent, while promotion necessitates repeated applications of a promoting substance to be administered only after initiation. Most of the evidence currently available favors the hypothesis that initiation of cells occurs as a result of damage to DNA, perhaps leading to the fixation of a somatic mutation. However, the exact mechanism of tumor promotion is not known. Current evidence indicates that the cell membrane may be the initial site at which many, if not all, tumor promoters act.

Metabolic cooperation is a form of intercellular communication in which the mutant phenotype of enzyme deficient cells is corrected by normal wild type cells. In the Chinese hamster system described here, the recovery of 6-thioguanine resistant cells (deficient in hypoxanthine-guanine phosphoribosyl transferase activity), when cocultivated with large numbers of 6-thioguanine sensitive cells, will be greatly diminished. This reduction in recovery is due to metabolic cooperation, i.e. the 6-thioguanine sensitive cells possessing hypoxanthine-guanine phosphoribosyl transfer activity will transfer a toxic metabolite of

6-thioguanine to the resistant cells, thereby killing them. In this form of metabolic cooperation cell to cell contact is required.

The experiments reported here describe the elimination of metabolic cooperation between 6-thioguanine sensitive and 6-thioguanine resistant Chinese hamster cells by 12-0-tetradecanoyl phorbol-13-acetate (TPA), a powerful mouse skin tumor promoter. The effect of TPA, i.e. a reduction of metabolic cooperation manifested as an increase in the recovery of 6-thioguanine resistant cells, was observed at all densities of 6-thioguanine sensitive cells tested. In addition the effect of TPA was demonstrated to be dose responsive. A time course experiment indicated that the two cell lines need be exposed to TPA for as little as ten minutes to increase significantly the recovery of the 6-thioguanine resistant cells. In addition, excellent correlation was demonstrated between the in vivo tumor promoting activity of several structural analogues of TPA and their ability to reduce metabolic cooperation in the Chinese hamster system. The following known or strongly suspected tumor promoters also tested positively in the in vitro system: phenobarbital, butylated hydroxytoluene, Tween 80, mezerein, and melittin (bee venom).

There are no known short term in vitro assays to detect tumor promoters. As a result of the excellent correlation observed between in vivo tumor promoting ability and response in the previously described in vitro system, a large number of known and suspected tumor promoters was examined. Excellent correlation was observed between the in vivo and in vitro results. Consequently, the Chinese hamster metabolic cooperation assay may lend itself well as an in vitro short term test for the detection of tumor promoters; in addition to

providing information useful for the elucidation of the mechanism of tumor promotion.

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INTRODUCTION

Temin (112) has recently reviewed the hypotheses of carcinogenesis. These include the following: mutation, differentiation, oncogene, infection, and protovirus. Basically each of these hypotheses differs as to the origin of the genes responsible for neoplastic transformation. The mutation, infection, and protovirus theories of carcinogenesis propose that normal cells do not contain the genes for carcinogenesis. It is proposed that the genes responsible for the tumor cell phenotype can arise by mutation (mutation hypothesis), infection by oncogenic viruses (infection hypothesis), or by misevolution of the normal system of DNA to RNA to DNA information transfer, leading to the appearance of oncogenic ribodeoxyviruses (protovirus hypothesis). However, the differentiation and oncogene hypotheses of carcinogenesis presume that normal cells constitutively contain the genes for neoplastic transformation and during carcinogenesis these genes are activated leading to the phenotypic expression of the malignant state (differentiation) or to expression of a strongly transforming tumor virus (oncogene hypothesis).

Trosko and Chang have recently (115) discussed each of these theories within the framework of either a mutational or epigenetic interpretation. Since the tumor cell phenotype is a consequence of the tumor cell genome, these authors propose that the alteration of gene activity characteristic of tumor cells must be due either to mutation or gene modulation (epigenesis). Consequently, Trosko and

Chang have synthesized an integrative theory of carcinogenesis from: (a) the mutation and epigenetic views of cancer; (b) the two-stage theory of carcinogenesis; and (c) Comings' general theory of carcinogenesis. The reader is referred to Trosko and Chang (115) for a complete description of the integrative theory of carcinogenesis; however the essential, salient point is that alterations of gene activity leading to the tumor cell phenotype can occur either through gene mutation or gene modulation (epigenesis). It is within this framework (specifically the two-stage component of the integrative theory) that the experiments described below are conducted.

The two-stage theory of carcinogenesis was originally proposed almost 40 years ago (13) and to this day the exact mechanisms underlying initiation and promotion remain to be elucidated. Evidence has accumulated which tends to favor the hypothesis that initiation is a mutagenic event (114). Most "initiators" are chemical or physical agents which directly or indirectly damage DNA (72). Much of that DNA damage is the substrate for DNA repair enzymes. A considerable proportion of what is known about DNA repair synthesis in mammalian cells is based upon observations made in prokaryotic systems where DNA repair has been classified into error free and error prone modes of repair. Although somewhat arbitrary, a similar classification has been applied to mammalian cells (54). The error free type of DNA repair is thought to involve excision repair enzymes (repair replication), while some forms of post replication repair are thought to be error prone.

If one assumes that the fixation of a mutation is instrumental in the initiation phase of two stage carcinogenesis, and if the

assumption is also made that the error prone repair of DNA is important for the fixation of a mutation, then any factor influencing the repair of DNA would be important in the carcinogenic process. Using these assumptions, Gaudin et al. (47, 48) suggested that the general mechanism of action of cocarcinogens (promoters) was the inhibition of DNA repair replication, thought to be a relatively error free process.

In light of the Gaudin hypothesis, and given the assumption that the repair of damaged DNA may play a role in the fixation of a mutation, we decided to examine the effects of tumor promoters (specifically 12-0-tetradecanoylphorbol-13-acetate, TPA) upon the recovery of induced somatic mutant cells. Utilizing ultraviolet light and X-irradiation with V79 Chinese hamster cells, we noted a significant increase in the recovery of 6-thioguanine resistant and ouabain resistant mutant cells if TPA was present after mutagen treatment. At first consideration it would appear as if these results corroborate the Gaudin hypothesis, i.e. TPA inhibits error free excision repair forcing the cells to rely upon more error prone repair pathways, the consequence of which is an increase in the recovery of mutant 6-thioguanine resistant and ouabain resistant cells. However, in this series of experiments TPA was effective in increasing the recovery of mutant cells only if it was present after the mutation expression time. Since this represents a period of time well beyond the period of DNA repair, it is clear that TPA is not acting by inhibiting excision repair. Alternatively the hypothesis was proposed that TPA was acting epigenetically, i.e. repressing or derepressing genes which had undergone mutations but were not in a transcribable condition. In an attempt to investigate more fully the mechanism through which

TPA increased the recovery of 6-thioguanine and ouabain resistant mutants, reconstruction experiments were performed which corroborated the initial observation but which offered a somewhat different interpretation of the data.

In the HG-PRT, 6-thioguanine mutation system there is a form of intercellular communication (requiring cell to cell contact), which if not prevented, can significantly reduce the recovery of 6-thioguanine resistant mutant cells. This is known as metabolic cooperation and is a consequence of culturing 6-thioguanine resistant and 6-thioguanine sensitive cells in densities which allow high levels of cell to cell contact. Apparently the sensitive cells are capable of transfering a toxic metabolite of 6-thioguanine (phosphoribosylated 6-thioguanine) to the resistant cells. The result of this communication is the death of the 6-thioguanine resistant cell.

The results of reconstruction experiments demonstrated dramatically that the experimental conditions under which the UV- and X-ray-induced mutation frequencies were determined included a considerable amount of metabolic cooperation. The effect of the tumor promoter TPA, therefore, was not to modulate the activity of the HG-PRT gene but rather was to eliminate or block metabolic cooperation and "rescue" 6-thioguanine resistant cells which would have been killed were TPA not present. The block of metabolic cooperation by TPA is hypothesized to be a modification of membrane structure and/or function.

The elimination of metabolic cooperation by the powerful mouse skin tumor promoter, TPA, led to the speculation that this system may be capable of detecting other chemical compounds known or suspected to be tumor promoters. As will be described below, the prediction

was correct; many known tumor promoters, structurally and functionally dissimilar, have tested positively in this system, i.e. they have blocked or reduced metabolic cooperation between 6-thioguanine sensitive and 6-thioguanine resistant Chinese hamster cells.

LITERATURE REVIEW

Initial Demonstration of Cocarcinogenesis

Berenblum in 1941 (12), working with white mice, observed that croton oil, a pharmaceutical product obtained from seeds of the Croton tiglium plant, caused a marked augmentation of benzpyrene carcinogenesis when it was applied at weekly intervals to the skin of mice. The croton oil needed to be applied in conjunction with a very dilute solution of the benzpyrene, a known carcinogen, in order to measure the enhancement of carcinogenesis. Furthermore, it was demonstrated that croton resin, the active constituent of croton oil, was even more effective than croton oil in augmenting the carcinogenicity of benzpyrene. Berenblum designated this phenomenon as cocarcinogenesis, identifying croton resin as a cocarcinogen, i.e. an agent capable of enhancing the carcinogenicity of a weak carcinogen only when applied at periodic intervals concurrently or after administration of the carcinogen. Later in 1941 (13) Berenblum refined his experiments and demonstrated that croton resin was effective only in augmenting the carcinogenesis of a dilute solution of a potent carcinogen (3,4 benzpyrene), itself incapable of inducing skin tumors in mice. Additionally, Berenblum demonstrated the importance of sequence in the administration of the two chemicals. Prior treatment of the skin of mice for extended periods (26 weeks) with croton resin had no effect upon the response of the skin to subsequent applications of benzpyrene. Croton oil also appeared to facilitate

the conversion of papillomas previously established to a malignant state. Berenblum had clearly designed an interesting experimental protocol which was capable of demonstrating the phenomenon of cocarcinogenesis; however it was Friedewald and Rous in 1944 (46) who defined in operational terms the phenomenon Berenblum had demonstrated in the animal model system. These authors were the first to define the concepts of initiation and promotion: initiation conceptualized as a process which changes normal cells to preneoplastic cells while promotion is a process caused by a promoter, an agent or condition which is in itself incapable of initiating neoplastic change but which will stimulate previously initiated cells to divide and proliferate. Working with rabbit skin, these authors demonstrated that the carcinogenicity of coal tar could be divided into the initiating potential of benzpyrene, a constituent of coal tar, and the promoting potential of coal tar itself. Friedewald and Rous had elegantly set the stage for many years of investigation in many different systems by defining carcinogenesis as a two stage event, initiation and promotion.

A most remarkable finding which proved to be of immense importance in the understanding of the process of initiation was made by Mottram in 1944 (75). He was able to demonstrate that a single painting of a dilute solution of benzpyrene to the flank of a mouse was sufficient to initiate the tissue such that subsequent repeated exposure to croton oil produced large numbers of benign and malignant epidermal tumors. Mottram also stressed the importance of hyperplasia (cellular proliferation) in the process of tumor promotion. In effect, then, the work of Mottram demonstrated conclusively that the initiation phase of carcinogenesis did not require repeated exposure to the

initiating agent; a single short exposure to a subcarcinogenic dose of an initiator, followed by repeated exposure to a tumor promoter which, by necessity, was a hyperplastic agent, resulted in the appearance of epidermal tumors in the skin of mice. Berenblum and Shubik (14) confirmed the observation of Mottram, i.e. a single application of a carcinogen followed by repeated exposure to croton oil was sufficient to induce tumors. Additionally, they concluded that "the initial action in carcinogenesis constitutes a sudden and irreversible process, whereby a few normal cells are changed into permanently altered 'latent tumor cells,' which lie dormant among the non-neoplastic cells." (14) These investigators also hypothesized that the mechanism by which these latent tumor cells are promoted into frank tumor cells was altogether different from initiation. The implications of these observations were of crucial importance. The brief, irreversible nature of the initiation phase of carcinogenesis provided good evidence that initiation might involve a mutagenic event (to be discussed below), while promotion which necessitates longer time periods and repeated exposures might operate in a non-mutagenic manner.

Two Stage Carcinogenesis in Organs Other Than Mouse Skin

The original demonstrations of two stage carcinogenesis were conducted with epidermal tissue of mice or rabbits. While these observations were interesting, they did not provide evidence that such a view of malignancy could be extrapolated to other organ systems or other organisms.

Hall in 1948 (53), in an elegant but relatively unquoted series of experiments, was able to demonstrate two stage carcinogenesis in

thyroid tissue of Wistar rats. He initiated thyroid tissue with small doses of 2-acetylaminofluorene (A.A.F.) which were insufficient to induce malignant adenomata of the thyroid. However, treatment of the rats with methyl thiouracil (a goitrogenic agent which directly stimulates thyrotropic hormone) after A.A.F. treatment results in the appearance of large numbers of thyroid tumors in the rats. The interpretation of this experiment was clear. A.A.F. initiated cells of the thyroid gland to a preneoplastic state and thyrotropic hormone served as the tumor promoter much the same as croton oil does in the pathogenesis of the papillomata of mice skin. These observations were of immeasurable significance, in that they established the credibility of two stage carcinogenesis in a system other than skin. They also provided a strong theoretical base for the observation that human beings, exposed to X-irradiation as children, are much more prone to develop thyroid tumors in young adulthood than are unirradiated individuals (39). Perhaps X-rays are initiating cells of the thyroid gland to a preneoplastic state which can be promoted to an active state of cellular proliferation upon exposure to thyrotropic hormone.

Following the experiments of Hall with thyroid tumors in rats, there was a hiatus in the progress of establishing the credibility of two stage carcinogenesis. However, there were some important reports suggesting that tumors initiated with chemical carcinogens could be promoted by agents other than croton oil or its resin. Glinos et al. (49) were able to demonstrate the promoting ability of a partial hepatectomy in a rat liver system. Rats treated with an azo dye, 4-dimethylaminoazobenzene, a known liver carcinogen were subsequently given a partial hepatectomy in order to induce liver regeneration.

Those rats treated with surgical intervention, leading to increased liver cell division, exhibited an accelerated rate of appearance of tumors. It was clearly demonstrated that liver regeneration was capable of promoting the growth of latent liver tumor cells in much the same way as croton oil promotes polycyclic hydrocarbon initiation of mouse epidermis.

Hormones had not been implicated strongly in the two stage carcinogenic process until Dao and Sunderland (35) were able to demonstrate that pregnancy promoted the growth rate of 3-methylcholanthrene induced mammary carcinomas in female rats. Furthermore, it was demonstrated that increased progesterone stimulation was an essential factor in the induction of mammary tumors during pregnancy. In addition, prior hormone treatment had essentially no effect upon carcinogen induced mammary tumors. The authors concluded that carcinogen induced mammary carcinogenesis was a striking confirmation of "two-stage" carcinogenesis: the initiating stimulus provided by 3-methylcholanthrene in conjunction with a hormone regulated promotion stage.

Two-stage carcinogenesis had been clearly established in the laboratory by the decade of the 1960's as an important conceptualization of experimental, primarily chemical carcinogenesis. However, it was the decade of the 1970's before laboratories began to employ the sequential protocol of initiation and promotion in experiments designed to test chemical compounds which were widely distributed in the human environment. Although it is obviously impossible to analyze human tumorigenesis in experimental terms, epidemiological evidence had been accumulating (22) which implicated many environmental agents

as potential cancer risks to human populations. It was considered efficacious to test many of the chemicals in animal systems, particularly within the framework of two-stage carcinogenesis, in order to establish their potential risk to human populations.

Peraino et al. (84, 85, 86, 87) began their work by testing the effects of phenobarbital, DDT, and butylated hydroxytoluene (BHT) on the appearance of 2-acetylaminofluorene (AAF) induced liver tumors in rats. Each of these drugs, all of which are ubiquitous in the human environment, enhanced liver tumorigenesis (significantly increased the number of rats with tumors). It was clearly concluded that these three chemicals when included in a rat's diet after AAF treatment were acting as tumor promoters in a two-stage process analagous to that occurring in skin tumorigenesis.

Pitot et al. (88) were able to more fully characterize initiated populations or islets of liver cells which had been treated with chemical carcinogens. Through an examination of enzyme markers such as glucose-6-phosphatase and γ-glutamyl transpeptidase within morphologically altered islands these authors concluded that the foci represented the immediate progeny of one or a few initiated cells. Furthermore, treatment of rat liver with phenobarbital after exposure to diethylnitrosamine (DEN) dramatically increased the number of enzyme altered foci and also increased the probability that any one focus would progress to the stage of malignant hepatocellular carcinoma. Thus it was apparent that in liver tissue it was possible to identify the population of initiated cells and, more importantly, to follow their journey from enzyme altered foci to malignant carcinomas.

In an attempt to examine the problem of extremely high colorectal

cancer rates in the U.S. Cruse et al. (34) performed an experiment testing the hypothesis that the Western high fat diet resulting in high levels of fecal bile acids, cholesterol, and cholesterol metabolites may be instrumental in the development of colon cancers. Accordingly, they treated rats with the potent mutagen-carcinogen dimethylhydrazine (DMH), after which the rats were placed on a diet with and without cholesterol. The results clearly indicated that cholesterol facilitated the development, growth, and spread of DMH-induced colon carcinogenesis; therefore one could conclude from this study that dietary cholesterol has the capability of acting as a promoter of carcinogen induced colon cancers. However, a later series of reports (92,93,94) indicated that cholesterol itself is not the actual promoter of carcinogen-induced colon tumorigenesis. Utilizing germ free animals, essentially free of intestinal flora, it was determined that various bile acids (taurodeoxycholic acid and lithocholic acid) and cholesterol metabolites, which are produced as a result of intestinal bacterial action, are the actual tumor promoters. Consequently, germ free animals demonstrated no enhancement of colon carcinogenesis when administered cholesterol or sodium deoxycholate. This situation, therefore, can be viewed analagously to the metabolic activation of a procarcinogen to a reactive electrophile; dietary cholesterol present in high levels in the intestine as a result of a high fat diet is acted upon by endogenous bacteria to be converted to a metabolic form strongly suspected as being a tumor promoter.

Butylated hydroxytoluene has also been implicated in mouse lung as a tumor promoter (128). A single dose of urethan, a potent lung carcinogen, followed by repeated injections of BHT, significantly

increased the yield of lung tumors. There was no effect of BHT stimulated lung cell growth if the BHT was administered before urethan treatment. Therefore, the data seemed to strongly implicate BHT as a classic tumor promoter in a two-stage carcinogenic scheme.

Another organ system which appears to lend itself to the experimental induction of tumors in a two-stage manner is rat bladder (56). If nitrosamide N-methyl-N-nitrosourea (MNU) is injected by urethral catheter into the lumen of the rat urinary bladder, tumors of the urothelium will soon result. If saccharin or cyclamates are included in the diet of a rat previously treated with a single dose of MNU (in itself not capable of inducing bladder tumors), there is a significant increase in the incidence of bladder tumors (tumors per bladder examined). Therefore, it is apparent that rat bladder tumorigenesis can proceed according to the mechanism of two-stage carcinogenesis, i.e. initiation with a subcarcinogenic dose of the powerful mutagen MNU and promotion resulting from saccharin or cyclamate treatment. This observation is extremely relevant to the human population in that tremendous quantities of cyclamates were consumed in previous years and large amounts of saccharin are currently used. These two chemicals, both suspected tumor promoters, are excellent examples of chemicals which would be missed in most short and long term assays of carcinogenic risk. However, the fact that their consumption is of a repetitive nature meets one of the criteria for experimental tumor promotion, i.e. repeated exposure to the promoter in question. Therefore, cyclamates and saccharin should be evaluated fully in light of their ability to promote experimental bladder tumors in rats.

Modifications of Two-Stage Carcinogenesis

It is perfectly clear that the two-stage sequential view of experimental carcinogenesis has major relevance if one attempts to discern the mechanism of tumorigenesis, particularly within the human being. In the previous section evidence was provided which attempted to lend credibility to such a view of carcinogenesis; evidence which tended to implicate certain environmental agents (most of which are extremely prevalent in the human environment) as potential tumor promoters.

There have been reports in the literature which describe modifications of the classic two-stage protocol. These alterations are of great importance as they increase the experimental conditions under which two stage carcinogenesis can be demonstrated and consequently increase the probability of detecting suspected tumor promoters.

Armuth and Berenblum (4) were able to demonstrate unequivocally an example of systemic tumor promotion. They used newborn male and female AKR mice which had received a single subcutaneous injection of dimethylnitrosamine (DMN). If these mice were maintained for a two week interval and then given repeated intraperitoneal injections of phorbol, the parental alcohol of TPA (inactive as a mouse skin promoter), the mice exhibited high frequencies of lung and liver tumors. DMN control mice exhibited very few tumors. Therefore, phorbol conclusively was acting as a tumor promoter in the classical sense and, in addition, it was demonstrated that tumor promotion can occur systemically, far from the original point of application of the promoter. This phenomenon has obvious implications to the onset of tumorigenesis in the human population.

In a later report, Armuth and Berenblum (5) were able to demonstrate the promotion of 7,12-dimethylbenz(a)anthracene (DMBA) induced mammary carcinogenesis by phorbol in virgin female Wistar rats. A single feeding of DMBA (6 mg.) resulted in a mammary tumor incidence of 21% while twice weekly intraperitoneal injections of phorbol for ten weeks led to a mammary tumor incidence of 78%. Two conclusions are quite clearly suggested by these data: (1) mammary carcinogenesis can be promoted by agents other than hormones, most particularly the hormones of pregnancy, and (2) the systemic promoting action of phorbol includes not only lung and liver but also mammary tissue. On the basis of these results, one is tempted to speculate that virtually all tissue is susceptible to the process of tumor promotion merely because almost all tissue is systemically supplied. Interestingly, this report also describes an experiment in which long treatment with phorbol alone (9 months) resulted in 94% of the animals exhibiting leukemia. The spontaneous incidence of leukemia in untreated control animals was 2%. The authors were unable to interpret these results.

Goerttler and Loehrke in 1976 (50) initiated pregnant female NMRI mice with the mutagen-initiator dimethylbenzanthracene (DMBA), followed by topical treatment of F-1 mice with 12-0-tetradecanoyl-phorbol-13-acetate. The results of such treatment were the appearance of benign and malignant tumors on the backskin of the mice and also in various internal organs. Treatment of the pregnant mice with the same relatively small dose of DMBA alone resulted in the appearance of no tumors within the first year of life of the F-1 mice. Therefore, diaplacental initiation of pregnant mice with a dose of a known carcinogen insufficient to induce tumors, if followed by treatment

with TPA of F-1 mice, will result in offspring with tumors not only of the skin but also of various internal organs. The authors correctly point out the implications of these observations to human medicine. Exposure of a fetus to doses of carcinogens insufficient to terminate the pregnancy are sufficient to initiate populations of fetal cells which can be subsequently promoted during the life span of the animal. Also of importance is the close association between the initiation of the carcinogenic process and the onset of a teratological event. If the initiator (usually a powerful mutagen) is administered diaplacentally to a fetus at a point in its ontogeny that is critical for the normal development of a particular tissue, one can envision a disruption of development sufficient to produce a teratological event.

Biochemical Response of Mouse Skin to Tumor Promoters

Two-stage carcinogenesis had been clearly demonstrated in a wide range of tissues within a number of experimental protocols. Its applicability not only to experimental chemical carcinogenesis in the laboratory but also tumorigenesis in human populations has been clearly documented. However, the exact biochemical mechanism of the initiation-promotion scheme has yet to be deciphered. As stated earlier, initiation appears to be a mutagenic event (114). The evidence for such an interpretation lies in the observation that most, if not all, initiators are known mutagens which directly interact with nuclear DNA (28). Also, the exposure of a cell to an initiator need be of a very short duration and usually involves a dose of initiator which is in itself subcarcinogenic, i.e. incapable of producing a

malignant response.

However, tumor promotion is much less well understood. The early observations of tumor promotion in mouse skin invariably included a description of the hyperplastic response of the epidermal stem cells to the treatment by promoter. Hyperplasia, an increase in cellular proliferation, appeared to be the most outstanding response of mouse skin to tumor promoter treatment. The question, of course, remained as to whether hyperplasia was both a necessary and sufficient condition for tumor promotion. Again the mouse skin system was chosen and TPA was the promoter of choice in an attempt to elucidate the biochemistry of tumor promotion.

The first interesting observation was that TPA was capable of acting as a gene derepressor, i.e. an agent capable of stimulating transcription and translation of genes not usually expressed to such an extent within the experimental conditions. Hennings and Boutwell (55) demonstrated a stimulation of incorporation of tritiated cytidine into mouse skin RNA within two to six hours after croton oil treatment. They reported a subsequent increase in overall protein synthesis as measured by tritiated leucine incorporation and also a significant increase in DNA synthesis (a peak specific activity at 18 hours after croton oil treatment). Additionally, it was noted that there was an increase in mitotic activity resulting in an increased epidermal cell number, reaching a peak of thickness by 72 hours after treatment (hyperplastic response). All of these responses were essentially reversible; the skin slowly resumed its normal appearance. Baird et al. (7) extended these observations by demonstrating excellent correlation between the tumor promoting ability of a series of

synthetic phorbol esters and their ability to stimulate macromolecules (RNA, DNA, and proteins).

Raineri et al. (90) provided additional evidence for the hypothesis that TPA may be acting by derepressing genes. They noted two peaks of histone phosphorylation following TPA treatment of mouse skin; one peak 2 hours after TPA treatment and a second peak 1 to 3 days later. Additionally it has been reported that TPA treatment stimulates the synthesis of histones (91) in mouse epidermis. Rohrschneider et al. (95) reported a significant increase in the synthesis of phospholipids following tumor promoter treatment. Within 1 hour after TPA treatment there was a rapid and dramatic increase in the incorporation of ^{32}P-inorganic phosphate into each of six different skin phospholipids.

The responses listed above are not specific responses induced only by tumor promoters. On the contrary, any agent which has the ability of stimulating cells to divide will stimulate the synthesis of the macromolecules listed above. Consequently, the search for a specific biochemical response peculiar to tumor promoters continued.

Probably the most significant biochemical observation linking tumor promoters to the process of gene derepression was the work of O'Brien et al. (80,83) who reported that a single topical application of TPA resulted in a large, very rapid increase in mouse epidermal ornithine decarboxylase activity. Ornithine decarboxylase (ODC) is the first and rate limiting enzyme in the polyamine biosynthetic pathway. Within 5 hours after TPA application the ODC activity was nearly 250 times greater than the level in untreated cells. The consequence of such a large increase in ODC activity in TPA treated

epidermis is an accumulation of the polyamines putrescine, spermine, and spermidine. The significance of the TPA stimulation of ODC was more fully appreciated when O'Brien et al. were able to demonstrate that the magnitude of the ODC induction correlated extremely well with the promoting activity of graded doses of TPA and also with the promoting activity of a series of structural phorbol ester analogues. In addition, these authors conclusively demonstrated that other tumor promoters, structurally and chemically different from the phorbol esters, i.e. anthralin, Tween 60, and iodoacetic acid were capable of stimulating the production of ornithine decarboxylase. Of crucial importance was the observation that non-tumor promoting agents which are known to be hyperplastic, i.e. acetic acid, cantharidin, and ethyl phenylpropriolate had little or no effect on ornithine decarboxylase activity. The results of these experiments strongly suggested that the induction of epidermal ornithine decarboxylase by tumor-promoting agents may be an essential prerequisite for skin carcinogenesis in mice.

Additional evidence implicating ornithine decarboxylase induction as a critical event in skin tumorigenesis has been recently reviewed by Boutwell (20). The most compelling evidence is as follows: (1) retinoic acid known to inhibit the TPA promotion effect will inhibit the TPA induction of ornithine decarboxylase; (2) completely carcinogenic doses of polycyclic aromatic hydrocarbons are effective inducers of ODC while initiating doses are completely ineffective; and (3) epidermal mouse skin tumors exhibit constitutively high levels of ODC with malignant tumors higher than benign tumors.

The mouse skin system has proved to be the most propitious in

terns of its response to the phorbol ester series and in terms of the relative ease with which the biochemistry of skin tumor promotion can be analyzed. Consequently, there has been a plethora of reports dealing with a number of in vivo biochemical responses in mouse skin following tumor promoter treatment. A number of these responses will be reviewed below.

The cyclic nucleotides 3':5'-cyclic AMP (cyclic AMP) and 3':5'-cyclic GMP (cyclic GMP) have been strongly implicated as second messengers in the hormone effect upon gene expression. Since TPA shares some striking similarities with hormones, i.e. produces a number of biological responses other than tumor promotion at very low concentrations ($< 1\mu g$) and appears to act initially at the plasma membrane, an examination of the levels of cyclic nucleotides in mouse epidermis was suggested. Bellman and Troll (10) reported that TPA lowered the level of cyclic AMP in mouse skin; Grimm and Marks (51) corroborated this observation. However, subsequent reports (76,78) have demonstrated that the diminution of cyclic AMP levels in response to TPA might be artifactual, due to the method of skin preparation. In fact, Mufson (76) could find no evidence that TPA influenced the epidermal levels of either cyclic AMP or cyclic GMP. However, because it is difficult to measure cyclic nucleotides in vivo, no definitive conclusion can be made at this time. Therefore, it appears as if the mode of action of TPA might be different from the way in which hormones alter gene expression, i.e. intracellular cyclic nucleotides do not appear to function as 2nd messengers. However, Grimm and Marks (51) and Mufson et al. (78) did demonstrate a dramatic TPA effect upon the β adrenergic receptor of the outer cell membrane. Normally, a cell

will accumulate cyclic AMP in response to treatment by a β agonist such as isoproterenol. Apparently the β adrenergic receptor is coupled to adenyl cyclase and administration of a β agonist will result in the stimulation of adenyl cyclase with the subsequent accumulation of cyclic AMP. TPA, as well as other tumor promoters, will rapidly reduce the ability of mouse epidermal cells to accumulate cyclic AMP in response to isoproterenol. This effect of TPA upon the cell membrane is of the utmost significance particularly in light of later reports dealing with TPA alterations of membrane structure and/or function (to be discussed below).

Balmain (8) has reported the synthesis of two new, previously undetected, proteins in mouse epidermis after the application of TPA. These two proteins are apparently "new" in that they are not seen as a consequence of normal stimulation (proliferation) of mouse epidermis. Balmain has hypothesized that these two specific proteins, neither of which is detected in normal adult mouse epidermis but both of which are major proteins of the newborn mouse epidermis, represent biochemical evidence of the dedifferentiation process which has been described morphologically (89) after TPA administration.

Slaga et al. (102) have provided extremely useful information concerning the mechanism of tumor promotion by examining the effects of antiinflammatory steroids upon the action of the phorbol esters. They were able to desmonstrate that the relative antiinflammatory potency of a group of fluorinated glucocorticoids correlated extremely well with their ability (a) "to inhibit mouse skin tumor promotion by TPA, (b) to counteract TPA-induced skin hyperplasia, and (c) to inhibit epidermal DNA synthesis." Interestingly, the antiinflammatory

steroids do not counteract the ornithine decarboxylase induction by TPA. These observations led Slaga et al. to speculate that the mechanism by which the glucocorticoids inhibit mouse skin tumor promotion is through their inhibition of DNA synthesis, thereby abolishing the induction of hyperplasia as a result of TPA treatment.

In Vitro Analyses of Tumor Promoter Action

It was a very logical step from experiments with mouse skin epidermis to mouse cells in culture. In addition to ease of handling cell cultures, they represent a relatively homogeneous population of target cells with which one can measure the activity of tumor promoters.

Mondal et al. (73) were able to demonstrate initiation and promotion in cultured C3H/10T 1/2 mouse embryo cells. Initiation of the cells with non-transforming doses of strong initiators (3-methylcholanthrene, benzo(a)pyrene and ultraviolet light) followed four days later by non-transforming doses of TPA resulted in cell transformation. Also of significance was the observation that TPA did not appear to stimulate DNA synthesis, prompting Mondal et al. to suggest that the ability of TPA to promote initiated cells is in addition to its stimulation of cell division.

Kennedy et al. (60), also using C3H/10T 1/2 cells, clearly demonstrated the enhancement of X-ray transformation by TPA. The enhancement of transformation frequency was most pronounced when a minimally transforming dose of X-rays was followed by TPA treatment. These authors speculated that perhaps TPA was reversing the suppression of the X-ray induced transformed cells by high normal cell densities. This interpretation will be discussed more fully below.

Yuspa et al. (131), using primary mouse epidermal cells in culture, have demonstrated that TPA and other phorbol derivatives cause a dose responsive stimulation of ornithine decarboxylase which correlates well with their in vivo tumor promoting ability and their ability to stimulate DNA synthesis in vivo and in vitro.

However, in a later report, Yuspa et al. (132) demonstrated that the synthetic antiinflammatory steroid, fluocinolone acetonide (FA), was capable of inhibiting tumor promotion in vivo and promoter stimulated DNA synthesis in vivo and in vitro (mouse epidermal cells). Of interest though was the observation that FA could inhibit epidermal proliferation (promotion) and at the same time stimulate promoter induced ODC activity. This finding led Yuspa et al. to speculate that the products of ornithine decarboxylase are not involved in promoter-stimulated epidermal proliferation. This could, of course, be a highly tissue specific response not at all related to tumor promotion in other systems.

Another response of a variety of cells in culture to TPA treatment has been described by Wigler and Weinstein (126). It appears that TPA treatment results in the production of the protease, plasminogen activator. This finding is of considerable interest in light of the fact that a number of chemically or virally transformed cell lines exhibit constitutively high levels of plasminogen activator. This observation led to the speculation that perhaps the protease was destroying a postulated repressor protein which was regulating a series of genes whose expression would lead to the tumor cell phenotype.

Troll et al. (113) on the basis of the observation that TPA was able to induce a protease in mouse skin and plasminogen activator in

cell culture systems (126) have proposed a fascinating hypothesis to explain two-stage carcinogenesis. According to this hypothesis, proteases induced by tumor promoters derepress the genes of an SOS system of DNA repair. This is accomplished by proteolytic hydrolysis of repressor protein, the end result of which is derepression of genes which may confer survival of a cell but at the price of a mutagenic event.

One view of tumorigenesis which has gained rather broad support is that which states that the malignant cell phenotype represents a retrogression or dedifferentiation to a less differentiated, more stem cell-like appearance (120). In light of this concept, the work of Rovera et al. (96) is of major significance. Friend erythroleukemia cells will spontaneously differentiate in cell culture; eventually producing globin chains and hemoglobin. However, if TPA is added to the medium, the terminal differentiation of these cells is markedly diminished. In addition, TPA also effectively prevented the induced (dimethylsulfoxide or hypoxanthine) terminal differentiation of these same cells. There was excellent correlation between the in vivo tumor promoting ability of a series of phorbol diesters and their ability to inhibit cellular differentiation. In a later report (36) Diamond et al. demonstrated the TPA inhibition of adipose conversion of 3T3 mouse fibroblasts. Kasukabe et al. (59) were able to demonstrate the inhibition of both functional and morphological differentiation by TPA in cultured mouse myeloid leukemia cells. These three instances of inhibition of cellular differentiation by tumor promoters led to the hypothesis that tumor promoters may be acting by blocking stem cell differentiation thereby allowing amplification of initiated

but not expressed stem cells.

There have been a number of other cellular responses to phorbol esters which have received much less attention in the literature. These will be briefly reviewed below. Evidence implicating tumor promoters, specifically the phorbol esters, in the modification of cellular membranes has recently been reviewed (124). Phorbol esters within 5 to 10 minutes produced a stimulation of $^{86}Rb^{+}$ and $^{32}P_i$ uptake in mouse 3T3 cells. In another report (38) TPA treatment resulted in an increase in the uptake of 2-deoxyglucose in chicken embryo fibroblasts. An observation made recently (18) may be of major significance in the elucidation of the mechanism of tumor promotion. TPA treatment will reduce the amount of fibronectin (LETS protein) on the cell surface. Fibronectin is a cell surface protein very much involved in organization of cell to cell and cell to surface adhesions. Transformed cells deficient in cell surface fibronectin (LETS) can be converted back to a more normal spread morphology by the administration of fibronectin (2). Therefore, TPA has the ability of reversibly mimicking one of the characteristics of the transformed cell phenotype.

Finally, previous work from this laboratory (116) has indicated that TPA if administered to Chinese hamster cells after ultraviolet light treatment and after completion of the DNA repair period will increase the recovery of 6-thioguanine resistant and ouabain resistant mutant cells. These observations were later confirmed by Lankas et al. (64).

In conclusion, it is apparent that tumor promoters, specifically the phorbol esters, can have major effects upon cellular biochemistry and biology. It is equally obvious that the mechanism through which

tumor promoters act remains unknown. Most recent evidence suggests that the initial site of action of tumor promoters is the cell membrane. The major thrust of the experiments to be described below relates to a modification of the structure and/or function of the Chinese hamster cell membrane by the strongest of all tumor promoters, TPA.

Review of Metabolic Cooperation

Metabolic cooperation is a form of intercellular communication in which the mutant phenotype of enzyme deficient cells is corrected by normal or different mutant cells. Two types of metabolic cooperation have been observed: one requires cell to cell contact, the other does not. A typical example of the former type is the HGPRT (EC 2.4.2.8) system first described by Subak-Sharpe and his colleagues (109). Subsequent investigations have indicated that metabolic cooperation also occurs with the products and functions of the enzymes coded by genes for APRT (32), TK (32), Na^+-K^+-ATPase (30) and β-adrenergic receptor (27). The second type of metabolic cooperation is exemplified by the different syndromes of mucopolysaccharidosis (44,127). Here cell to cell contact is not required since cooperation appears to be mediated via a diffusable product. Metabolic cooperation has been shown to be influenced by various factors, e.g. different analogues (37), cell lines (29), and membrane modifications (33). Cell to cell communication, thought to be involved in metabolic cooperation, has also been postulated to be involved in a variety of biological processes, including normal and abnormal growth control (68).

Review of the Role of the Calcium Ion in Cell Division

A series of reports which may have relevance to the molecular mechanism of tumor promotion has recently appeared in the literature. The hypothesis that an increase in the intracellular level of calcium during a specific stage in the cell cycle may provide the primary stimulus for cell division was proposed almost five years ago (15). In the period following the introduction of this hypothesis a large volume of literature has appeared which has stressed the absolutely essential role of Ca^{++} ions in the control of cell division, as it occurs normally and as it occurs in the process of neoplastic growth. Some of the more salient references will be mentioned below. The importance of the calcium ion concentration upon the biochemical reactions involved in energy production and macromolecular biosynthesis both thought to be of critical importance in the onset of cell division has recently been stressed (24). Extracellular calcium ions have been demonstrated to be an absolute necessity for the initiation of DNA synthesis and cell division in a number of cell systems, including rat hepatocytes in vivo (125), primary mouse epithelial cells (134), and C3H mouse skin cells (21). However, it has recently been demonstrated that tumorigenic rat liver cell lines have a considerably reduced extracellular calcium requirement in order to proliferate (as measured by the colony forming ability) than do non-tumorigenic cell lines (normal epitheliod rat liver cells) (110). In fact, the reduction of the extracellular calcium requirement observed in neoplastic epitheliod liver cell lines has prompted these authors to propose that the loss of proliferative calcium dependence may be a simple and sensitive in vitro test of the tumorigenicity of a cell

line. Two additional observations implicating calcium ions as important elements in the normal and abnormal functioning of the cell have recently been reported: (1) Ca^{++} ions are capable of inducing major morphological changes in V79 Chinese hamster cells, inducing a more fibroblast-like and less epithelial-like morphology (52). Such a major alteration of the cell membrane could be of major importance in cell function in light of a recent report (23) stressing the importance of "membrane impression," i.e. the information flux from the cell membrane to the genome; and (2) Ca^{++} ions are responsible for the liberation of Dengue virus from BHK-21 cells in culture (71). These two reports are particularly interesting in light of some recent work demonstrating quite similar results upon cell membrane morphology and virus induction by TPA. It has recently been demonstrated that TPA induced major changes in cell morphology in KB cells, a transformed human epithelial cell line (123). In addition, TPA has been shown to be a very effective inducer of Epstein-Barr virus in P3HR-I cells (derived from Burkitt's lymphoma tissue) and marmoset 95-8 cells (133).

MATERIALS AND METHODS

Cell Strains

The majority of the experiments described below were conducted with V79 Chinese hamster lung fibroblasts. This transformed, aneuploid cell line was originally derived from lung tissue of a male Chinese hamster, Cricetulus griseus, 2n=22 (42). This cell line is capable of forming colonies from single cells which have attached to the bottom surface of a cell culture vessel, i.e. glass bottle, plastic flask, or plastic tissue culture dish. The plating efficiency (colony forming ability of single cells) is consistently 75 per cent or higher.

The V79 strain has been cloned in our laboratory to result in a cell line designated 743x. 743x is a cell line resulting from cloning V79 cells which were quite low in spontaneous mutations; specifically mutations conferring resistance to the purine analog 6-thioguanine. In the text of this communication, 743x will be referred to as wild type or 6-thioguanine sensitive; this designation implies that these cells are sensitive to the killing action of 6-thioguanine. A second cell strain, to be discussed below, is designated 6-thioguanine resistant. This cell strain was constructed in our laboratory by exposing 743x cells to a mutagenic dose of X-irradiation (700R at 184 R/min, 250 kV, 20 mA with 3 mm Al filtration). After seven days of growth the surviving cells were exposed to 6-thioguanine (10 μg/ml) to select for those cells which were induced 6-thioguanine resistant. Approximately 100 surviving

colonies were pooled and designated as a "cell line" resistant to 6-thioguanine. The resistance to 6-thioguanine exhibited by this cell strain appears to be quite stable. During the course of this entire series of experiments, control cultures consistently exhibited plating efficiencies in 6-thioguanine containing medium which were comparable to plating efficiencies in normal medium. All cells (6-thioguanine sensitive and 6-thioguanine resistant) were suspended in PBS with 10% dimethylsulfoxide, sealed in glass ampules, and frozen in liquid nitrogen until needed for experimentation.

The experiments conducted with human cells utilized three separate diploid fibroblast cell lines. Cell strain 73-6, derived from the foreskin of a normal human male was provided by Dr. David J. Segal, University of Alberta, Edmonton, Alberta, Canada. Cell strain NFS-3 was also derived originally from foreskin of a normal, newborn male. GM 1362 is a cell strain of Lesch-Nyhan fibroblasts derived from a 10 year old male and purchased from the Human Genetic Mutant Cell Repository of Camden, New Jersey. All of the human fibroblasts utilized in these experiments were non-transformed cells which exhibited typical human fibroblast life spans (approximately 50 population doublings). As with the Chinese hamster fibroblasts the human cells were suspended in freezing medium (10% dimethylsulfoxide in PBS), sealed in glass ampules, and frozen in liquid nitrogen until needed for experiments.

Culture Medium

All cells in the experiments to be described below were grown in modified Eagle's Minimum Essential Medium (MEM) (40) with Earle's

salts (GIBCO, Grand Island, New York). This medium was supplemented with a 50% increase of all essential amino acids except glutamine, a 100% increase of all non-essential amino acids, a 50% increase of all vitamins, and 1 mM sodium pyruvate. The concentration of bicarbonate was altered to a final concentration of 1.5 g/l. Sterilization of the medium was accomplished by filtration with positive pressure through Nucleopore filters (Nucleopore Corporation, Pleasanton, California). Sterile medium was stored in a dark, cold room at 4^{o} C. Prior to using the medium for experimentation or stock culturing, it was supplemented with fetal calf serum (5% v/v for Chinese hamster cells and 10% v/v for human cells). The fetal calf serum (GIBCO, Grand Island, New York or Flow Laboratories, Inc., Rockville, Maryland) was stored at -20^{o} C, thawed, and heat inactivated at 56^{o} C for 25 minutes prior to use. In addition, all medium was supplemented with penicillin G (100 units/ml.) and streptomycin (100 μg/ml.).

Culture Vessels and Incubation Conditions

Stock cell cultures were grown in sterile glass bottles or plastic flasks (Corning Glass Works, Corning, New York). The Chinese hamster cells were subcultured the day before an experiment in a ratio sufficient to produce enough cells for the following day. The human cells were subcultured in a 1:2 or 1:4 ratio one to two days prior to an experiment. All cells were subcultured with 0.01% crystalline trypsin (Sigma Chemical Company) in PBS without calcium and magnesium ions. For experimentation, all cells were cultured in 9 cm. plastic tissue culture dishes (Falcon Plastics, Oxnard, California or Corning

Glass Works, Corning, New York). Stock cultures and experimental plates were incubated in humidified air at 37° C with 5% CO_2. In these conditions, non-contact inhibited Chinese hamster cells had a mean generation time of approximately 12 hours while human cells exhibited a generation time of approximately 24 hours.

Cell and Colony Counts

In order to assure the proper cell dilution a stock culture was trypsinized, diluted with fresh cold medium, and placed on ice for a short period of time. The trypsinized individual cells were counted using a hemacytometer. Serial dilution series were utilized throughout all experiments. Individual macroscopic colonies were rinsed with 0.85% saline, fixed with 95% ethanol, and stained with 2.5% Giemsa stain. The colonies were then scored visually using a colony counter (American Optical Company).

Chemicals

6-Thioguanine (2-amino-6-mercaptopurine) was purchased from the Sigma Chemical Co. (No. A-4882) and was used for both Chinese hamster and human experiments. It was prepared by dissolving the commerical powder in NaOH (1N) and then in double distilled water (final pH of 11.35). The 6-thioguanine solution was filter sterilized with a disposable, negative pressure plastic filter (Falcon Plastics, Oxnard, California). The final concentration of 6-thioguanine delivered to Chinese hamster cells was 10 μg/ml; human cells received 5 μg/ml.

Known or suspected tumor promoters were dissolved in appropriate solvents and delivered to the tissue culture plates at non-cytotoxic

concentrations. All solutions were delivered in microliter quantities utilizing Eppendorf pipettes.

Plating Efficiencies and Cell Survival Determinations

Plating efficiencies, replating efficiencies, and cell survival determinations were determined by plating relatively small (100-200) numbers of cells in 10 ml. of medium in 9 cm. plates. There were usually four to six plates used for each treatment group. For mutation experiments large quantities of cells were X-irradiated, subcultured during the mutation expression period, and replated in 9 cm. tissue culture dishes. Replating efficiency plates were handled identically to treatment plates with the exclusion of the selective medium.

For the metabolic cooperation experiments, four to six plating efficiency plates were utilized for each treatment groups (including control plates containing "vehicle"). These plates served two purposes: (1) they served as plating efficiency plates necessary to calculate per cent recovery among treated groups and (2) they were used to determine if any cytotoxicity had occurred. These "plating efficiency" plates contained 6-thioguanine and, therefore, were identical to treatment plates with the exclusion of the wild type cells.

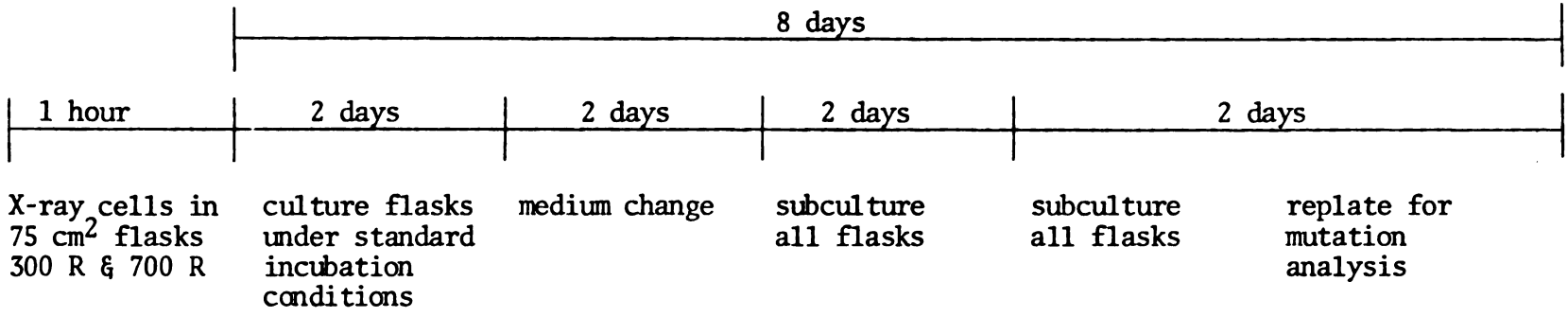
Establishment of Mutation Frequencies

Previous investigations (43,122) had established that a period of 7-9 days after mutagen treatment was sufficient time to allow for the expression of 6-thioguanine resistant mutants at the hypoxanthine

guanine phosphoribosyl transferase (HG-PRT) locus. Therefore, 8 days was chosen as a mutation expression time following X-irradiation of the Chinese hamster cells. Plastic flasks (75 cm.^{2}, Falcon Plastics, Oxnard, California) were seeded with extremely large numbers (5 - 50 x 10^6) of wild-type V79 cells. The cells were irradiated within the flasks with two doses of X-irradiation (300 and 700R). The dosimetry is as follows: 184 R/min., 250 kV, 20 mA with 3 mm. of Aluminum filtration. The cells were quickly incubated under standard conditions in the same flasks within which they were irradiated. TPA was added to the flasks at the pre-designated times. The flasks were subcultured twice during mutation expression. All surviving cells were reseeded into new flasks each time. Eight days after X-ray treatment the cells were trypsinized, pooled, counted, diluted, and delivered to 9 cm. plates (2 x 10^5 cells/plate). TPA (1μg/ml.) was added to the plates after cell attachment (approximately 4 hours). 6-Thioguanine was added to each individual plate following completion of TPA administration. Seven days later the colonies were treated as previously described. Figure 1 illustrates the complete protocol.

Unscheduled DNA Synthesis

The effect of TPA upon unscheduled DNA synthesis was determined exactly according to the protocol of Trosko and Yager (118). Through the combined use of deficient medium and hydroxyurea the cells were arrested in G1 of the cell cycle; at which time they were exposed to doses of TPA ranging from 0.1μg/ml. to 3.0μg/ml. Two small doses of ultraviolet radiation (0.5 joules/m^2 and 2.5 joules/m^2) were included in the experiment as internal controls. Specific activity was



Replating Protocol:

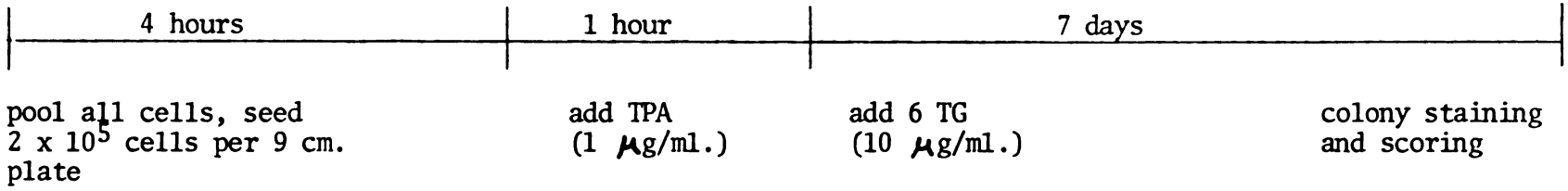


Figure 1. Protocol for the influence of TPA upon X-ray induced 6 TG^R mutations in V79 Chinese hamster cells.

expressed as disintegrations per minute per microgram of DNA.

Metabolic Cooperation Protocol - Chinese Hamster Cells

The experiments designed to test the effects of various known or suspected tumor promoters were all patterned after the protocol in Figure 2. A large number (9×10^5) of 6-thioguanine sensitive cells was plated in 9 cm. tissue culture dishes (10-21 dishes per treatment group). Immediately following the addition of the wild type cells, 100 6-thioguanine resistant cells were plated in the same dishes. Both cell lines were given four hours for cell attachment. The tumor promoter was then administered to the plates followed immediately by the addition of 6-thioguanine. Both the tumor promoter and the 6-thioguanine were delivered to the tissue culture dishes in microliter quantities utilizing Eppendorf pipettes. Three days later the medium was changed on all plates. The suspected tumor promoter was excluded at this time and growth was continued in selective medium. Growth of the colonies was continued for four more days with periodic monitoring of the cells. When the colonies were of a size to be scored visually they were fixed, stained, and scored (as previously described).

Metabolic Cooperation Protocol - Human Cells

The protocol utilized for the human cell experiments was essentially identical to that used for Chinese hamster cells. Human cells require longer periods of time to attach to the tissue culture dishes and to begin cell division. Consequently, the periods of time allotted for cell attachment and subsequent colony growth were protracted. Six to twelve hours were allowed for cell attachment, at

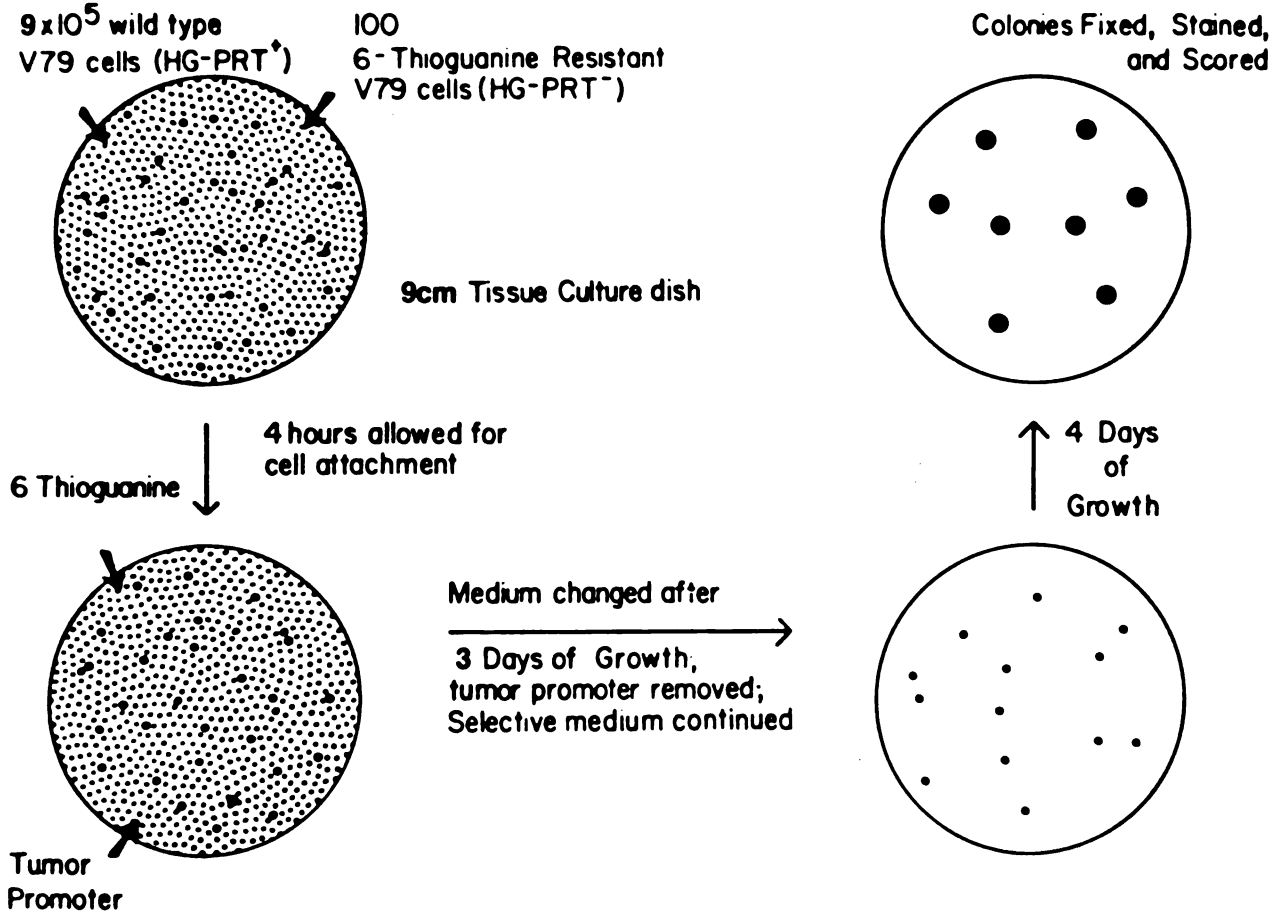


Figure 2. Protocol designed to test for known or suspected tumor promoters.

which point the suspected tumor promoter was added. Immediately after addition of the tumor promoter, 6-thioguanine was added. Media changes were completed 4, 11, and 17 days after cell plating. Visible colonies were fixed, stained, and scored at day 21 of the experiment.

RESULTS

Modification of the Recovery of Induced Mutations by TPA

The primary aim of this report was to test the hypothesis that TPA, the most powerful tumor promoter in the mouse skin system, was exerting its effect through an inhibition of some mode of DNA repair (47,48). If such an hypothesis were true, one of the major biological consequences of the inhibition of DNA repair would be the formation of somatic mutations in those cells previously exposed to the action of an initiator/mutagen. A previous report in the literature (116) had clearly demonstrated that TPA was capable of significantly increasing the recovery of both 6-thioguanine resistant and ouabain resistant V79 cells if it were present during the mutation expression period (that period following ultraviolet light treatment), well after the completion of the period of DNA repair. Since the repair of ultraviolet light induced DNA damage is thought to be different from the repair of X-ray induced damage (54), the effect of TPA could possibly be different if administered after X-irradiation. Therefore, an attempt was made to examine the influence of TPA upon the recovery of X-ray induced 6-thioguanine resistant V79 cells.

Prior to determining the effect of TPA upon the recovery of specific somatic mutant cells, a number of preliminary experiments were conducted to characterize better the response of V79 cells to TPA. Figure 3 represents a cytotoxicity experiment intended to measure V79 cell survival with increasing doses of TPA. The % survival falls quite rapidly at doses of TPA above 3μg/ml, consequently the dose of TPA

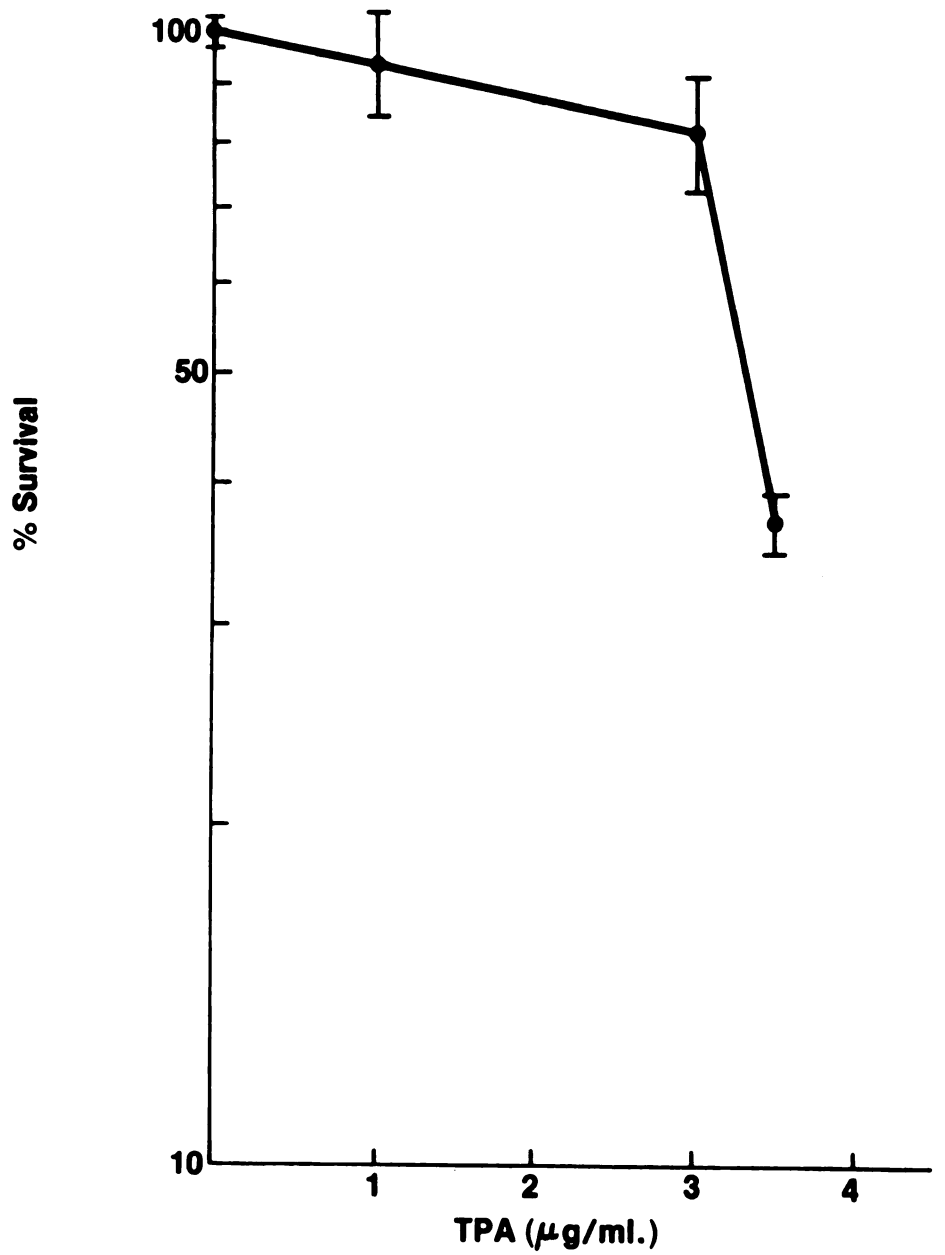


Figure 3. Survival of V79 cells to increasing doses of TPA.

chosen for subsequent mutation experiments was 1 μg/ml. (approximately 94% survival).

If one attempts to determine the effects of TPA on the recovery of X-ray induced mutations it should first be demonstrated that TPA is not in itself mutagenic. Utilizing auxotrophic strains of Salmonella typhimurium with single-base substitution and frame-shift mutations to histidine dependence, Soper and Evans (104) detected no mutagenic or toxic effects on the bacterial text strains with TPA at 1 μg/ml.

In order to rule out fully the possibility that TPA was acting as a mutagen, an experiment examining the effect of TPA upon "unscheduled" DNA synthesis was performed. Most mutagens, including ultraviolet radiation and activated chemical carcinogens (72), interact covalently with DNA; the alteration of which serves as a substrate for excision repair enzymes. Therefore a determination of "unscheduled" DNA synthesis (excision repair) can be good evidence of a potential mutagenic event. Figure 4 illustrates the results of the experiment examining the effect of TPA upon unscheduled DNA synthesis. It is clearly evident from Figure 4 that TPA even at doses which are cytotoxic (3 μg/ml.) cannot significantly enhance the level of unscheduled DNA synthesis. Even at 3 μg/ml. the amount of excision repair is not significantly elevated above control levels and is well below a very small dose of UV (0.5 J/m^2), included as an internal control.

From the preceding discussion it is clear that TPA is not capable of interacting covalently with DNA to the extent that a cell recognizes the interaction as a substrate for DNA excision repair enzymes. Additionally, TPA is clearly not mutagenic itself; bacterial data support this claim.

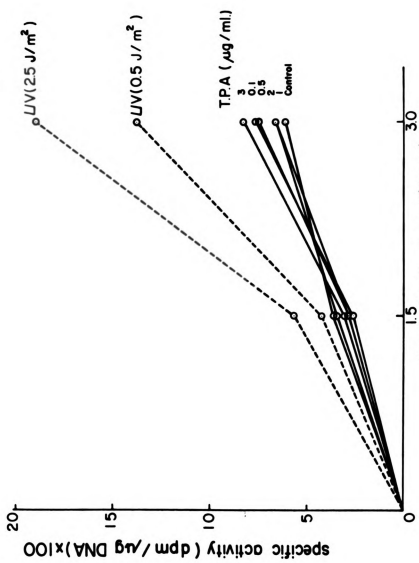


Figure 4. Unscheduled DNA synthesis following exposure of V79 cells to TPA.

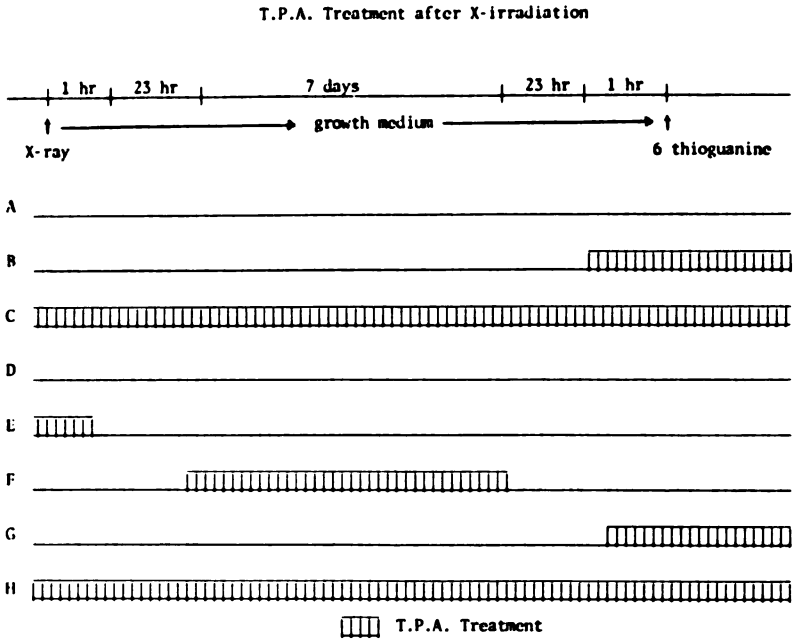
However, to explicate the mechanism of tumor promotion by TPA and other promoters, one must still consider the hypothesis proposed by Gaudin et al. (47,48), i.e. all tumor promoters may be acting, in part, by inhibiting the relatively error free process of DNA excision repair. Support for such an hypothesis was provided by Teebor et al. (111) who demonstrated that TPA was very effective at inhibiting the removal of pyrimidine dimers induced by high doses of UV in HeLa cells. However, molecular evidence refuting the contention that TPA inhibits DNA repair was provided by Trosko et al. (119), who demonstrated a negative effect of TPA on DNA repair synthesis occurring after low doses of N-acetoxy-acetylaminofluorene and ultraviolet light in human amnion cells. Additionally, TPA had no effect upon post-replication repair synthesis following UV in V79 cells. The work of Trosko et al. was more relevant than the previous reports in that the doses of carcinogens were well within the range of biological survival. Previous reports had either not indicated dosage or had utilized doses of carcinogens which allowed very low levels of cell survival. It would seem quite obvious that tumor promoters can promote only living (surviving) cells and that the biological relevance of the inhibition of DNA repair can only exist in those cells which are not killed by the action of the mutagen. Consequently, the credibility of the hypothesis that tumor promoters inhibit DNA repair had been called into question.

As a further test of the Gaudin hypothesis, Trosko et al. (116) examined the effects of TPA upon mutagenesis (resistance to ouabain and 6-thioguanine) in V79 Chinese hamster cells. The rationale for this series of experiments was as follows: if TPA inhibits excision

repair and if excision repair is an error free process, then the consequence of inhibiting an error free process would be increased reliance of the cells upon more error-prone types of repair, i.e. post-replication, "gap filling" repair. Error-prone repair while conferring cell survival would be manifested as an increase in the number of cells harboring mutant alleles. Therefore, TPA should increase the frequency of induced mutations. This is exactly the result observed by Trosko et al.; however, TPA was effective in increasing the recovery of ouabain resistant and 6-thioguanine resistant cells only if it was present after the mutation expression time, following completion of the DNA repair period. This result coupled with the observation that TPA did not sensitize the cells to the killing effect of ultraviolet light led the authors to conclude that the mechanism through which TPA acts is unrelated to the repair of nuclear DNA. Alternatively, the hypothesis was proposed that TPA was acting epigenetically, i.e. repressing or derepressing mutated and non-mutated genes. This alternative hypothesis would be much more compatible with the observation in whole animals that a tumor promoter can be applied up to several months after initiation and still effectively promote tumor cell growth.

Consequently, it had become increasingly obvious that the inhibition of DNA excision repair was not a viable explanation for the action of tumor promoters, specifically TPA. However, the observation that TPA was indeed capable of modulating (increasing) the recovery of specific somatic mutant cells after mutagen treatment was of considerable interest. Lankas et al. (64) confirmed this observation utilizing chemical carcinogens as mutagens and TPA as the gene

modulator. It was, therefore, apparent that TPA if applied to cells at the time of mutation selection could increase the recovery of specific induced mutants. Consequently, the experiment illustrated in Figure 5 was performed to determine the effects, if any, of TPA upon the recovery of X-ray induced 6-thioguanine resistant Chinese hamster cells. The complete protocol of this experiment is presented in Figure 5; including both spontaneous and induced mutation frequencies. Groups A, B, and C were not X-irradiated and, as can be seen, groups B and C, both of which were treated with TPA have a lower spontaneous mutation frequency than the non-treated cells. This reduction of spontaneous mutation frequency by TPA has not been consistently observed (data not included) and, as such, is given little significance. The cells in groups D-H have all been X-ray treated (700R, nine days prior to addition of selective medium). Group D was a control group treated with absolute ethanol (vehicle for TPA), while Groups E-H were all treated with TPA (1 μg/ml.) during the time periods indicated. Figure 5 clearly indicates that TPA treatment at all time periods with the exception of the period of DNA repair was effective in elevating the induced mutation frequency. TPA treatment only during mutation selection (Group G) resulted in the largest increase in mutation frequency; however Groups F and H each exhibited mutation frequencies which were significantly ($p < 1\%$) elevated above the non-treated cells. The most important point to be made is that TPA was effective in increasing the recovery of 6-thioguanine resistant cells only if it was present during the time of mutation selection, i.e. that period of growth in 6-thioguanine containing medium. There was a slight elevation of mutation frequency in Group F in which the TPA was removed 24



Survival and recovery of 6 thioguanine resistant mutants

X-ray	T.P.A. Treatment	Total No. of cells in 24 plates	Survival (Replating efficiency)	No. of Mutants	Mutation Frequency/ 10^6 survivors
0	A	4.8×10^6	1.1	358	67
0	B	4.8×10^6	1.1	192	36
0	C	4.8×10^6	1.1	177	34
700R	D	4.8×10^6	0.8	539	133
700R	E	4.8×10^6	1.0	495	103
700R	F	4.8×10^6	0.7	508[a]	155
700R	G	4.8×10^6	0.8	645	174
700R	H	4.8×10^6	0.6	484	166

[a] mutation frequencies of T.P.A.-treated cells (F,G,H) were highly significant, $p < 1\%$ when compared to the control cells (D).

Figure 5. Protocol and results of TPA modification of X-ray induced mutations to 6-thioguanine resistance in V79 cells.

hours prior to the addition of 6-thioguanine. This effect, I feel, represents a lingering effect of TPA treatment upon the cell membrane of those cells treated during the period of mutation expression. The significance of these observations will be discussed below (see section concerning Metabolic Cooperation).

The results discussed above with X-rays closely parallel the experiments previously reported with UV and TPA. TPA is capable of increasing mutation frequencies only if it is administered to mutagenized cell populations after the DNA repair period, preferably during the period of mutation selection.

In an attempt to determine if the TPA effect was discernible at increasing doses of X-irradiation the experiment illustrated in Figure 6 was conducted. The protocol for this experiment was identical to that of Group G in Figure 5. TPA was added to the cells after replating, one hour prior to the addition of 6-thioguanine. It is clearly apparent that the ability of TPA to increase the recovery of 6-thioguanine resistant mutant cells is quite pronounced at all three doses of X-rays (300R, 700R, and 1000R). At this point, therefore, the observation that TPA can increase the recovery of X-ray induced mutant cells appears sound and reproducible.

The interpretation of the above results presented some serious problems. Clearly, TPA was not inhibiting the repair of X-ray damaged DNA. This conclusion was made on the basis of the observation that TPA was effective in terms of increasing mutation frequencies only if it was administered to X-irradiated cells well beyond that period of DNA repair. As an alternative hypothesis to explain the TPA effect, one could evoke the hypothesis previously proposed by Trosko et al. (116),

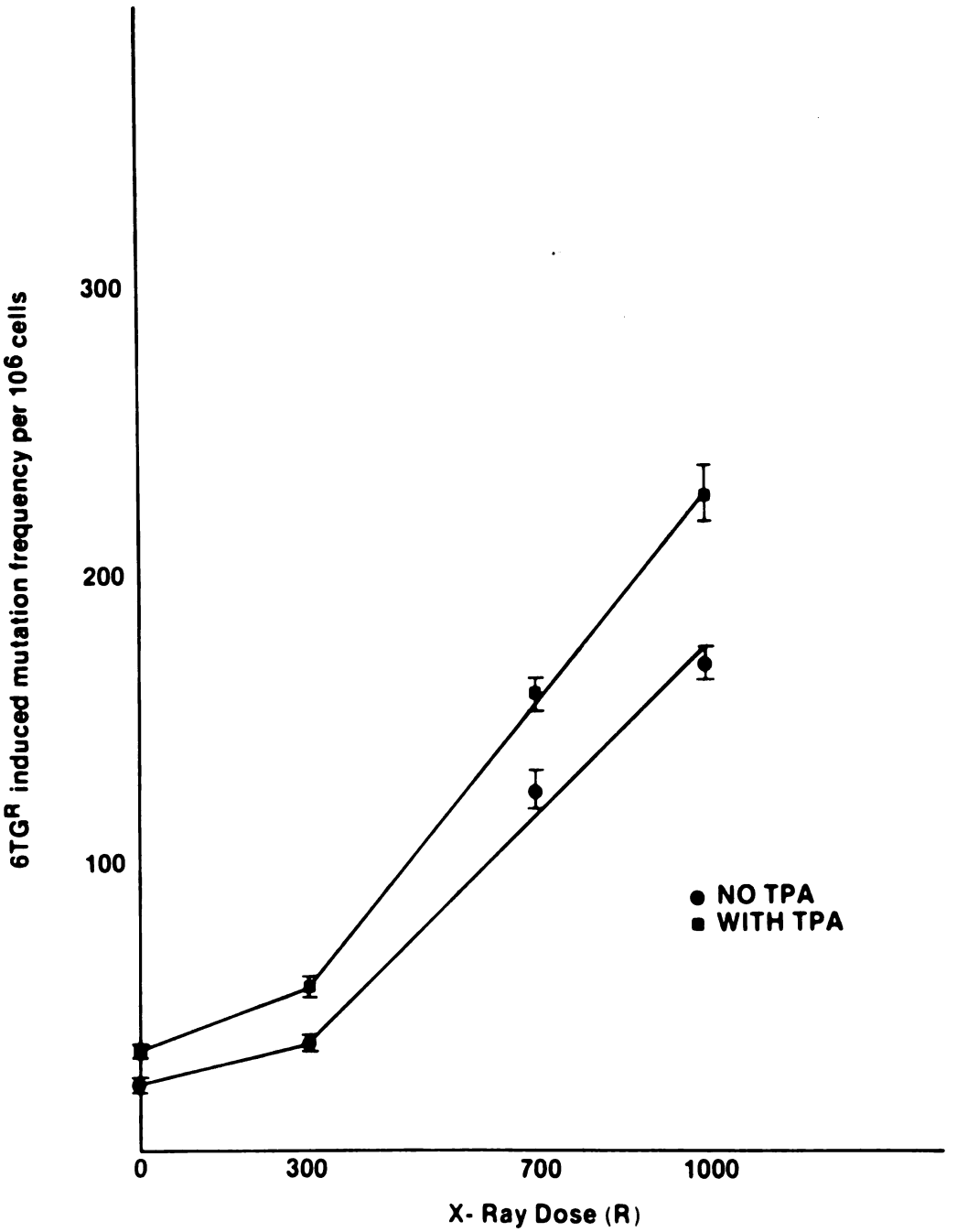


Figure 6. X-ray induced mutations to 6-thioguanine resistance in V79 cells: dose response.

i.e. TPA is capable of modulating gene activities by repressing and derepressing genes. This interpretation when applied to UV-induced mutants (6-thioguanine resistant and ouabain resistant) is quite consistent on the basis of UV inducing point mutations (base substitutions). Derepression of such mutated, but non-expressed, genes by TPA could explain the increase in mutant recovery. However, the experiments under discussion in this report utilized X-rays as the mutagen source. There is ample evidence in the literature stating that X-rays induce primarily deletion type mutations and not point mutations in higher organisms (74,106,107). This observation has recently been confirmed by Chang et al. (25) who report the failure of both "hard" and "soft" X-rays to produce ouabain resistant mutants in Chinese hamster cells. Ouabain resistance requires a point mutation of the gene coding for the membrane bound enzyme Na^+, K^+ATPase. X-rays are apparently incapable of producing base substitution type mutations which would result in the ouabain resistant phenotype. Therefore, it would seem that the data under consideration do not lend themselves to the interpretation that TPA is modulating gene activity. The locus in question (HG-PRT) is not essential for cellular growth; the deletion of this gene by X-irradiation would result in a perfectly viable 6-thioguanine resistant cell. It is difficult to conceive of a TPA derepression of a deleted gene. If TPA were either derepressing or, equally likely, repressing the HG-PRT locus, one would expect to observe a consistent and significant alteration (decreasing if there is gene derepression and increasing if there is gene repression) of spontaneous mutation frequencies with TPA treatment. Such fluctuations in spontaneous mutation frequencies at the HG-PRT locus in V79 cells

are not seen (data not included). Therefore, one is left without a valid hypothesis to explain the increase in the recovery of X-ray induced 6-thioguanine resistant mutants with TPA treatment.

TPA Modification of Metabolic Cooperation

Inherent problems exist when one attempts to measure mutation frequencies in mammalian cells. One of the most important factors to consider is the effect of cell density upon the recovery of the mutant cells. One type of intercellular communication which must be taken into consideration prior to establishing proper experimental conditions is metabolic cooperation. Metabolic cooperation is a form of cell to cell communication in which the phenotype of enzyme deficient cells can be corrected by wild type cells. The most extensively studied example of metabolic cooperation is the HG-PRT (EC 2.4.2.8) system first described by Subak-Sharpe and his colleagues (109). In this system, mutant cells deficient in the HG-PRT enzyme when co-cultivated with wild type cells ($HG\text{-}PRT^{+}$) will incorporate the phosphoribosylated purines of hypoxanthine or guanine into their nuclei. On the basis of radioautographic data it appears as if metabolic cooperation in this system requires cell to cell contact and an enzyme product or its derivative (inosine monophosphate) is transferred from wild type cells to mutant ($HG\text{-}PRT^{-}$) cells (31). Figure 7 graphically illustrates the process of metabolic cooperation as it occurs with the natural substrates for the HG-PRT enzyme. The HG-PRT system can be adapted for use as a mutation assay simply by supplying the cells with the purine analogue, 6-thioguanine. 6-Thioguanine can serve as a substrate for the HG-PRT enzyme. Its incorporation as 6-thioguanylic acid into

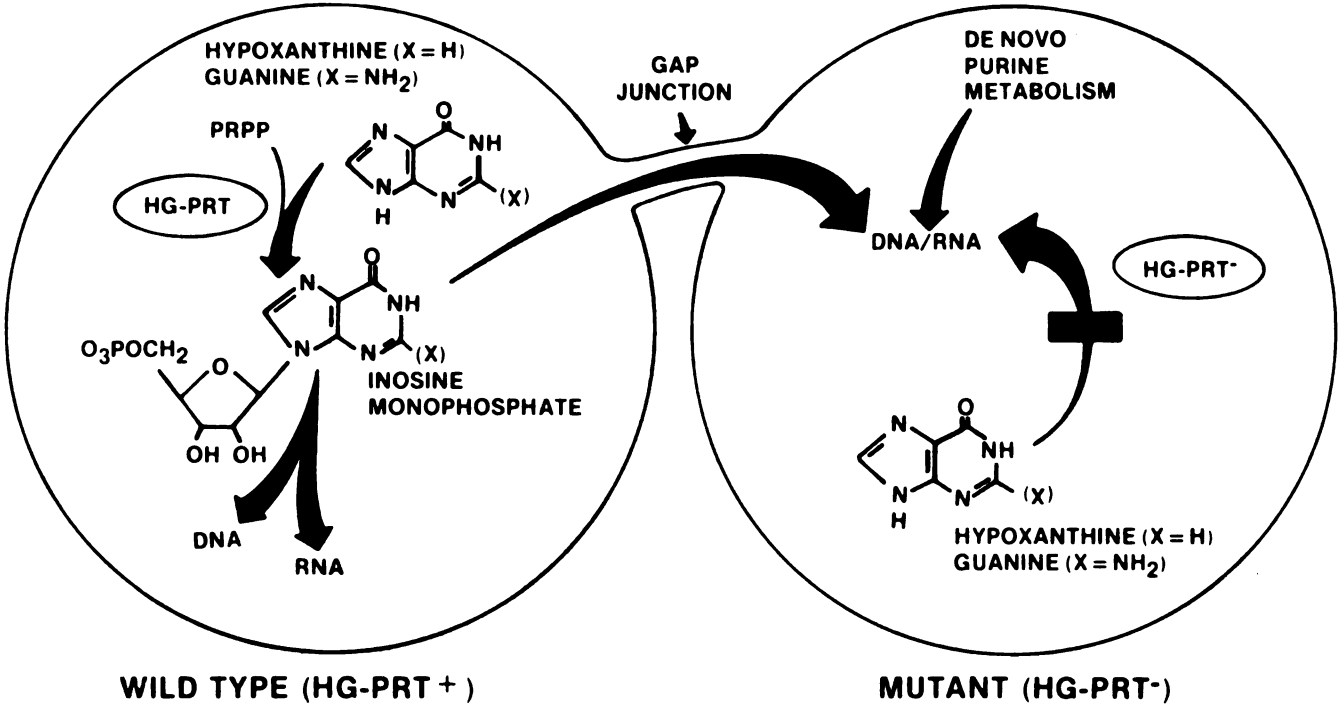


Figure 7. Metabolic cooperation in V79 cells at the HG-PRT locus.

nuclear DNA and RNA will result in cell death. Figure 8 illustrates the mechanism through which 6-thioguanylic acid (6-thioguanosine monophosphate) can be transferred to a mutant cell (HG-PRT$^-$) resulting in its death. As is apparent in Figure 8 the mutant cell, if it were not communicating with a wild type cell, would be resistant to the cytotoxic effect of 6-thioguanine; however if the cell density (ratio of wild type cells: mutant cells) is above a critical point there will be a considerable amount of cell to cell contact resulting in the communication which will eventually destroy a mutant cell. Therefore, it is absolutely critical that a proper protocol be developed which eliminates cell density effects (metabolic cooperation). Only under these conditions is it possible to compare two cell lines or treated versus untreated cells in terms of their mutation frequencies.

One experiment which will provide the data to rule out the effects of cell density upon mutation frequency is a reconstruction experiment. As the name implies, the experiment is intended to reconstruct the conditions under which the mutation frequency was originally derived. This is accomplished by seeding a known, small number (usually 100) of preexisting mutant cells (6-thioguanine resistant) with increasing numbers of wild type cells in the same dishes. Figure 9 illustrates the results of a reconstruction experiment conducted with and without TPA. It is apparent that the recovery of the 6 TG^R mutant cells falls off quite precipitously as the number of wild type cells increases. For example, the recovery of the mutant cells falls to approximately 75 per cent if there are 2×10^5 wild type cells seeded in the same dishes. However, if TPA is added to the plates immediately following attachment of the two cell lines the recovery of the 6 TG^R

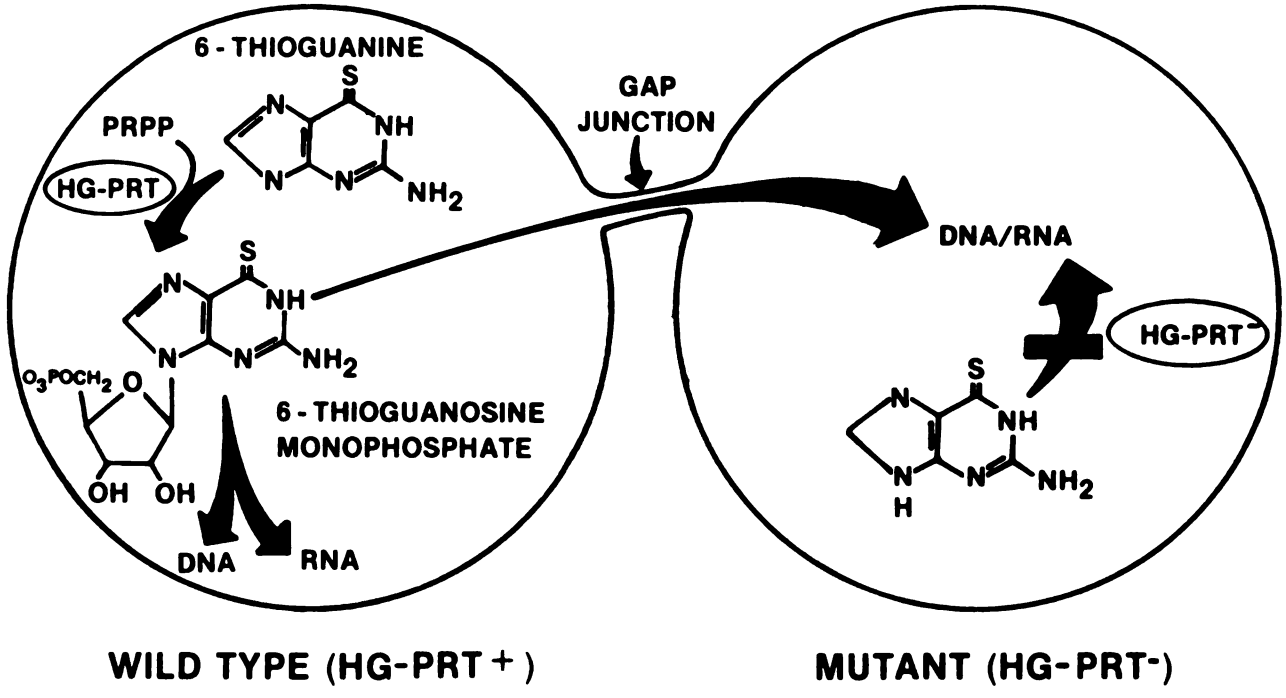


Figure 8. Metabolic cooperation in V79 cells at the HG-PRT locus: toxic substrate.

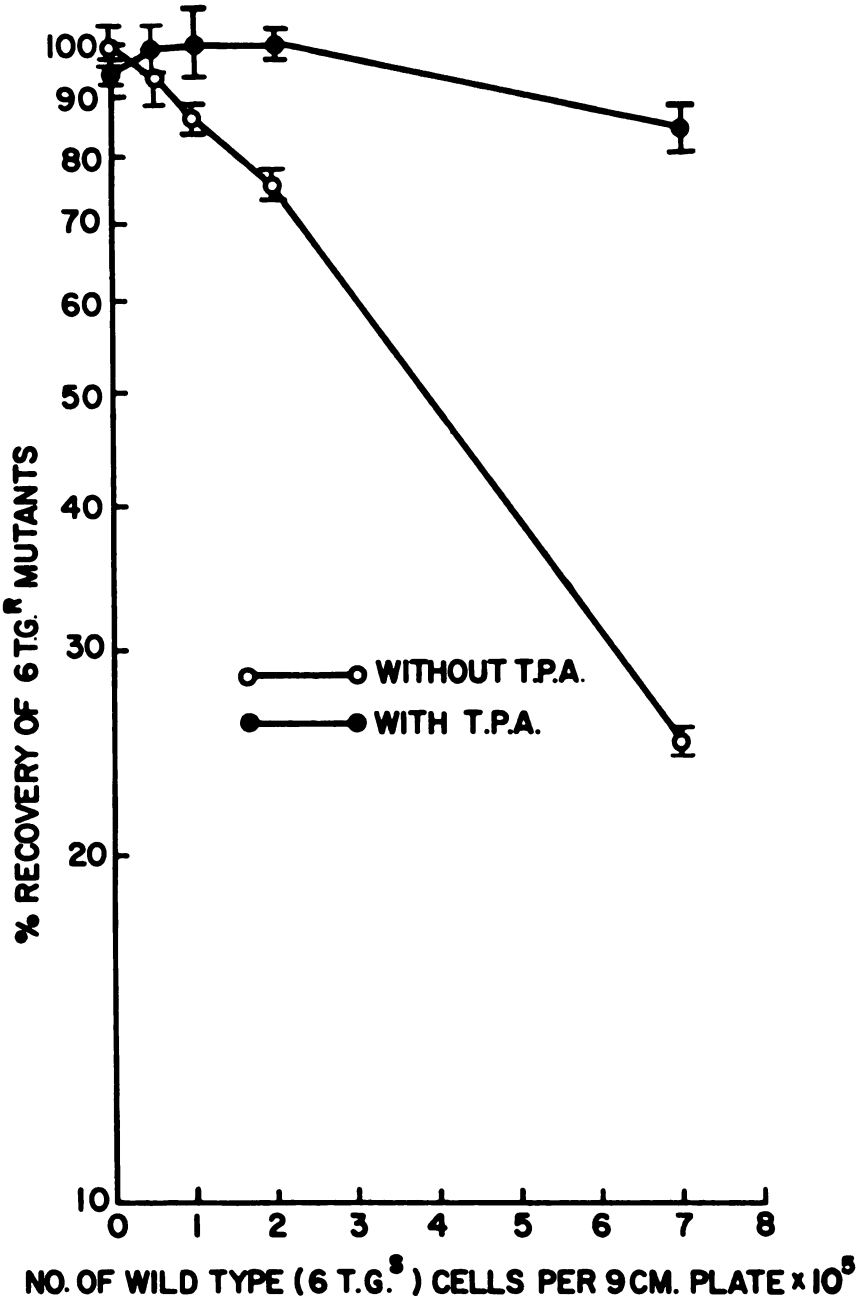


Figure 9. Reconstruction experiment with and without TPA: V79 cells.

mutant cells remains at nearly 100 per cent. In fact the recovery of mutant cells remains quite high (85%) even in the presence of 7×10^5 wild type cells, if TPA is present. Apparently, TPA is, in some unknown manner, blocking the transfer of the toxic metabolite of 6-thioguanine from the wild type to the 6 TG^R mutant cell, thereby blocking metabolic cooperation between the two cell types. Two observations need be made concerning Figure 9. First, TPA is not providing a selective growth advantage for the 6 TG^R cells; control plates are included with every treatment (data not included) which clearly indicate no increase in the colony forming ability of 6 TG^R mutant cells in the presence of TPA. Secondly, TPA is most effective when it is added to the treatment plates immediately following attachment of the two cell lines just prior to the addition of the 6-thioguanine. Since the TPA is removed three to four days after cell plating at a time when the wild type cells have died it would appear that the TPA is exerting its effect at the time of maximum intercellular communication.

However, the most important observation to be made from the experiment illustrated in Figure 9 relates to the experiments discussed above (Figures 5 and 6). In these experiments the observation was made that TPA was capable of increasing the recovery of X-ray induced 6-thioguanine resistant mutants if the drug were present during the time of mutation selection. These experiments were conducted by seeding 2×10^5 cells per plate. The cells had been X-rayed eight days prior to cell plating; sufficient time for the expression of any induced 6-thioguanine resistant mutants. However, as is clearly seen in Figure 9, there is a reasonable amount of metabolic cooperation at

a density of 2 x 10^5 cells per plate. Therefore, the original observation (TPA will increase the recovery of induced 6-thioguanine resistant mutants), is correct. The interpretation of this observation is not to be found in the gene modulatory potential of TPA; instead it seems clear that the effect of TPA can be explained on the basis of its ability to reduce metabolic cooperation, thereby recovering all of the 6-thioguanine resistant mutants induced by the X-ray treatment. The magnitude of the TPA effect (increase) upon mutation recovery (approximately 25-30 per cent, see Figure 5) correlates well with the amount of cell to cell communication (metabolic cooperation) which occurs at a density of 2 x 10^5 cells per plate. Therefore, it may be concluded that the effect of the tumor promoter TPA upon mutations induced by X-irradiation at the HG-PRT locus is not a gene modulatory one, i.e. not at the level of gene transcription. Rather, TPA acts at the level of the cell membrane possibly by modifying membrane structure and/or function in such a way as to reduce the amount of metabolic cooperation.

In order to more fully examine the TPA effect on cell to cell communication as it occurs in the HG-PRT, 6-thioguanine system the experiment illustrated in Figure 10 was conducted. This experiment was designed to provide information as to the length of time of TPA exposure which was necessary to increase the recovery of the 6 TG^R mutant cells. As can be seen in Figure 10, ten minutes exposure of the two cell lines (6-thioguanine sensitive and 6-thioguanine resistant) to TPA was sufficient to increase the recovery of the mutant cells from approximately 25% to approximately 75%. Additional time of exposure to TPA gradually increased the percent recovery of

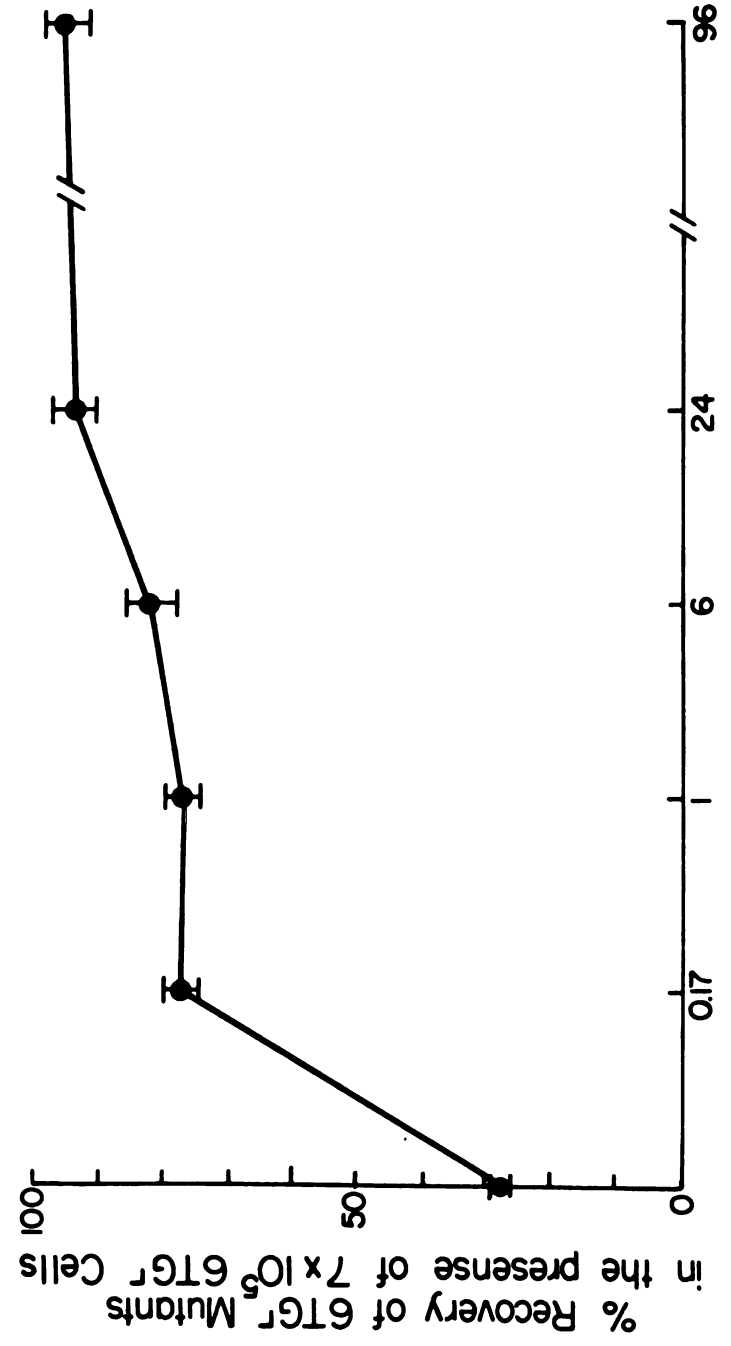


Figure 10. Recovery of 6 TG^r V79 cells as a function of length of TPA treatment.

the mutant cells to nearly 100. It is clear from this experiment that ten minutes exposure of the two cell lines to TPA is sufficient to induce membrane modifications which are sufficiently enduring to reduce intercellular communication and, consequently, increase the recovery of the mutant cells.

Table 1 illustrates the results of an experiment intended to determine if the TPA reduction of metabolic cooperation were dose responsive, i.e. did increasing doses of TPA result in increasing percent recoveries of the 6 TG^R mutant cells. As is apparent in Table 1 all doses of TPA were maximally effective at increasing the recovery of the mutant cells. Of major significance, however, was the result obtained with phorbol, the parent alcohol of TPA and the phorbol esters. Phorbol which has little or no mouse skin in vivo tumor promoting ability at a dose equimolar to the highest concentration of TPA proved to be entirely negative in this system. The implications of this observation are of clear cut importance. If a known non-tumor promoter is incapable of modifying intercellular communication (metabolic cooperation) while a strong promoter tests very positively in this in vitro system, then perhaps the system could be useful for the in vitro detection of tumor promoters. This modification of the TPA effect upon metabolic cooperation will be discussed more fully below.

Figure 11 demonstrates a definite dose response relationship with TPA. A dose of 0.01 ng/ml. (0.00001 μg/ml.) has very little effect upon the recovery of the 6 TG^R cells while 1 ng/ml. (0.001 μg/ml.) is of maximum effectiveness.

Table 1. Recovery of 6 TG^R cells in the presence of varying doses of TPA and phorbol.

No. of Cells per 9 cm. plate				
6 $T.G.^S$	6 $T.G.^R$	T.P.A.	Phorbol	% Recovery of 6 TG^R
-	100	-	-	106
-	100	1 μg/ml.	-	98
7×10^5	100	-	-	50
7×10^5	100	1 μg/ml.	-	90*
7×10^5	100	0.5 μg/ml.	-	95*
7×10^5	100	0.1 μg/ml.	-	102*
7×10^5	100	0.01 μg/ml.	-	103*
7×10^5	100	-	0.59 μg/ml.	30

* All doses of TPA were statistically elevated over control at the $p < .01$ level according to the Student-Newman-Keuls' test.

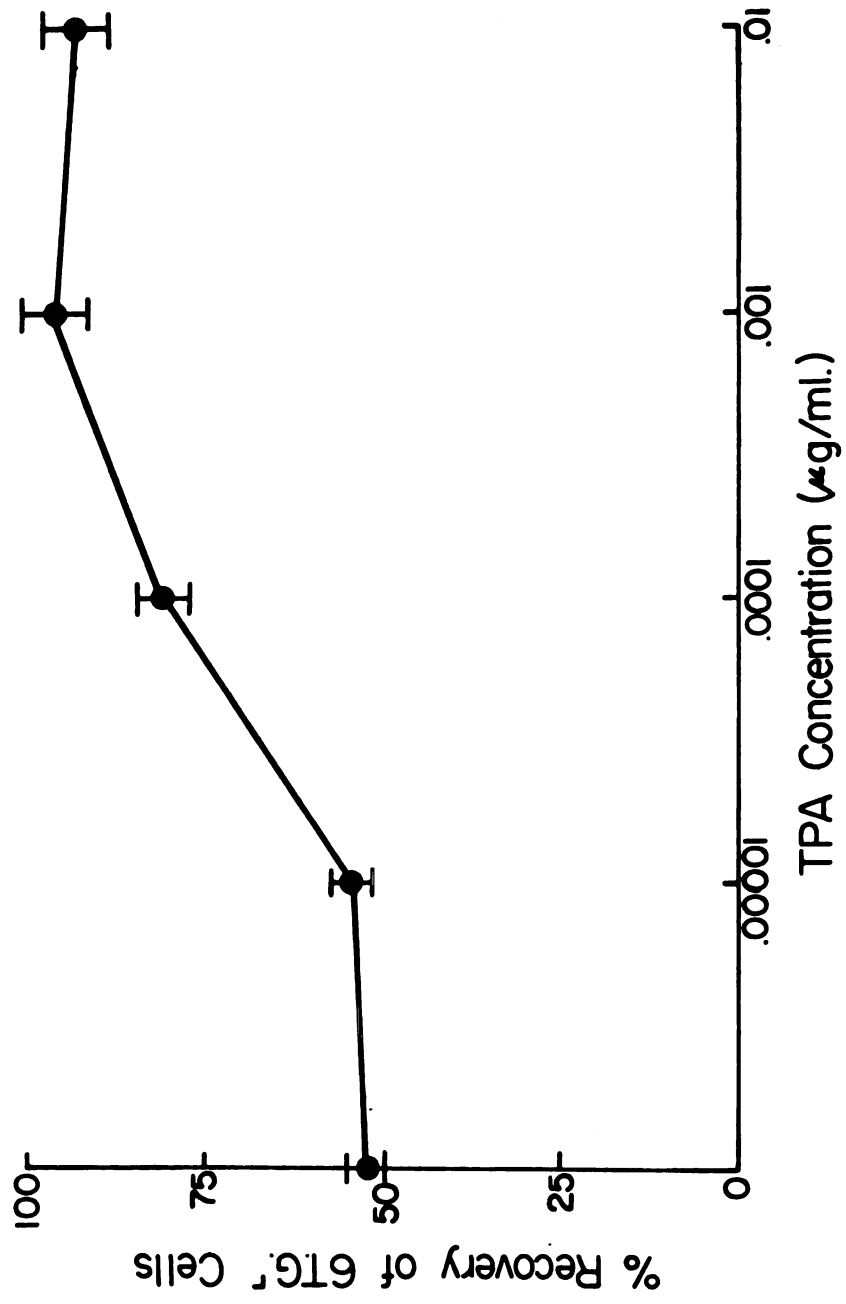


Figure 11. TPA modification of the recovery of 6 TG^r cells: dose response.

Phorbol Ester Analogue Modification of Metabolic Cooperation

At this point it is clear that 12-0-tetradecanoyl phorbol-13-acetate (TPA), the most powerful mouse skin tumor promoter yet identified, is capable of reducing communication between 6-thioguanine sensitive and 6-thioguanine resistant cells. The effect of TPA, which is manifested as an increase in the recovery of the 6-thioguanine resistant cells, has also been demonstrated to be dose responsive. However, only phorbol, a known ineffective mouse skin tumor promoter has been shown to be negative in this system. Therefore, it was deemed necessary to validate the system with a series of graded phorbol derivates, synthetically produced, each of which exhibited a degree of in vivo tumor promoting activity. Table 2 illustrates the results of the experiment. Excellent correlation was observed between the in vivo tumor promoting activity and the in vitro percent recovery for seven separate phorbol derivates (including phorbol and TPA). In addition, the assay system demonstrated excellent precision, in that the in vitro assay, rank order of promoters identically paralleled the in vivo tumor promoting rank order. This observation provides considerable credibility for the claim that this system is capable of detecting known whole animal tumor promoters.

Tumor Promoter Modification of Metabolic Cooperation

The series of phorbol derivatives is certainly of major importance in any discussion of tumor promoters; however any system which intends to detect tumor promoters must examine a series of proven whole animal tumor promoters. Consequently, a group of experiments was undertaken in an attempt to refine and further validate the system.

Table 2. Recovery of 6 TG^R cells in the presence of phorbol ester analogues.

No. of Cells per 9 cm. plate				
6 $T.G.^S$	6 $R.G.^R$	Phorbol Analogue	In Vivo Tumor Promoting Activity	% Recovery ± Standard Error
8×10^5	100	control (ethanol)	-	26.7 ± 1.3
8×10^5	100	phorbol	-	27.5 ± 1.3
8×10^5	100	4 -phorbol 12,13-didecanoate	-	31.6 ± 1.5
8×10^5	100	phorbol 12, 13-diacetate	+	32.8 ± 1.1
8×10^5	100	4-0-methylphorbol 12-myristate 13-acetate	+	33.5 ± 1.5
8×10^5	100	phorbol 12,13-dibutyrate	++	51.0 ± 1.9
8×10^5	100	phorbol 12,13-didecanoate	+++	91.5 ± 2.2
8×10^5	100	phorbol 12-myristate 13-acetate	++++	100 ± 2.3

The drugs chosen for examination in the in vitro assay system were chosen on the basis of their ability to represent a specific class of tumor promoter. Just as there are specific and different classes of tumor initiators, i.e. UV, chemical carcinogens, and ionizing radiation; there are various classes of tumor promoters. Among the various classifications of tumor promoters are (1) exogenous chemicals, (2) wounding, (3) cytotoxicity, (4) hepatectomy, and (5) growth stimuli (45,114). The particular in vitro system under discussion obviously lends itself best for the detection of exogenous chemical promoters. Chemical promoters themselves can be classified into specific types, i.e. skin promoters such as the phorbol ester series and Tween 80, butylated hydroxytoluene as a representative promoter of lung tumors in mice, and phenobarbital as a representative rat liver tumor promoter. Clearly these categories are only arbitrary and do not imply that there are specific chemical configurations which determine the class into which any tumor promoter falls; however such categories are useful in that they better characterize an individual promoter and allow a more meaningful interpretation of the results of tumor promoter administration.

Table 3 represents the results of an experiment intended to measure the response of two well documented rodent tumor promoters, phenobarbital and butylated hydroxytoluene, in the in vitro tumor promoter assay. As is apparent in Table 3 the average percent recovery of 100 6-thioguanine resistant cells when seeded with 7×10^5 6-thioguanine sensitive cells is approximately 41%. TPA, included as an internal control, more than doubled the control percent recovery and both phenobarbital and butylated hydroxytoluene significantly

Table 3. Recovery of 6 TG^R cells in the presence of butylated hydroxytoluene and phenobarbital.

No. of Cells per 9 cm. plate			
6 T.G.^{S}	6 T.G.^{R}	Tumor Promoter	% Recovery $\pm$ Standard Error[4]
7×10^5	100	control (ethanol)	41.4 ± 2.67
7×10^5	100	TPA[1]	92.6 ± 3.23
7×10^5	100	phenobarbital[2]	77.0 ± 2.43
7×10^5	100	butylated hydroxytoluene[3]	62.1 ± 2.16

1. TPA is 12-0-tetradecanoyl phorbol-13-acetate, 0.1 μg/ml.
2. Phenobarbital, 100 μg/ml.
3. Butylated hydroxytoluene, 40 μM.
4. The percent recovery of each of the three treated groups was significantly elevated above non-treated cells, $p < .01$, SNK test.

($p < 1\%$) increased the recovery of the thioguanine resistant cells. This result is consistent with published reports in the literature which indicate that phenobarbital is a more effective promoter of rat liver tumors than is butylated hydroxytoluene (86). Therefore, the in vitro assay system identified as positive two well researched chemical promoters and, in addition, was precise enough to rank them in order of effectiveness.

Table 4 reports the data testing a weak mouse skin tumor promoter (47), the common laboratory detergent Tween 80. The weakness of this promoter in whole animals is reflected in the in vitro assay. TPA more than doubled the average percent recovery of the thioguanine resistant cells while Tween 80 produced a much smaller, but statistically significant, increase above non-treated cells.

Table 5 illustrates the results of an in vitro test of mezerein. Mezerein is a phorbol-like diterpene which shares with TPA the ability to act synergistically with phytohemagglutinin in the mitogenic stimulation of bovine lymphocytes (61). Mezerein is nearly as proficient as TPA in this system and is an excellent candidate as a mouse skin tumor promoter (studies now in progress, T. Slaga, personal communication). The results of the in vitro test certainly parallel the in vivo results; mezerein increases the recovery of 6-thioguanine resistant cells from 43% to 95%. This increment of increase is identical to the response of TPA; therefore on the basis of the results obtained in this in vitro assay of tumor promotion one would predict that mezerein would be an excellent mouse skin tumor promoter.

Another drug that has yet to be tested in a whole animal tumor promotion system is melittin, a 26 amino acid polypeptide which is

Table 4. Recovery of 6 TG^R cells in the presence of Tween 80.

No. of Cells per 9 cm. plate				
6 $T.G.^S$	6 $T.G.^R$	TPA^1	Tween 80^2	% Recovery of 6 TG^r
-	100	-	-	111
7×10^5	100	-	-	41
7×10^5	100	+	-	96
7×10^5	100	-	+	52^3

1. TPA is 12-0-tetradecanoyl phorbol-13-acetate, 1 μg/ml.
2. Tween 80, 0.002% v/v.
3. Significantly increased above non-treated cells, $p < 0.01$, SNK test.

Table 5. Recovery of 6 TG^R cells in the presence of mezerein.

No. of Cells per 9 cm. plate				
6 $T.G.^S$	6 $T.G.^R$	TPA[1]	Mezerein[2]	% Recovery of 6 TG^r
-	100	-	-	77
8×10^5	100	-	-	43
8×10^5	100	+	-	95[3]
8×10^5	100	-	+	95
8×10^5	100	+	+	85

1. TPA is 12-0-tetradecanoyl phorbol-13-acetate, 0.01 μg/ml.
2. Mezerein, 0.1 μg/ml.
3. TPA and Mezerein treated groups are statistically elevated above controls ($p < 0.01$) according to the SNK test.

the major constituent of bee venom. In a C3H/10T½ mouse embryo cell culture system melittin has been shown to act very similarly to TPA, i.e. to inhibit differentiation of mouse melanoma cells, to enhance anchorage-independent growth of virus-transformed rat embryo cells, and to induce prostaglandin synthesis (77). Therefore, melittin was chosen to be tested in the in vitro system. The results appear in Table 6. The lowest concentration of melittin effectively doubled the recovery of the thioguanine resistant cells and increasing doses of melittin resulted in increasing recovery of the thioguanine resistant cells. Therefore, on the basis of the in vitro test results one would predict that melittin would be an effective whole animal tumor promoter. The whole animal studies are currently in progress (T. Slaga, personal communication).

PBB, a compound of considerable interest to those of us living in Michigan, was the next drug to be tested in the in vitro system. PBB, a widely distributed fire retardant, is a complex of polybrominated biphenyls known as Firemaster BP-6. There have appeared in the literature reports possibly implicating PBB as a tumor promoter (1). Table 7 indicates the results. PBB at two non-cytotoxic concentrations did not statistically significantly increase the average percent recovery of the thioguanine resistant cells. Therefore, on the basis of these results, one would predict that PBB is not a tumor promoter. However, technical problems arising from the handling the drug (possible photosensitivity) necessitate repeat testing.

In my judgment the in vitro system at this point had been adequately verified as an assay capable of detecting known or strongly suspected tumor promoters. The system was further characterized in

Table 6. Recovery of 6 TG^R cells in the presence of increasing doses of melittin.

No. of Cells per 9 cm. plate				
6 $T.G.^S$	6 $T.G.^R$	TPA[1]	Melittin[2]	% Recovery of 6 TG^r
-	100	-	-	78
9×10^5	100	-	-	15
9×10^5	100	+	-	80
9×10^5	100	-	+	30[3]
9×10^5	100	-	+	34[4]
9×10^5	100	-	+	36[5]

1. TPA is 12-0-tetradecanoyl phorbol-13-acetate, 0.001 μg/ml.
2. Melittin, bee's venom.
3. Melittin concentration, 0.01 μg/ml. Statistically elevated ($p < 0.01$) above control, SNK test.
4. Melittin concentration, 0.1 μg/ml. Statistically elevated ($p < 0.01$) above control, SNK test.
5. Melittin concentration, 1 μg/ml. Statistically elevated ($p < 0.01$) above control, SNK test.

Table 7. Recovery of 6 TG^R cells in the presence of a complex of polybrominated biphenyls.

No. of Cells per 9 cm. plate			
6 $T.G.^S$	6 $T.G.^R$	Tumor Promoter	% Recovery
7×10^5	100	control (acetone)	40.9
7×10^5	100	TPA (0.1 μg/ml.)	81.3
7×10^5	100	PBB (0.1 μg/ml.)	43.4
7×10^5	100	PBB (1.0 μg/ml.)	43.1

1. TPA is 12-0-tetradecanoyl phorbol-13-acetate.
2. PBB is a complex of polybrominated biphenyls, Firemaster BP-6.

this laboratory (117). Chemical compounds as structurally and functionally dissimilar as DDT, anthralin, saccharin, Tween 60, deoxycholic acid, lithocholic acid, and cytochalasin B have all tested positively in the in vitro system. All of these substances are either known or strongly suspected tumor promoters. Therefore, the validity of the in vitro assay system has been substantially demonstrated.

One class of compound not yet discussed is the anti-tumor promoter. These compounds when applied concurrently with known tumor promoters are capable of inhibiting the hyperplastic, proliferative response of the promoter. As a consequence the early onset and increased frequency of induced tumors is effectively prevented.

Two of the most extensively discussed anti-tumor promoters are the retinoids (synthetic analogs of Vitamin A) and dibutyryl adenosine 3',5'-monophosphate (dibutyryl cyclic AMP). The anti-tumor promoting ability of the retinoids has been thoroughly documented (105). In addition there have been a number of reports verifying the anti-tumorigenic capacity of cyclic adenosine 3',5'-monophosphate and its derivatives (26,58,97,130).

In an attempt to determine if the in vitro tumor promoter assay were capable of detecting anti-tumor promoters the experiment illustrated in Table 8 was conducted. Retinoic acid and dibutyryl cyclic AMP were each applied to the cells concurrently with TPA. Neither compound proved to be effective in eliminating the TPA effect. The recovery of the 6-thioguanine resistant cells in the presence of TPA was essentially unchanged by retinoic acid and dibutyryl cyclic AMP.

Table 8. Recovery of 6 TG^R cells in the presence of TPA, retinoic acid, and dibutyryl cAMP.

No. of Cells per 9 cm. plate					
6 $T.G.^S$	6 $T.G.^R$	TPA[1]	Bt_2 cAMP[2]	RA[3]	% Recovery of 6 TG^r
-	100	-	-	-	92
7×10^5	100	-	-	-	41
7×10^5	100	+	-	-	94
7×10^5	100	+	+	-	96
7×10^5	100	+	-	+	100

1. TPA is 12-0-tetradecanoyl phorbol-13-acetate, 1 μg/ml.
2. Bt_2cAMP is dibutyryl cAMP, 0.05 mM.
3. RA is retinoic acid, 1 μM.

Characterization of Metabolic Cooperation in Human Diploid Fibroblasts

The experiments discussed above were all conducted with V79 Chinese hamster fibroblasts. There is, however, no a priori reason to suspect that such a system would not function in human diploid fibroblasts. If such a system were developed it would eliminate the extrapolation one is forced to make from transformed rodent cell line to human cell line. Of course the extrapolation from cell culture results to the in vivo condition still must be made.

Metabolic cooperation at the HG-PRT locus exists and is well documented in human diploid fibroblasts (31,121). The form of metabolic cooperation in diploid human fibroblasts is identical to that of V79 cells, i.e. wild type ($HG\text{-}PRT^+$) cells can transfer phosphoribosylated 6-thioguanine to mutant 6-thioguanine resistant cells ($HG\text{-}PRT^-$). The result of this cross-feeding is the suppression and eventual death of the 6-thioguanine resistant cells. In the human system the 6-thioguanine resistant cells are derived from patients with Lesch-Nyhan syndrome. These diploid fibroblasts are genetically deficient in the HG-PRT enzyme and, consequently, are constitutively resistant to 6-thioguanine.

Given these considerations one would predict that co-cultivation of Lesch-Nyhan cells with increasing numbers of 6-thioguanine sensitive cells would result in decreasing recovery of the mutant cells. Figure 12 confirms this prediction, i.e. 100 Lesch-Nyhan cells plated with 8×10^4 6-thioguanine sensitive cells allows for the recovery of approximately 45% of the mutant cells and there is less than 1% recovery of the Lesch-Nyhan cells when they are grown in the presence of 3.2×10^5 wild type cells. However, as is apparent in Figure 12, TPA does not affect the human fibroblasts in the same manner as it

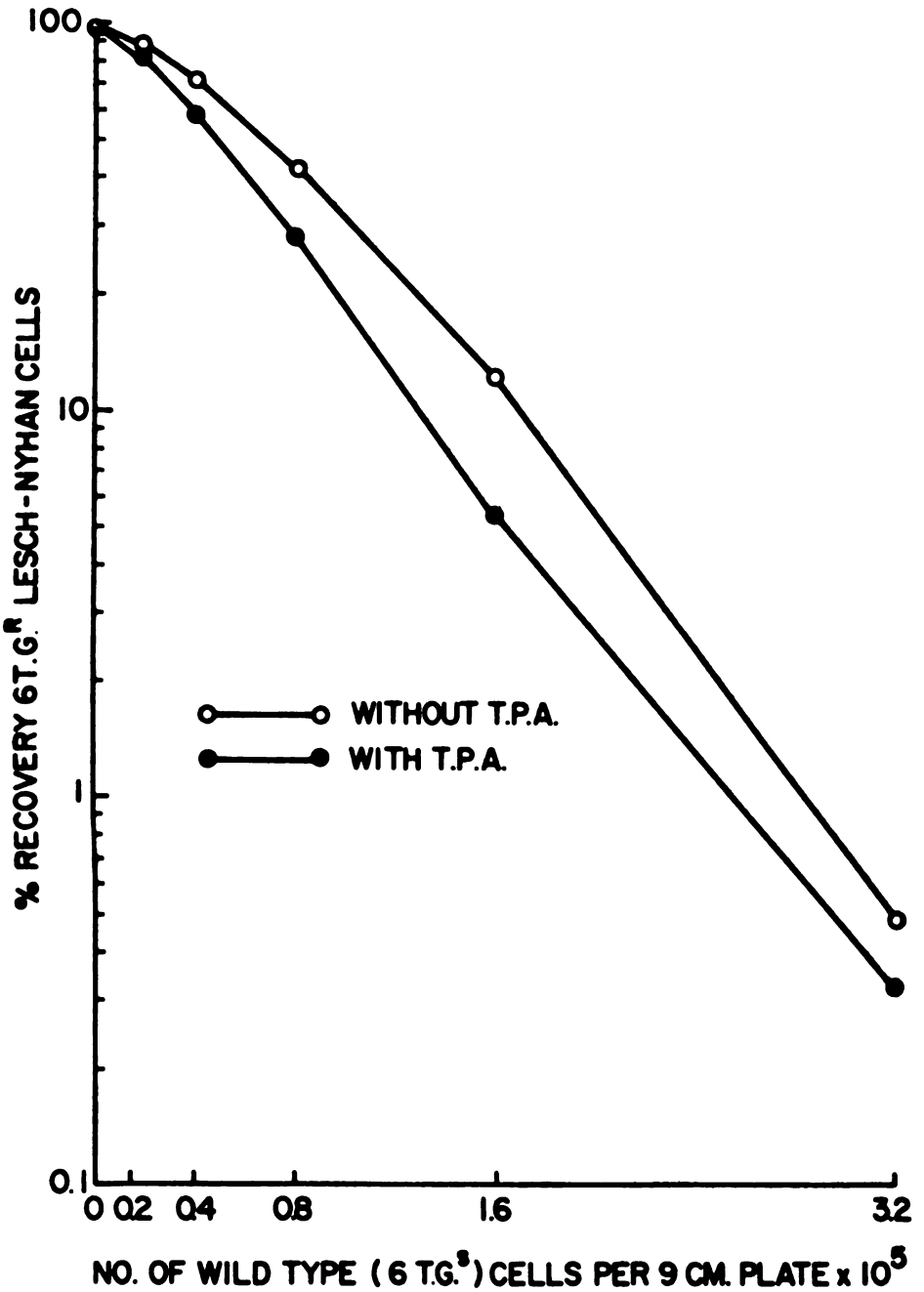


Figure 12. Reconstruction experiment with and without TPA: human diploid fibroblasts.

does the V79 Chinese hamster cells. Quite the contrary, TPA actually reduces the recovery of the 6-thioguanine resistant Lesch-Nyhan cells. At all densities of wild type cells TPA slightly lowers the average percent recovery of the Lesch-Nyhan cells. The experiment was conducted twice and the results were identical each time.

Two additional chemicals, phenobarbital and butylated hydroxytoluene, were tested in the in vitro system using diploid human fibroblasts. Preliminary results (data not included) would indicate that neither of these two strong rodent tumor promoters tested positively in the human fibroblast system.

However, the single chemical which tested positively in the human fibroblast system was cytochalasin B. Cytochalasin B is a compound known to disrupt microfilaments and to bind to cell membranes (31). It has also been demonstrated to significantly reduce metabolic cooperation (measured autoradiographically) between BHK (baby hamster kidney) fibroblasts and Lesch-Nyhan human fibroblasts (31). Table 9 clearly indicates that cytochalasin B effectively increases the recovery of 6-thioguanine resistant diploid human fibroblasts when co-cultivated with 6-thioguanine sensitive cells. The increase in the recovery of the Lesch-Nyhan cells is from 5% to almost 37%.

Table 9. Recovery of Lesch-Nyhan cells in the presence of cytochalasin B.

No. of Cells per 9 cm. plate			
6 T.G.^{S}	6 T.G.^{R}	Selected Promoter	% Recovery $\pm$ Standard Error
1.6×10^{5}	200	control (ethanol)	$5.2 \pm .03$
1.6×10^{5}	200	cytochalasin B	$36.9 \pm .34$

DISCUSSION

The hypothesis which generated the experiments discussed above was that tumor promoters may be acting by inhibiting the repair of DNA. One would predict that one of the major biological consequences of the inhibition of DNA repair would be an increase in the appearance of mutant cells (69). The experiments described above have provided evidence which helps rule out the possibility that TPA, as a representative tumor promoter, is acting by inhibiting DNA repair. Two lines of evidence support the contention that the effect of TPA is not related to an influence upon DNA repair. First, TPA has been demonstrated to have no specific inhibitory effect upon UV-induced unscheduled DNA synthesis in human cells or upon post-replication repair in Chinese hamster cells (119), and secondly, TPA is effective at increasing the recovery of specific somatic mutant cells only if it is present during the time of mutation selection (116), well beyond the period of DNA repair. In addition, the work described above has provided quite reasonable information as to the mechanism through which TPA increases the recovery of 6-thioguanine resistant cells. TPA has been demonstrated to be influencing the ability of two cell lines to communicate with one another, i.e. to cooperate metabolically with one another in the 6-thioguanine - HG-PRT mutation system. Although it is beyond the scope of this report to demonstrate directly the alteration of intercellular communication by TPA, good circumstantial evidence has been presented which suggests strongly that TPA acts at

the level of the membrane by altering structure and/or function.

The best evidence that TPA operates in our system at a time when cells are communicating with one another is the observation that TPA is effective in increasing the recovery of the mutant 6-thioguanine resistant cells only if it is present when wild type and mutant cells are alive and communicating. TPA had no specific inhibitory or stimulatory effect upon the colony forming ability of the 6-thioguanine resistant cells when they were grown alone. This observation is consistent with previous mutation studies (64,116) which indicated a TPA effect upon mutation frequencies only if the drug were present during the time of mutation selection, i.e. when wild type and induced mutant cells were seeded together in the presence of selective medium.

The literature recently has tended to support the view that the cell membrane may be an important target site for the phorbol ester class of tumor promoters. TPA is a highly lipid soluble molecule (57) which has been demonstrated to cosediment with nuclear membranes in CsCl equilibrium density gradients (63). In addition, TPA has been shown to associate rapidly with a cell membrane-rich fraction isolated from 3T3 cells previously exposed to the drug (100). Rapid changes in membrane morphology and permeability (99) following TPA treatment of cells in culture have also been observed. TPA has also been shown to reduce significantly and reversibly a transformation-sensitive membrane protein (LETS) (18). The putative early changes in cell membrane permeability caused by TPA are reflected in a stimulation of $^{86}Rb^{+}$ and $^{32}Rb^{+}$ uptake within 5 to 10 minutes of incubation of mouse 3T3 cells (124). Also implicating the cell membrane as a target site for phorbol ester tumor promoters is the observation that phorbol

myristate acetate (TPA) is an effective mitogen for cultured human peripheral blood lymphocytes (41). And finally there is evidence that TPA is capable of stimulating certain membrane bound enzymes (NA^+,K^+-ATPase and 5'-nucleotidase) (98), while at the same time modulating membrane protein conformation (129). Considered collectively these observations offer convincing evidence that the cell membrane is a critical target site for TPA and its related phorbol ester tumor promoters.

The fact that TPA is known to interact with cell membranes is of particular importance in any discussion of the TPA perturbation of metabolic cooperation. The type of metabolic cooperation under discussion is a form of junctional transmission that allows the direct flow of matter between cell interiors (103). These permeable junctions appear to be membrane protein appositions which are highly infiltrated with aqueous channels. The channels are thought to be contained within the membrane and have been referred to as gap junctions (70). Current thought is that the passage of small molecules through gap junctions is responsible for tissue homeostasis, perhaps by regulating ionic and metabolic pools (11,67). In addition, gap junctions are thought to be the channels through which the "signals" responsible for the induction of developmental processes, as well as for the control of growth and replication, may be transmitted (29).

Therefore, it appears reasonable to suggest that TPA is in some unknown manner altering membrane structure and/or membrane function in such a way as to significantly reduce the transfer of toxic nucleotide(s) from a wild type (6-thioguanine sensitive) cell to a mutant (6-thioguanine resistant) cell. As a consequence of this disruption

of membrane integrity, which is postulated to include gap junction protein irregularities, the mutant cells will be rescued and will proliferate in the 6-thioguanine containing medium. This is the most reasonable hypothesis to explain the tumor promoter induced reduction of intercellular communication seen between wild type and mutant Chinese hamster cells. The speculation could be offered that tumor promoters in general may operate in vivo by disrupting the flow of regulatory substances from normal cells to initiated, preneoplastic cells, eventually allowing initiated cells to express their transformed phenotype. This speculation is consistent with a number of experimental and theoretical reports, each of which points to the importance of intercellular communication between normal or wild type cells in physical contact with mutant or transformed cells. Borek and Sachs (19) have demonstrated that non-transformed hamster or rat cells could inhibit the replication of transformed cells when they were cultured together. Sivak and VanDuuren (101) noted that a purified fraction of croton resin enhanced the recovery of virus-transformed cells co-cultivated with high densities of non-transformed mouse 3T3 fibroblasts. Bertram (16) and Lloyd et al. (66) showed that untransformed 10T½ cells can by co-cultivation suppress the expression of transformed C3H 10T½ cells. Recently, Bertram (17) has demonstrated that the mechanism, by which non-transformed 10T½ cells cause the inhibition of growth of co-cultivated malignant cells, seems to involve the modulation of cyclic nucleotides in mediating intracellular communication between normal and transformed cells. Three additional observations may be taken into consideration: (1) transformed human cells exhibit reduced contact mediated communication when compared to normal cells

(29); (2) malignant mouse melanoma cells do not form tumors when mixed with cells of a non-malignant clone (79); and (3) TPA has been demonstrated to directly alter the intercellular connections (including the membrane and desmosomes) of mice epidermal cells (62). Clearly these reports demonstrate the ability of normal (non-transformed) cells to suppress the transformed or malignant phenotype of cells with which they are in physical contact. Additionally, it would appear that tumor promoters, specifically TPA, are capable of disrupting the flow of regulatory information from normal to "initiated" cells. The result of such disruption may possibly be proliferation of the initiated cell population until it reaches a critical mass, at which point it could become promoter independent. Recently (9), a mathematical model was postulated to explain just how a single transformed cell could proliferate until a "critical mass" was reached. The theory upon which the model was based depended upon the postulation of a locally reduced mitotic inhibitor (chalone) allowing proliferation of the transformed cell. It is, therefore, unequivocal that the transformed phenotype may remain unexpressed as long as there is an uninterrupted flow of "information" from the surrounding environment of normal cells. In addition, since tumorigenicity has been shown to be a recessive trait (108) in all but virally transformed cells, it is likely that tumorigenic cells can be converted into normal cells when regulatory substances from normal cells are transferred by metabolic cooperation.

The work described in this report provides evidence that one consequence of tumor promotion administration may be a disruption of intercellular communication, eventually leading to proliferation of

the transformed cell.

Speculatively it may be suggested that TPA might possibly be acting by altering membrane permeability and thereby influencing the flux of calcium ions. Any alteration in the intracellular level of calcium ions would be predicted to have major consequences on the rate at which a cell divides and on the physical integrity of the plasma membrane. Perhaps the effect of TPA upon metabolic cooperation is mediated by calcium ions, such that TPA perturbations of membrane permeability are reflected in calcium dependent alterations in the manner in which cells communicate with one another.

As should be obvious, the exact mechanism of tumor promotion has yet to be reported. The experiments reported above do, however, provide some evidence, albeit circumstantial, that tumor promoters alter the manner in which cells communicate with one another. Whether this alteration of intercellular communication by tumor promoters is operative in vivo will have to be determined.

As noted in the Results section above, TPA was completely ineffective in terms of altering metabolic cooperation in a diploid human fibroblast system. For the following reasons these results were not entirely unexpected. The vast majority of all human cancers are carcinomas (epithelial in origin); therefore observing a negative response to tumor promoters using diploid human fibroblasts is not surprising. Attempts are currently underway in our laboratory to characterize a human epithelial cell line with which we could repeat the metabolic cooperation experiments with TPA. However, TPA may not be the most appropriate tumor promoter to test in any human in vitro test. There is evidence that TPA is metabolized completely

differently in four human fibroblast cell lines in comparison to Syrian hamster embryo cells (81,82). The search for a human system to detect tumor promoters must, therefore, continue.

SUMMARY

The rationale behind the experiments reported above was that tumor promotion in the sequential two-stage view of carcinogenesis was molecularly different from the initiation step. Evidence at this point in time would favor the view that tumor initiation is probably as a result of a somatic mutation in a gene whose product is essential to the regulation of cell division. However, the exact molecular mechanism of tumor promotion has proven to be much more elusive. In this report, it has been demonstrated that 12-0-tetradecanoyl phorbol-13-acetate (TPA), the most powerful mouse skin tumor promoter, is not acting as a mutagen. Quite the contrary, it appears as if the initial target site of TPA, at least in a Chinese hamster fibroblast system, is the cell membrane. If a genetic marker (6-thioguanine resistance) is introduced into V79 Chinese hamster cells such that a phenotypically distinct cell line can be cloned, it is possible to observe the consequences of the phenomenon of intercellular communication between the two cell lines. The consequence of the type of intercellular communication occurring between 6-thioguanine resistant and 6-thioguanine sensitive Chinese hamster fibroblasts is a reduction in the recovery of the mutant 6-thioguanine resistant cells. This reduction in recovery can be essentially completely prevented by TPA. Since it has been demonstrated in other laboratories that the type of intercellular communication we are observing requires cell to cell contact and is postulated to occur through membrane bound gap junctions, we have postulated that the initial consequence of TPA is a disruption

of membrane structure and/or function such that communication between the two cells is disturbed. The TPA effect has been demonstrated to be dose responsive and excellent correlation has been observed between the *in vivo* tumor promoting ability and the *in vitro* response of a graded series of phorbol ester derivatives. In addition, several other known tumor promoters (phenobarbital, BHT, Tween 80, mezerein and melittin) have tested positively in the *in vitro*, "intercellular communication" system.

The speculation that tumor promoters *in vivo* may act by disrupting the flow of regulatory "information" from normal cells to previously initiated cells has been made. In addition, the role of the calcium ion has been discussed as it relates to the phenomenon of transformation and tumor promotion.

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