ENHANCEMENT OF BOVINE PAPILLOMAVIRUS DNA-MEDIATED TRANSFORMATION OF C3H/10T1/2 CELLS BY CARCINOGENIC METALS AND SUPPRESSION OF TRANSFORMATION BY ASCORBATE AND RETINOL

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Enhancement of Bovine Papillomavirus DNA-mediated Transformation of C3H/10T½ Cells by Carcinogenic Metals and Suppression of Transformation by Ascorbate and Retinol

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ABSTRACT

Tumor promoters such as phorbol esters increase the numbers of transformed foci in BPV DNA-transfected C3H/10T½ cells in a dose dependent manner. In this respect, BPV DNA-transfected C3H/10T½ cells model the interaction between a DNA tumor virus and tumor promoting environmental agents. One objective was to investigate whether carcinogenic metals could enhance neoplastic transformation in BPV DNA-transfected C3H/10T½ cells. Exposure to vanadyl or vanadate for 21 days enhanced numbers of transformed foci by 25-50 fold. Arsenic and chromium enhanced numbers of foci by 6- and 1.5-fold respectively.

A second objective was to determine whether anticarcinogenic agents could suppress metal-mediated transformation. Ascorbate at 500 μM suppressed mezerein-mediated transformation by 36%. Ascorbate at 100 μM suppressed vanadium-mediated transformation by approximately 50%. Retinol at 1.74 μM suppressed mezerein and vanadium-mediated transformation completely. Retinol at 1.74 μM suppressed mezerein and vanadium-mediated transformation by approximately 80% even if exposed to retinol 11 days after commencement of promoter treatment.

BPV and human papillomavirus are integrated in some cloned cell lines and in tumors. Therefore, another objective was to determine the extent to which BPV DNA was integrated, deleted or rearranged in lines of cells subcloned from foci which developed in BPV DNA-transfected C3H/10T½ cells exposed to vanadate, vanadyl or mezerein. Approximately 80 cell lines were cloned and subcloned. The resulting lines contained BPV DNA that was, in various lines, episomal, integrated, and contained deletions and rearrangements.

A final objective was to identify cell lines in which focus formation was enhanced by vanadate or mezerein and suppressed by retinol or ascorbate for use in future experiments. Out of 80 clones and subclones, four subcloned cell lines were found which produced more foci in the presence of promoter. In a preliminary experiment, five cell lines, two of which produced more foci in the presence of promoter, were passaged 10 times in promoters, ascorbate and retinol. Integration of BPV DNA was not altered by retinol, ascorbate or promoters during passaging. This suggests that enhanced neoplastic transformation by promoters or suppression by ascorbate or retinol occur by mechanisms that are largely independent of viral integration.

DEDICATION

I would like to dedicate this thesis with gratitude and appreciation to the memory of my father, William Kowalski, and to my mother, Antoinette Maria (Longo) Kowalski, of Albany, New York.

Come, my friends,
'Tis not too late to seek a newer world.
Push off, and sitting well in order smite
The sounding furrows; for my purpose holds
To sail beyond the sunset, and the baths
Of all the western stars, until I die.

A.L. Tennyson, *Ulysses*

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ABBREVIATIONS USED IN THIS THESIS:

BPV:	Bovine papillomavirus
HPV:	Human papillomavirus
ORF:	open reading frame
kb:	kilobase
bp:	base pair
CAT:	catalase
SOD:	superoxide dismutase
pML2d:	plasmid pML2d
pdBPV-1:	pML2d plasmid containing the complete BPV type-1 genome inserted at the Bam HI site
BPV-1	Bovine papillomavirus, type 1

TERMS USED IN THIS THESIS:

Focus: a multilayered group of cells which have overgrown a confluent monolayer.

Colony: a single group of cells, which is distinct and separated from surrounding cells and most likely arose from a single cell.

Transfected: untransformed cells into which viral DNA has been inserted.

Transformed: C3H/10T½ cells which form foci. C3H/10T½ cells which are transformed to focus formation are tumorigenic in nude mice.

Co-cultures: mixtures of 2000 untransformed C3H/10T½ cells and 200 cells from a subclone cultured together in the same dish.

Promoter dependent cell line: a cell line which produces at least twice as many foci in co-culture in the presence of promoter than in its absence and produces at least 10 foci in the presence of promoter

Promoter independent cell line: a cell line which produces the same number of foci in co-culture in the presence and absence of promoter

Clone: a line of cells isolated from a single focus induced in BPV DNA-transfected cells by a tumor promoter.

Subclone: a line of cells isolated from a promoter-induced clone.

STATISTICAL METHODS

Significance of data determined from counting foci or colonies (toxicity testing) was determined either by t-tests or by one-way analysis of variance. The methods used are indicated in the table or figure legends along with the p values. For t-tests, two-tailed tests were used because it was not known beforehand whether the various treatments would increase or decrease the numbers of foci or numbers of colonies formed, relative to the untreated controls.

CHAPTER 1

INTRODUCTION AND OVERVIEW

INTRODUCTION

DNA viruses interact with carcinogens

Cancer viruses are responsible for about 15% of human cancer (1). Human papillomaviruses 16, 18 and 33 (HPV) are most commonly associated with anogenital carcinomas, particularly cervical carcinoma (1). Many women, however, test positive for HPV but are asymptomatic for cervical neoplasia (2). This suggests that HPV infection is necessary but not sufficient to cause benign papillomas to progress to malignant carcinomas, other factors are required (3). These factors are, epidemiologically, if not causally, identified as environmental risk factors. Examples include smoking (4) or arsenic (5) in the activation of HPV. In one model system, nitrosomethylurea with 12-O-tetradecanoylphorbol-acetate converts non-tumorigenic HPV-18-immortalized human keratinocytes to tumorigenic cells (3). However, the interaction between DNA tumor viruses and environmental factors such as carcinogens and tumor promoters has not been extensively investigated in part because of the lack of an *in vitro* model.

Bovine papillomavirus type 1 (BPV-1) forms benign fibrosarcomas in cattle and sarcoid tumors in horses (6).

BPV-type 4 also causes benign fibropapillomas in cattle. However, when the cattle browse on bracken, these progress to carcinomas. This was one of the earliest demonstrations of tumor promotion by an environmental agent (7).

BPV-1 contains a 7946 base pair (bp) circular, double-stranded DNA genome in an icosahedral protein coat (8, 9) and is genetically similar to HPV. Other types (strains) of BPV are genetically distinct from each other and have different hosts.

The molecular biology of BPV is complex and serves as a system in which papillomavirus replication, transcription and translation can be explored. The genome contains a total of 10 open reading frames (ORF's) (E1 - E8) which are expressed early in the infective cycle and are responsible for neoplastic transformation. Two late ORF's (L1 and L2) are involved with production of the coat proteins and are not expressed in non-productive cells such as cell lines in tissue culture (10) which do not produce infective particles. In addition to the 10 ORF's, the BPV genome contains a "long control region" This contains several promoters (10) and the origin of replication. It plays a role in viral transcription and replication.

Transcription in BPV involves several regions of the DNA. A feedback loop involves the products of the E2 ORF and the long control region. Interplay between enhancing sequences in the long control region and the E2 ORF control transcription of the E5 and E6 ORF's which are the major transforming ORF's (10, 11). The E8 ORF forms a combined mRNA product with part of the E2 ORF which also plays a role in regulation of transcription (12).

The E5 protein is primarily responsible for transformation of rodent cells (13). Products of the E5 ORF may activate the PDGF receptor (14) leading to enhanced cell proliferation. The E6 protein contains sequences which suggest that it is a nucleic acid binding protein which may bind to cellular tumor suppressor genes (15). The E2 ORF, in conjunction with the E8 ORF and the long control region, controls transcription of these two major transforming genes. The functions of the E3 and E4 ORF's are under investigation (12).

Like transcription, replication of BPV DNA is also controlled by complex feedback loops. BPV DNA contains one origin of replication (16) and two "plasmid maintenance sequences" within 2000 bp of the origin (12) in the long control region. Viral replication depends on the plasmid maintenance sequences and gene products from

all ORF's except the E3 and E4 ORF's (12). The E1 ORF produces two proteins which can amplify copy number or decrease it (17). Disruption of parts of other ORF's also impairs replication.

Expression of the transforming genes, E5 and E6 are controlled by the E2 ORF. However, expression of the transforming genes themselves is required for control of replication. Therefore, control of transcription and replication are closely linked in BPV. This coordinated regulation of transformation and replication is a common characteristic of papillomaviruses (12).

BPV-1 is a novel virus in which its ability to transform cells in tissue culture is enhanced by tumor promoters. In the usual scenario, BPV-1 DNA transfected into C127 cells transforms them to multilayered focus formation (18) in the absence of tumor promoters. However, unlike C127 cells, when BPV DNA is transfected into C3H/10T½ mouse embryo fibroblasts, very few foci develop. When tumor promoters such as phorbol esters (19), betel quid ingredients (20), teleocidin and okadaic acid (21) are applied to BPV DNA-transfected C3H/10T½ cells, the number of multilayered foci increases, in a manner that is proportional to the dose of tumor promoter (19). Therefore, BPV DNA-transfected C3H/10T½ cells

constitute an effective model of the interaction between a DNA tumor virus and tumor promoting agents.

In this system, however, transfection produces a combination of transfected C3H/10T½ cells, which form foci, and untransfected cells which form a monolayer. An objective of this project was to isolate subcloned lines of cells which produced an increased number of foci when exposed to tumor promoters from foci which developed in BPV DNA-transfected C3H/10T½ cells exposed to tumor promoters. This would produce a line of cells in which transformation, as measured by focus formation, would be enhanced by promoters; uncontaminated by variable numbers of untransfected cells.

Metals are environmental pollutants and carcinogens

Metals such as vanadium, arsenic and chromium occur naturally in the earth, as airborne pollutants, and as industrial pollutants. Chromium and arsenic (22, 23) are known human carcinogens, though their mechanism of action is still under investigation. Vanadium is a suspected human carcinogen (24). A second objective of this project was to investigate whether carcinogenic metals enhance

the formation of multilayered foci (transformation) in BPV DNA-transfected C3H/10T½ cells.

Anticarcinogenic agents inhibit transformation

Tumor promoters enhance transformation of BPV DNA transfected C3H/10T½ cells. Retinol (25) and ascorbate (26) inhibit both virally-mediated and chemically-mediated neoplastic processes. A third objective of this project is to investigate whether retinol or ascorbate can inhibit metal or phorbol ester mediated focus formation in BPV DNA-transfected C3H/10T½ cells and in cloned cell lines carrying BPV DNA.

BPV DNA is integrated in cloned cell lines

Integration of papillomaviruses may be a mechanism in virally-mediated neoplastic transformation. BPV DNA is usually episomal in tumors and when transfected into C127 cells (27-29). It is integrated in cloned cell lines (30, 31). HPV DNA is usually integrated in malignant tumors and episomal in benign lesions (32). The integration of episomal viral DNA is thought to be one of the mechanisms in transformation of benign to malignant tumors by HPV (33). This leads to the hypothesis that BPV DNA is initially episomal when transfected into C3H/10T½ cells. It becomes integrated when treated with tumor promoters,

and leads to enhanced transformation. Therefore, a fourth objective is to determine whether BPV DNA is integrated in cell lines cloned from foci which developed in BPV DNA-transfected C3H/10T½ cells exposed to tumor promoters.

BPV DNA transfected into C127 cells is stably carried as 60-120 copies per genome (28). In cells exposed to retinoic acid, copy number decreases (34, 35). Therefore, alteration of copy number may serve as a mechanism for control of transformation. A fifth objective is to determine whether copy number and integration of BPV DNA in cloned lines established from transfected cells exposed to tumor promoters is altered by passaging in retinol, ascorbate or promoters.

SUMMARY OF THESIS OBJECTIVES

1. To isolate cloned lines of cells which produce an increased number of foci when exposed to tumor promoters from foci which develop in BPV DNA-transfected C3H/10T½ cells exposed to tumor promoters.

2. To investigate whether carcinogenic metals enhance transformation of BPV DNA-transfected C3H/10T½ cells.

3. To determine whether retinol or ascorbate can inhibit metal or phorbol ester mediated focus formation in BPV DNA-transfected C3H/10T½ cells and in cloned cell lines carrying BPV DNA.

4. To determine whether there is evidence for integration, deletion or rearrangement of BPV DNA in cell lines cloned from foci which develop in BPV DNA-transfected C3H/10T½ cells exposed to tumor promoters.

5. To determine whether copy number and integration of BPV DNA in cloned lines from transfected cells exposed to tumor promoters is altered by passaging in retinol, ascorbate or promoters.

OVERVIEW OF THE PROJECT

The first part (chapter 2) describes enhancement of transformation in tissue culture of BPV DNA-transfected C3H/10T½ cells exposed to various tumor promoters, emphasizing vanadium, arsenic and chromium. These were identified as vanadate, vanadyl and mezerein. Arsenic and chromium enhanced transformation to a lesser extent than vanadium and mezerein.

In the next section (chapter 3), retinol and ascorbate were investigated as possible inhibitors of metal mediated transformation. Retinol and ascorbate strongly inhibited enhancement of transformation by vanadium and mezerein.

The next goal (chapter 4) was to develop cloned lines of cells from foci which developed in BPV DNA transfected C3H/10T½ cells exposed to vanadate, vanadyl and mezerein. Approximately 80 clones and subclones were established. Of these cell lines, four lines formed more foci in the presence of mezerein or vanadate than in normal medium (promoter dependent). There was evidence for integration, deletion and rearrangement of BPV DNA in subcloned lines of cells. Five of the subcloned lines were selected. These were passaged in the presence and absence of ascorbate, retinol and the promoter (vanadate

or mezerein). Copy number and integration were investigated during the passaging.

Chapter 5 sets the study in perspective, summarizes the conclusions and suggests experiments to further explore the interaction of BPV DNA and tumor promoters or tumor suppressing agents. The major question remaining from this project is the mechanism by which tumor promoters enhance transformation of BPV DNA transfected C3H/10T½ cells. The most logical direction to explore would be viral gene expression, especially of the E5 and E6 ORF's, since these are most involved in cell transformation by BPV DNA (24).

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CHAPTER 2

VANADATE, ARSENIC AND CHROMIUM ENHANCE TRANSFORMATION OF BOVINE PAPILLOMAVIRUS DNA-TRANSFECTED C3H/10T1/2 CELLS

Vanadate data originally published as: Kowalski, L.A., Tsang, S.-S. and Davison, A.J. (1992) Vanadate enhances transformation of bovine papillomavirus DNA-transfected C3H/10T½ cells. *Cancer Letters* **64**, 83-90. Arsenic and chromium data added in thesis

ABSTRACT

Tumor promoters such as phorbol esters, teleocidin and okadaic acid enhance numbers of transformed foci produced by BPV DNA-transfected C3H/10T½ cells. In chapter 2, we explore whether the carcinogenic metals vanadium, arsenic and chromium also enhance transformation of BPV DNA-transfected C3H/10T½ cells. Vanadate at 4 μM enhanced numbers of transformed foci by 50-fold. Vanadate-induced enhancement of transformation was not inhibited by catalase or superoxide dismutase. If transfected cells were exposed to vanadate as late as 96 hours after subculturing, vanadate still enhanced transformation. Vanadate also induced reversible loss of adhesion of the cells. Chromium (III) at 200 μM enhanced transformation by 1.4-fold, but chromium (VI) did not enhance transformation. Both arsenite at 5 μM and arsenate at 25 μM enhanced neoplastic transformation by 6-fold. We conclude that the carcinogenic metals vanadate, arsenic and chromium all enhance numbers of transformed foci, to some extent, but vanadate enhances transformation more strongly. This suggests that enhancement of transformation occurs by mechanisms that are unique to certain tumor promoters such as vanadate or phorbol esters. These mechanisms may involve alteration of tyrosine phosphorylation.

INTRODUCTION

Vanadium is widely distributed in the environment in trace amounts. Although its carcinogenicity to humans is still under investigation (1), vanadium oxide (V_2O_3), vanadyl sulfate ($VOSO_4$) and ammonium metavanadate (NH_4VO_3) induce sister chromatid exchanges in mammalian cells (2). In bacterial assays, NH_4VO_3, $VOCl_2$ and V_2O_5 are mutagenic (3).

Several effects of vanadate on gene expression have been documented. In BC3HI mouse fibroblasts, vanadate induces the expression of c-*fos* and actin genes (4). In human ovary carcinoma cells, vanadate enhances the expression of the c-*myc* gene (5). Vanadate and vanadyl enhance expression of the actin and c-Ha-*ras* genes in C127 and C3H/10T½ mouse fibroblast cells (6). In C127 cells, vanadate increases the expression of the c-*jun* gene (6).

By changing oxidation states, among V(V), V(IV) and V(III), vanadium can participate in oxidation and reduction reactions (7). Entering the cells as vanadate, V(V), vanadium is reduced to vanadyl, V(IV), as it crosses the membrane. Intracellularly, vanadium is found mainly as vanadyl (8). Vanadate stimulates NADH oxidation (9), which produces $O_2^{\cdot-}$, H_2O_2 and •OH within the cell

(10). Addition of NADH further enhances expression of c-*jun* in C127 cells. Therefore, the genotoxicity of vanadium may reflect its oxidant or prooxidant qualities.

Vanadate is a mitogen (11 - 13). It induces DNA synthesis (11, 13) and ornithine decarboxylase activity (14). Like other mitogens such as epidermal growth factor and phorbol ester tumor promoters, vanadate stimulates phosphatidylinositol metabolism (15, 16). Vanadate also mobilizes intracellular calcium (17). Stimulation of phosphatidylinositol metabolism, ornithine decarboxylase activity, and activity of receptor protein kinases (18-20) suggests that vanadate may act through early signal transduction pathways.

Since vanadate is a phosphate analogue (21), it can inhibit or stimulate enzymes involved in phosphorylation and phosphate metabolism. Vanadate enhances phosphorylation of tyrosine residues of many enzymes (22) and enhances autophosphorylation of growth factor receptors which possess tyrosine kinase activity (18 - 20). Vanadate esterifies tyrosine and other hydroxyl groups (18, 21) and inhibits many tyrosine phosphatases (23). These effects stimulate metabolic pathways that are normally stimulated by phosphorylation.

Altered patterns of phosphorylation induce changes in cytoskeleton, cell morphology and cell adhesion. Rat hepatocytes (24) and baby hamster kidney cells treated with vanadate develop rounded morphology and cease to adhere to the substrate (25). Removal of vanadate reverses these effects and the cells return to normal morphology. Similarly, NRK-1 cells develop altered morphology. This correlates with an increase in phosphotyrosine as a result of inhibition of tyrosine phosphatases by vanadium (26). Vanadate induces multilayered foci in cells transformed by viral DNA coding for tyrosine kinases (27). The authors suggest that this transformation may be due to phosphatase inhibition by vanadate. Vanadate induces mitogenesis in SV40 large-T antigen-transformed 3T3 cells, but not in untransformed 3T3 cells. The mechanism involves changes in tyrosine phosphatase activities (28).

C3H/10T½ mouse embryo fibroblasts have been used in a well-established assay to examine carcinogenicity of environmental agents (29), including many metals (30, 31). We have previously shown that transformation of C3H/10T½ cells transfected with BPV DNA, is greatly enhanced by tumor promoters such as mezerein (a phorbol ester) (32), teleocidin (an indole alkaloid) and okadaic acid (an inhibitor of serine/threonine phosphatases)

(33). Because vanadate is a mitogen and phosphatase inhibitor, we chose to investigate whether vanadate would promote neoplastic transformation in this system. We report here that vanadate promotes BPV DNA-induced cell transformation.

This leads to the question of whether other metals which are carcinogenic or tumor promoting are also capable of enhancing transformation of BPV DNA-transfected C3H/10T½ cells. Vanadate and mezerein both generate oxygen-derived active species and they enhance transformation. Therefore, other metals which generate active oxygen may also be capable of enhancing transformation of BPV DNA-transfected C3H/10T½ cells.

There are many redox-active metals which generate active oxygen, such as iron and copper. However, iron (34) and copper (35), are not normally associated with human cancer. Molybdenum is an industrial metal which is redox-active and forms an oxyanion but evidence for molybdenum as a human carcinogen or toxin is very weak (36). Nickel is a human carcinogen, but it is not redox-active under physiological conditions. Cadmium is both redox-active and a human carcinogen (37). However, in preliminary studies, 0.1 μM of cadmium proved to be extremely toxic to C3H/10T½ cells. Therefore, it was not

chosen for this study. In order to limit the scope of this study, chromium, which occurs as an oxyanion, is redox-active, has no known function as a tyrosine phosphatase inhibitor, and is a common human carcinogen was chosen as a comparison to vanadate.

Cr(VI) generates •OH by a mechanism similar to vanadate. When microsomal NADPH reduces Cr(VI), reduction of molecular oxygen to H_2O_2 generates •OH, which reacts with a Cr(V)-NADPH complex (38). Chromium is synergistic with other carcinogens and with transforming viruses. Cr(VI) enhances transformation of SHE cells infected with SA7 virus (39) and enhances transformation of SHE cells induced by benzo[a]pyrene (40). Low doses of Cr(VI) stimulate DNA synthesis (41). Therefore, Cr(VI) may act as a tumor promoter (40).

In contrast to vanadium, there are no reports that chromium inhibits tyrosine phosphatases. The major action of Cr(III) lies in its ability to bind to DNA (42). It decreases fidelity of replication and repair causing single and double-strand breaks, interstrand crosslinks, gene amplification and ultimately altered gene expression (41). Therefore, both chromium and vanadium generate active oxygen species, but only vanadium inhibits tyrosine phosphatases and only chromium binds to DNA.

Comparison of the abilities of the two metals to enhance transformation could shed light on the mechanism of tumor promotion.

Like chromium, arsenic is a known human carcinogen and forms an oxyanion, but, in contrast to vanadium and chromium, its major mode of action does not involve tyrosine phosphatase inhibition or generation of active oxygen species. Arsenic is genotoxic, but not strongly mutagenic (43), unlike chromium which is clearly mutagenic. Arsenite (As(III)) induces morphological transformation of SHE cells (44, 45), despite its lack of mutagenicity. Like chromium, arsenic also functions synergistically with other agents to enhance transformation or to enhance viral transformation. Arsenite enhances viral transformation of SA7-infected SHE cells (39). It increases clastogenicity and mutagenicity of DNA cross-linking agents (46) and mutagenicity of ultraviolet light (47, 48). Arsenic also activates *Herpes simplex* and *Herpes zoster* (49). This suggests that arsenic may enhance transformation of BPV DNA-transfected C3H/10T½ cells.

Arsenic inhibits DNA repair and induces gene amplification. Arsenate (As(V)) and arsenite induce polyploidy, possibly due to endoreduplication, as well as

sister chromatid exchanges, gaps and breaks (45). They also inhibit post replication repair in *E. coli* (50), excision repair in human and in CHO cells (51), and repair after x-ray or ultraviolet light damage (52). These types of repair may be blocked by inhibition of DNA polymerase (53) activity.

Arsenate is a phosphate mimic, like vanadate. Since it uncouples mitochondrial oxidative phosphorylation via a phosphate-like intermediate, it is toxic (54). Although arsenate is a competitive inhibitor of alkaline phosphatase (55), there are no reports of its ability to inhibit tyrosine phosphatases or to enhance tyrosine phosphorylation. Unlike chromium, arsenic does not bind directly to DNA bases.

In summary, the neoplastic activities of arsenic may involve gene amplification and interference with DNA repair, but not DNA binding. Therefore, it constitutes a good foil against which to compare the transformation enhancement abilities of vanadium and chromium.

METHODS

Cell culture and transfection

C3H/10T½ mouse embryo fibroblasts from the American Type Culture Collection were grown at 37° C, 97% air/3% CO_2, in Dulbecco's modified essential medium (Flow) supplemented with 10% fetal calf serum (Gibco). Medium also contained 20 mM HEPES [N-2 hydroxyethylpiperazine - N'- 2 - ethanesulfonic acid] (Sigma), 9.96 U/ml. of penicillin G (Sigma) and 72.5 μg/ml. of streptomycin sulfate (Sigma). C3H/10T½ cells of passages 16-23 were used for our experiments. Medium was changed twice weekly.

We transfected C3H/10T½ cells with pdBPV-1 (142-6) containing the complete BPV-1 genome cloned into the plasmid pML2d at the Bam HI site, using the method described previously (32). Controls were transfected with only sheared calf thymus DNA (Sigma) (56).

For each experiment, cells were seeded on to plates of 100 mm diameter. After 48 hours, cells were transfected, medium removed, exposed to 15% glycerol in HBS buffer for 2 minutes, washed twice with medium, then left overnight in fresh medium as described previously (32).

After each experiment, foci were stained for 1-2 hours with 0.025% methylene blue in 50% methanol. Plates were air dried and foci scored.

Addition of compounds

K_2CrO_4, $NaAsO_2$, and Na_2HAsO_4 were prepared as 10 mM or 100 mM stock solutions in glass distilled water. $CrK(SO_4)$ was prepared as a 100 mM stock solutions in glass distilled water.

Na_3VO_4 (Fisher) was freshly prepared as a 10 mM stock solution in glass distilled water prior to each medium change and diluted with medium to the desired concentrations. Mezerein (Sigma) was dissolved in dimethylsulfoxide at a concentration of 0.2 mg/ml. and diluted with medium. Controls received 0.5 ng/ml. of mezerein for 21 days. Catalase (65,000 U/ml.) (Boehringer Mannheim) and superoxide dismutase (4050 U/mg.) (DDI Pharmaceuticals) were freshly prepared prior to each medium change as a stock solution of 50 U/μl in phosphate buffer and diluted with medium.

Inactivation of catalase

To inactivate catalase, a stock solution of 50 U/μl was boiled for 40 minutes. Superoxide dismutase was inactivated by incubation with 10 mM hydrogen peroxide for 1.5 hours followed by dialysis against phosphate buffer.

RESULTS

The number of foci in vanadate-treated C3H/10T½ cells transfected with BPV DNA increased in a dose-dependent manner to 50-fold for 4 μM vanadate (figure 1). Our results are consistent with a previous study in which vanadate at 1 to 4 μM caused a 50-fold increase in foci in NIH 3T3 cells infected by *fps/fes* viruses (27).

In untransfected BALB/3T3 cells, 3 μM vanadate for 72 hours followed by 5 weeks of vanadate-free medium induced 11 foci per 18 plates (0.6 foci per plate) (8). In our study, cells transfected with only calf thymus DNA and exposed to vanadate for 21 days produced a maximum of 1 focus per plate.

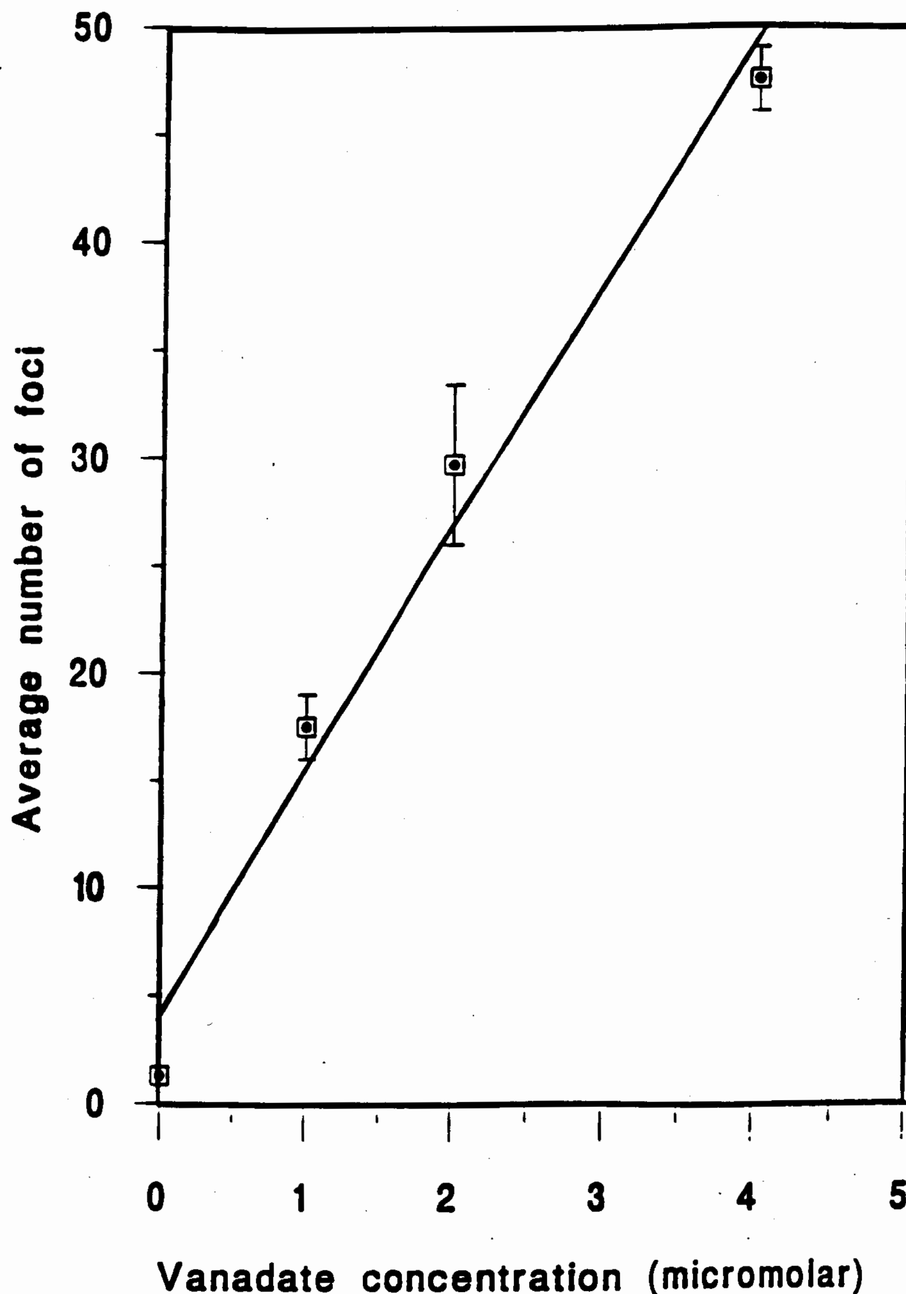


Figure 1: Number of foci in BPV DNA-transfected C3H/10T½ cells as a function of concentration of vanadate

Cells at passage 16 were transfected at a density of 5 x 10^5 cells/100 mm plate with 1 µg of BPV DNA per plate. Cells were subcultured 21 hours later and seeded at a density of 6.4 x 10^4 cells per 60 mm plate. Vanadate and mezerein were added and the cells were incubated for 21 days. Mezerein at 0.5 ng/ml yielded an average of 164 foci/plate. Cells which were transfected with BPV DNA, but not exposed to vanadate yielded an average of 1.7 foci per 60 mm plate. Cells transfected with only calf thymus DNA and exposed to vanadate yielded a maximum of 1 focus/plate. Values graphed are averages of triplicate plates. Error bars are standard errors of the mean. The line shows a linear regression. The experiment was repeated twice to 4 µM vanadate and 7 times to 2 µM vanadate with similar results. Values tested by analysis of variance. $p < 0.001$.

The plating efficiency of untransfected C3H/10T½ cells, not exposed to vanadate, measured as colony forming ability was 30% of subcultured cells. Vanadate at 20 μM reduced the plating efficiency to 3.2% after 5 days of exposure (figure 2, $p < 0.001$). Plating efficiency is not due solely to toxicity but reflects the ability of vanadate-treated cells to adhere to the plates and to form colonies of a sufficient size to be counted. Colony forming ability is decreased to 15% at 3 μM vanadate (figure 2) from 30% for untransfected cells, while number of foci increases about 30-fold (figure 1). This suggests that actual enhancement of focus production might be considerably higher after taking into account the toxicity of vanadate.

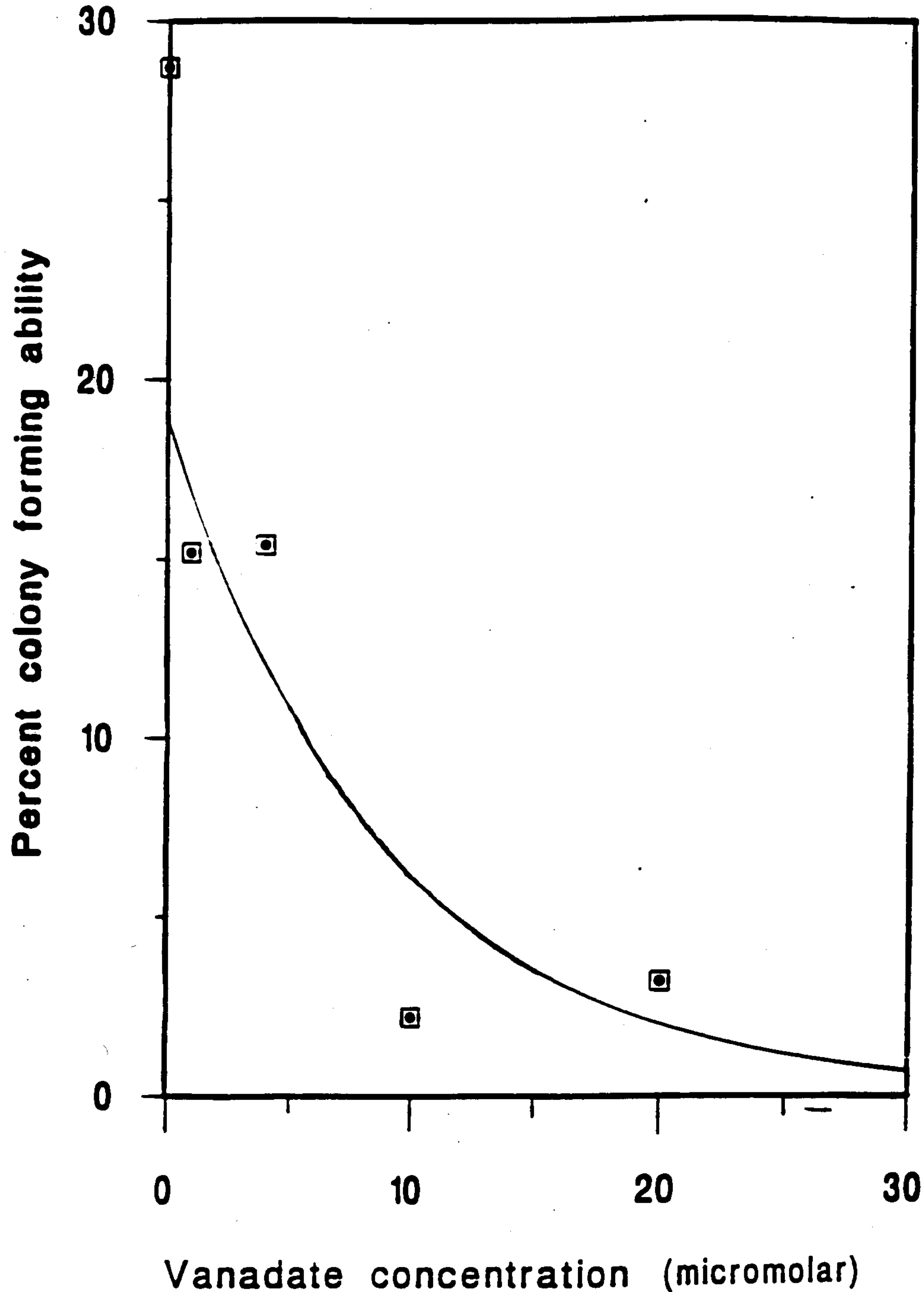


Figure 2: Cloning efficiency of C3H/10T½ cells at various concentrations of vanadate

Untransfected cells were grown for 8 days in various concentrations of vanadate in 100 mm dishes. Media were changed twice weekly. On the eighth day, treated cells were trypsinized. 200 cells were seeded from each vanadate treatment into 60 mm. dishes in vanadate-free medium. Cells were incubated without vanadium for 7 days, then stained and clones counted. Percent colony forming ability was calculated as: (# of clones > 80 cells / 200 cells) x 100%. Values graphed are averages of triplicate plates. $p < 0.001$ by analysis of variance for values >20 μM vanadate..

Vanadate may have enhanced the uptake of BPV DNA post transfection resulting in enhanced transformation. To test this hypothesis, we exposed BPV DNA-transfected cells to 2 μM vanadate for 24 hour intervals from the time the cells were subcultured up to 4 days after subculturing. Otherwise, cells were maintained in vanadate-free medium. Results and the various treatment schemes are shown in figure 3.

Cells exposed for 24 hour intervals from day 0 to day 4 developed, on average, 19 foci/plate. Cells exposed continuously to 2 μM vanadate from 0 to 21 days developed an average of 41 foci/plate. Cells transfected with BPV DNA but not exposed to vanadate developed an average of 13 foci/plate. BPV DNA- transfected cells that were not exposed to vanadate for the first 96 hours were still competent to produce foci when exposed to vanadate 96 hours after subculturing (figure 3).

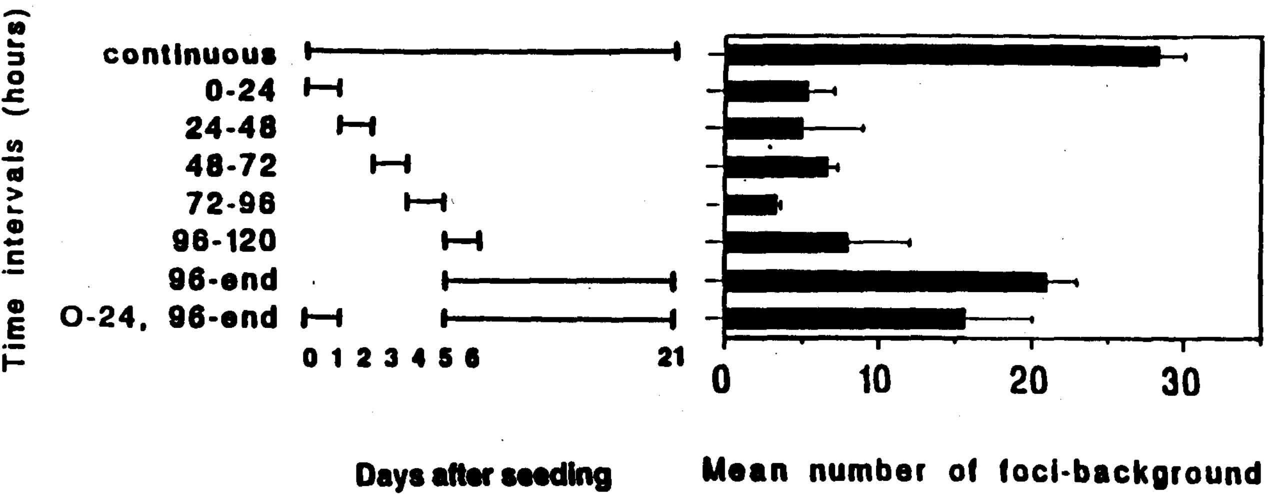


Figure 3: Number of foci in BPV DNA-transfected cells after exposure to 2 μM vanadate for various time intervals after transfection

Cells at passage 18 were transfected at a density of $2.4x10^5$ cells / 100 mm. plate with 1 μg of BPV DNA. They were subcultured 21 hours after transfection and seeded at $1.7x10^5$ cells / 60 mm. plate (time = 0). Cells were incubated for 21 days. Vanadate was added at various intervals after time 0 according to the protocol given below the graph. Values are averages of triplicate plates. Error bars are standard errors of the mean. 0.5 ng/ml of mezerein yielded an average of 86 foci/plate. Cells transfected with BPV DNA but not exposed to vanadate averaged 13 foci/plate.

Vanadate may generate active oxygen species in the medium or at the cell surface that could promote transformation. When active oxygen species generated in the medium act as tumor promoters, inhibitors of active oxygen added to the medium suppress promotion (57). In our study, addition of catalase, superoxide dismutase, the two together, or an equivalent amount of albumin did not significantly block the induction of transformed foci by vanadate ($p > 0.05$, Table 1).

TABLE 1

Number of transformed foci in vanadate-treated BPV DNA-transfected cells exposed to catalase (CAT) and superoxide dismutase (SOD)

Treatment	Mean foci
No inhibitor	26.0 ± 2.6
+SOD	33.0 ± 1.0
+CAT	24.7 ± 3.0
+SOD +CAT	28.0 ± 1.2
+inactivated SOD	20.0 ± 0.0
+inactivated CAT	28.5 ± 1.5
+albumin	25.0 ± 2.1

Cells at passage 20 were transfected at a density of $1.3x10^5$ cells / 100 mm. plate with 1 μg of BPV DNA. They were subcultured 21 hours later and seeded at a density of $7x10^4$ cells / 60 mm. plate. Catalase at 50 U/ml. and superoxide dismutase at 50 U/ml. were added. Bovine serum albumin (Sigma) was prepared as a stock solution in phosphate buffer and diluted with medium. It was added to controls at a final concentration of 2.8 μg/ml, which is equivalent to the highest protein concentration in experiments using superoxide dismutase and catalase. Vanadate at 2 μM was added and cells were incubated for 21 days. Mezerein at 0.5 ng/ml. yielded an average of 53 foci / plate. In the absence of vanadate, BPV DNA-transfected cells yielded an average of 10 foci / plate. Values are means ± standard errors of the mean of triplicate plates except for SOD alone (without added vanadate) which is only 1 plate. All values except SOD alone have $p > 0.05$ by t-test.

At vanadate concentrations above 6 μM another phenomenon was observed. Cells ceased to adhere to the plates, whether the cells were transfected with BPV DNA, calf thymus DNA, or untransfected. After the cells were detached from the plate, they formed multicellular aggregates of 3-4 mm diameter.

To investigate the viability of these detached cells, BPV DNA-transfected cells were grown in 10 μM vanadate for 8 days. Nearly all the cells were detached from the plates at the eighth day and formed into masses of 3-4 mm in diameter. These cells were collected, trypsinized, and washed in vanadium-free medium. We plated 200 of these cells in vanadium-free medium to determine their cloning efficiency. After 7 days, cloning efficiency was 24%. These cells were attached to the plate and appeared to be morphologically normal.

Conversely, we trypsinized and subcultured cells grown in 10 μM vanadate but plated them in 10 μM vanadate. After 7 days (2 changes of medium), they formed many small, multicellular masses, freely suspended in the medium. To change medium, cells were removed with medium and pelleted by centrifugation. The pellets were resuspended and added back to the plates with fresh medium.

In previous studies, BHK cells exposed to 50 μM vanadate for 12 hours assumed rounded shapes and detached from the plastic dishes. After the cells were removed to vanadate-free medium, they reattached to the dishes and recovered their normal, flattened shape (25). NRK-1 and 10T½ cells lost contact inhibition and developed the ability to grow in semi-soft agar after exposure to 37.5 μM vanadate. These changes were reversible (26). Our observations are similar to these. Since these morphological changes occur whether cells are transfected or not, they may reflect general effects of vanadate on cell adhesion and morphology not mediated by BPV DNA.

Arsenite stimulated a dose-dependent increase in transformed foci to 6-fold at 5 μM (figures 4 and 5). Percent colony forming efficiency decreased sharply above 7 μM (table 2). Arsenate increased transformed foci to 6-fold at 25 μM (figures 5 and 6). Percent colony forming efficiency decreased sharply above 25 μM (table 2).

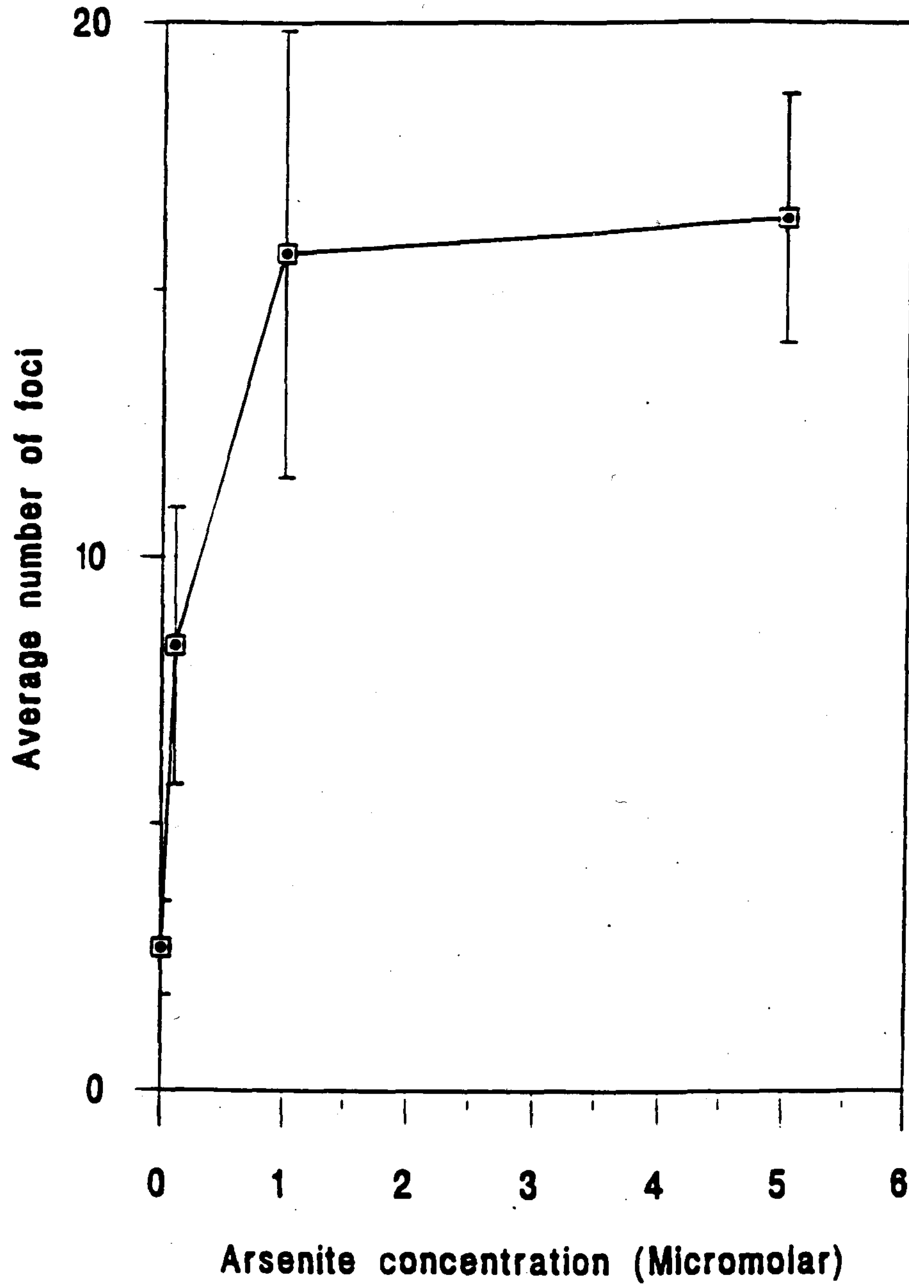
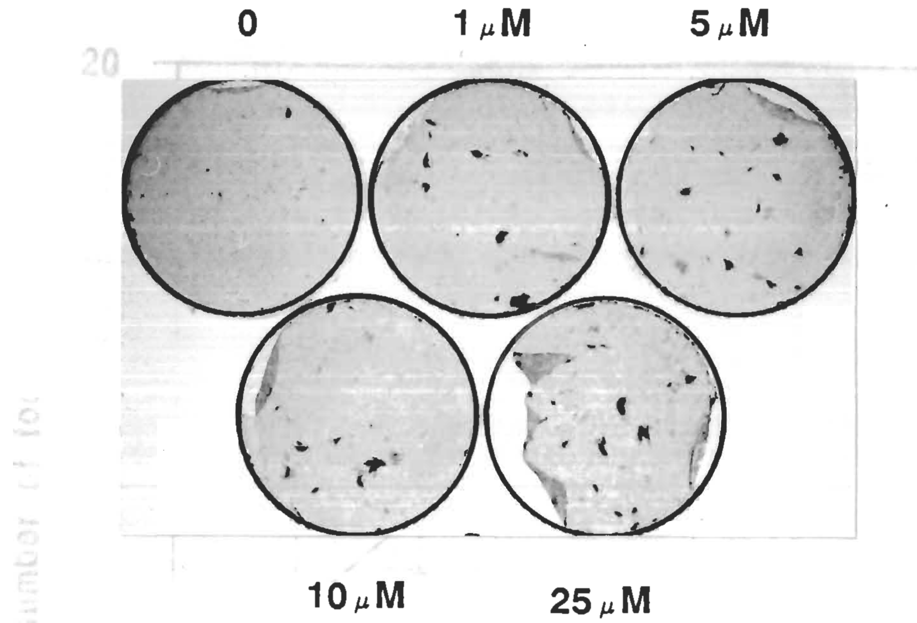
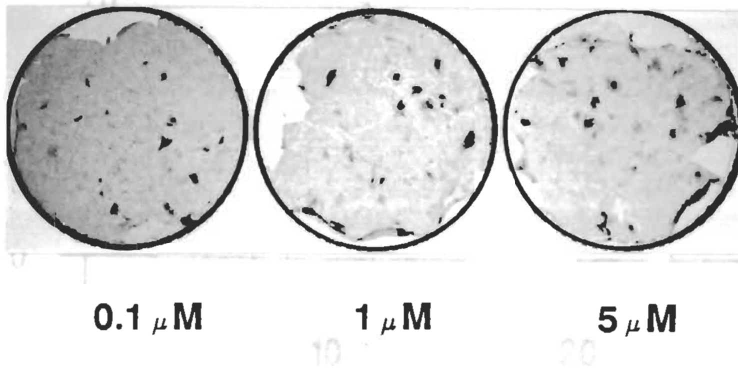


Figure 4: Average number of foci as a function of arsenite concentration

Cells at passage 17 were seeded at a density of 2.2×10^5 cells /100 mm plate and transfected with 1 µg of BPV DNA /plate. They were subcultured 21 hours later at 1:17, then exposed continuously to arsenite for 21 days. Cells transfected with BPV DNA but not exposed to arsenite developed an average of 2.7 foci/ 60 mm plate. Cells exposed continuously to 0.5 ng/ml of mezerein developed an average of 91 foci/60 mm plate. Values were determined in triplicate. Error bars are standard errors of the mean. $p < 0.001$ by analysis of variance



BPV DNA-transfected cells exposed continuously to various concentrations of arsenate for 21 days



BPV DNA-transfected cells exposed continuously to various concentrations of arsenite for 21 days

Figure 5: Enhancement of numbers of foci in BPV DNA transfected C3H/10T½ cells exposed to arsenite and arsenate.

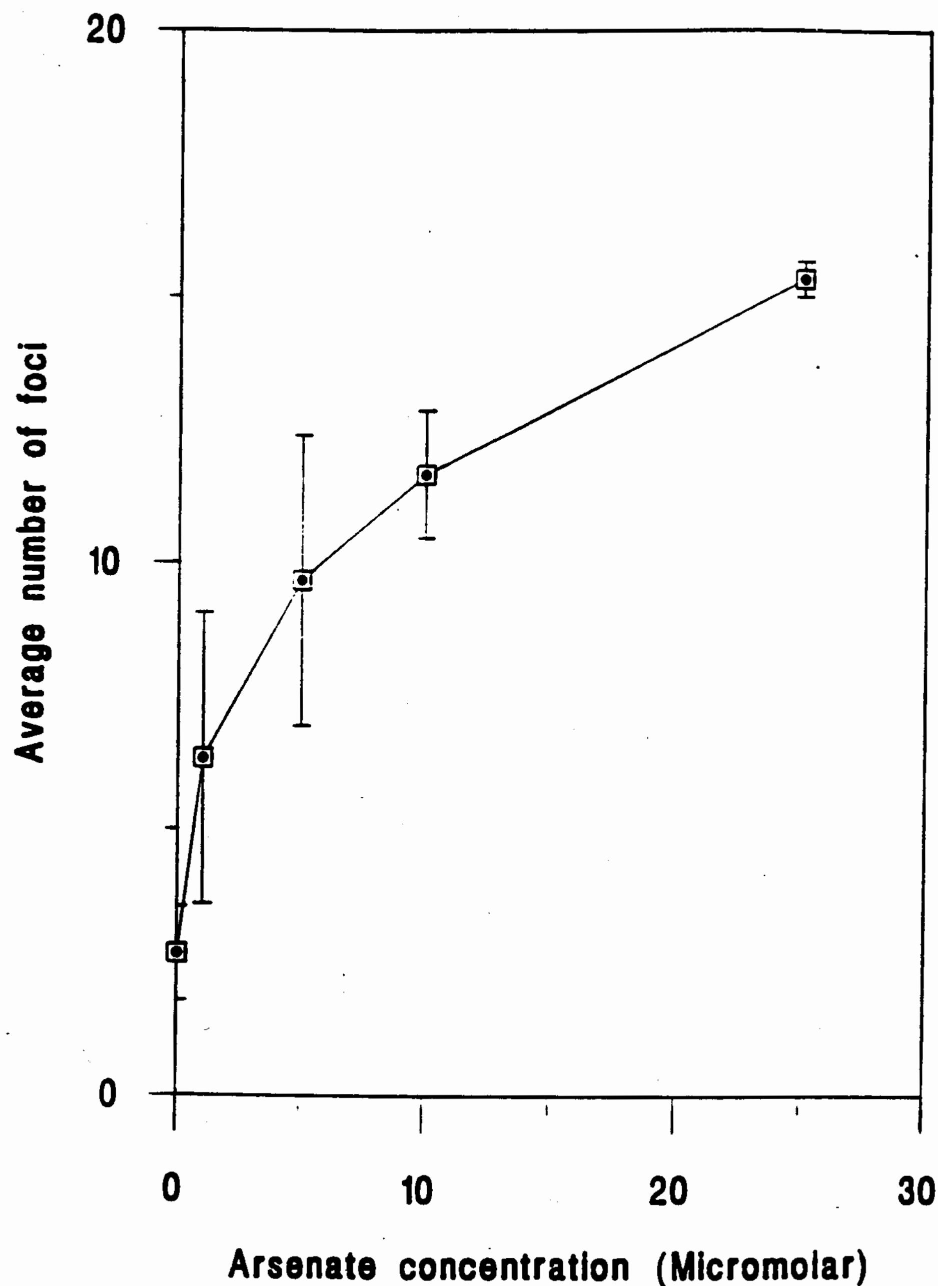


Figure 6: Average number of foci as a function of arsenate concentration

Cells at passage 17 were seeded at a density of 2.2×10^5 cells /100 mm plate and transfected with 1 µg of BPV DNA /plate. They were subcultured 21 hours later at 1:17, then exposed continuously to arsenate for 21 days. Cells transfected with BPV DNA but not exposed to arsenate developed an average of 2.7 foci/ 60 mm plate. Cells exposed continuously to 0.5 ng/ml of mezerein developed an average of 91 foci/60 mm plate. Values were determined in triplicate. Error bars are standard errors of the mean. $p < 0.001$ by analysis of variance.

TABLE 2

Number of colonies formed by 200 BPV DNA-transfected C3H/10T½ cells exposed to arsenic for 8 days

$NaAs(III)O_2$		$Na_2HAs(V)O_4$	
μM conc.	colonies	μM	colonies
0	55±2	0	55±2
0.1 μM	52±10	5 μM	58±10
1 μM	55±2	10 μM	55±3
5 μM	42±4	25 μM	45±3
10 μM	5±1	50 μM	5±2

C3H/10T½ cells of passage 16 were transfected with BPV DNA. One day later, 200 cells were seeded into 60 mm dishes in normal medium without arsenic. 24 hours after seeding 200 cells, various concentrations of arsenate and arsenite were added to the medium. Cultures were incubated for a further 8 days, with 2 changes of medium, continuously exposed to arsenite and arsenate. Cultures were then stained, dried, and colonies containing more than 25 cells counted. The experiment was done twice, each time in triplicate. Values reported are averages of 4-6 plates with standard errors of the mean. Toxicity for arsenite is significant above 5 μM, $p < 0.05$ and for arsenate above 25 μM, $p < 0.01$, determined by t-tests.

$Cr(III)K(SO_4)$ produced a 1.4-fold increase in transformed foci at 20-200 μM. (table 3). Percent colony forming efficiency was not altered by concentrations of Cr(III) compounds up to 250 μM (table 3) ($p > 0.01$). $K_2Cr(VI)O_4$ up to 2 μM did not enhance focus formation (table 3) ($p < 0.05$). Percent colony forming efficiency was significantly decreased at 0.1 μM and fell abruptly to 0% at 2 μM (table 3) ($p < 0.05$)

TABLE 3

Numbers of foci formed by BPV DNA-transfected C3H/10T½ cells exposed for 21 days to various concentrations of chromium compounds

$Cr(III)K(SO_4)$		$K_2Cr(VI)O_4$	
μM conc.	foci	μM conc.	foci
0	13±1	0	13±1
20 μM	16±0.3	0.5 μM	11±2
100 μM	19±0.3	1 μM	9±1
200 μM	19±1	2 μM	3±1

C3H/10T½ cells of passage 19 were transfected with BPV DNA. One day later, the cells were subcultured at a dilution of 1:17 and chromium compounds were added. Cultures were incubated for 21 days continuously exposed to chromium or mezerein with twice-weekly changes of medium. Positive controls of transfected cells exposed to 0.5 ng/ml of mezerein developed an average of 52 foci per 60 mm dish. The experiment was done three times, each time in triplicate with similar results. Values reported are averages of 3 plates with standard deviations. $p < 0.05$, determined by t-tests for all values as compared with untreated controls except for 20 μM Cr(III) and 0.5 μM Cr (VI) for which $p > 0.05$..

TABLE 4

Number of colonies formed by 200 BPV DNA-transfected C3H/10T½ cells exposed to chromium for 8 days

$Cr(III)K(SO_4)$		$K_2Cr(VI)O_4$	
μM conc.	colonies	μM	colonies
0	55±2	0	55±2
50 μM	56±2	0.1 μM	42±5
100 μM	54±5	0.5 μM	40±10
200 μM	52±10	1 μM	16±2
250 μM	53±7	2 μM	0

C3H/10T½ cells of passage 16 were transfected with BPV DNA. One day later, 200 cells were seeded into 60 mm dishes in normal medium without chromium. 24 hours after seeding 200 cells, various concentrations of chromium compounds were added to the medium. Cultures were incubated for a further 8 days, with 2 changes of medium, continuously exposed to chromium compounds. Cultures were then stained, dried, and colonies containing more than 25 cells counted. The experiment was done twice, each time in triplicate. Values reported are averages of 4-6 plates with standard errors of the mean. Toxicity of $Cr(III)K(SO_4)$ was not significant ($p > 0.05$) for 250 μM and toxicity of $K_2Cr(VI)O_4$ was significant at 0.1 μM ($p < 0.05$), both determined by t-tests.

DISCUSSION

Efficient neoplastic transformation of C3H/10T½ cells requires both vanadate and transfection with BPV DNA. If BPV DNA transfected cells are exposed to vanadate as late as 96 hours after transfection, vanadate still enhances neoplastic transformation (figure 3). Extended exposure to vanadate (greater than 24 hours) is necessary to promote cell transformation optimally (figure 3). Three possible mechanisms for this promotion of transformation are: (1) intracellular generation of oxygen-derived active species, (2) activation or amplification of the viral genome, and (3) kinase activation, phosphatase inhibition, or esterification of tyrosine by vanadate.

Compounds which generate free radicals or oxygen-derived active species promote neoplastic transformation (57, 58). Catalase and superoxide dismutase did not block the enhancement of foci by vanadate (Table 1). Catalase and superoxide dismutase added extracellularly have only limited access to intracellular H_2O_2 and $O_2^{\cdot-}$. Their failure to inhibit the effects of vanadate does not eliminate the possibility that vanadate may generate active oxygen species intracellularly.

Vanadate is a phosphatase inhibitor. It enhances transformation of BPV DNA-transfected C3H/10T½ cells similarly to cells transformed by c-*fes/fps* viruses (27) that vector tyrosine kinase genes. Enhancement of transformation in c-*fps/fes* virus-transfected cells is attributed to the inhibition of phosphatases by vanadate (27). Although BPV DNA is not known to encode a kinase gene, the E5 protein increases autophosphorylation on tyrosine of the PDGF receptor and its activity as a tyrosine kinases in C127 and NIH 3T3 cells (59). In an analogous mechanism, vanadate-mediated inhibition of the phosphatase which presumably dephosphorylates the PDGF receptor or other tyrosine kinases could result in enhanced transformation of BPV DNA-transfected C3H/10T½ cells.

Oxygen-derived active species or changes in phosphorylation of key proteins resulting from exposure to vanadate may activate transfected BPV DNA. Chromium generates active oxygen species, but does not enhance transformation to the same extent as vanadate. This suggests that the ability of vanadate to enhance transformation resides in its unique ability to alter phosphorylation.

Another explanation for the inability of chromium and arsenic to enhance transformation to the same extent as vanadate may lie in differences in permeability or uptake of the cations by the cells or differential binding of the metal cations by serum in the medium. These factors require further investigation.

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CHAPTER 3

ASCORBATE AND RETINOL SUPPRESS VANADIUM AND MEZEREIN-MEDIATED ENHANCEMENT OF TRANSFORMATION OF BOVINE PAPILLOMAVIRUS DNA-TRANSFECTED C3H/10T1/2 CELLS

ABSTRACT

Vanadate enhances transformation of BPV DNA-transfected C3H/10T½ cells (chapter 2). A next step was to ask whether anticarcinogenic agents can inhibit vanadium-mediated enhancement of transformation and, by comparison, whether anticarcinogenic agents can also inhibit mezerein-mediated enhancement of transformation. Vanadate generates active oxygen species. Ascorbate can scavenge active oxygen species and also inhibits chemically-induced transformation of C3H/10T½ cells. Therefore, we asked whether ascorbate could inhibit vanadate or mezerein-mediated enhancement of transformation. Retinol inhibits papillomavirus-mediated neoplastic transformation. For this reason, we investigated whether retinol could inhibit vanadate or mezerein-mediated enhancement of transformation in transfected cells. We also questioned the ability of retinol and ascorbate to inhibit formation of foci by transformed C3H/10T½ cells, carrying BPV DNA, subcloned from vanadium- or mezerein-mediated foci in transfected cells.

Supplementation of culture medium with vanadyl (V(IV)) or vanadate (V(V)) at 4 - 5 μM for 21 days increased numbers of multilayered foci 20- to 30-fold.

Ascorbate decreased the effect of vanadium by approximately 50% and the effect of mezerein by 36%. Retinol applied continuously with vanadate, vanadyl or mezerein for 21 days completely suppressed enhancement of BPV DNA-mediated transformation. Retinol applied after 11 days of treatment with vanadium or mezerein decreased vanadate- and vanadyl-enhanced transformation by 90% and mezerein-enhanced transformation by 66%. Retinol, but not ascorbate, significantly inhibited focus formation in co-cultures, of BPV DNA-carrying transformed cells by approximately 50%. Therefore, ascorbate inhibits BPV DNA-mediated transformation in transfected cells but not in the transformed cells which were tested. Suppression of the transformed phenotype (focus formation) in BPV DNA-transfected cells, rather than transformed cells, by ascorbate suggests that ascorbate inhibits transformation by scavenging active oxygen species. Retinol suppressed transformation of both transfected and transformed cells. These results suggest that alteration of viral gene expression is a possible mechanism for suppression of transformation by retinol.

INTRODUCTION

DNA tumor viruses such as human papillomavirus (HPV) account for approximately 15 % of human cancers (1). However, papillomaviruses alone are not sufficient to induce cancer. Other factors are required to advance benign lesions to malignancy (2). There is active interest in the mechanisms by which environmental factors or tumor promoters act synergistically with tumor viruses to enhance neoplastic cell transformation.

The current studies are a continuation of on-going research which has demonstrated that tumor promoters increase the numbers of multilayered transformed foci in bovine papillomavirus (BPV) DNA-transfected C3H/10T½ mouse embryo fibroblasts (3). This system provides a model in which to investigate the interaction between a DNA tumor virus and tumor promoters or other environmental risk factors.

In addition to phorbol esters, okadaic acid and teleocidin, vanadate also enhances transformation of BPV DNA-transfected C3H/10T½ cells (4). Vanadium is widely used as an industrial metal and occurs as a ubiquitous environmental pollutant. Steel workers are exposed occupationally to vanadium. It is a contaminant of motor vehicle exhaust and crude oil fires. Vanadium compounds

are genotoxic. Vanadium oxide (V_2O_3), vanadyl sulfate ($VOSO_4$) and ammonium metavanadate (NH_4VO_3) induce sister chromatid exchanges in mammalian cells (5). In bacterial assays, NH_4VO_3, $VOCl_2$ and V_2O_5 are mutagenic (6). Vanadium is a suspected human carcinogen (7).

Vanadate is reduced to vanadyl intracellularly (8). Vanadyl does not transform BALB/3T3 cells and is much less toxic than vanadate (9), but in Chinese hamster V79 cells it is more toxic than vanadate (10). Vanadyl induces aneuploidy in *Saccharomyces cerevesiae* (9). We therefore investigated whether vanadyl would also enhance transformation in BPV DNA-transfected C3H/10T½ cells.

Since vanadium can enhance BPV-mediated transformation, a related question is whether anticancer agents or antioxidants can suppress vanadium-promoted transformation of BPV-DNA-transfected C3H/10T½ cells. Vanadium is a source of active oxygen intermediates (11-13) that function to enhance neoplastic transformation. Ascorbate, on the other hand, can remove active oxygen intermediates. Ascorbate prevents initiation of lipid peroxidation in plasma (14). It scavenges •OH (15), $O_2^{\cdot-}$ (16) and singlet oxygen (17). Therefore, we sought to determine whether ascorbate would

suppress the transformation-enhancing action of vanadium in BPV DNA-transfected C3H/10T½ cells.

Retinol is another possible compound which may suppress vanadium-mediated transformation. Retinoids inhibit transformation and neoplastic processes mediated by chemicals, viruses and viral DNA (18). Retinol inhibits development of Shope papillomavirus-induced tumors (19). Retinoic acid suppresses growth of HPV 16-immortalized human keratinocytes (20) and decreases BPV copy numbers in murine C127 cells (21).

Retinol inhibits mezerein-promoted transformation of BPV DNA-transfected C3H/10T½ cells (22) and suppresses tumor promoter-mediated increases in ornithine decarboxylase activity (23). Conversely, vanadate enhances ornithine decarboxylase activity (24). The anti-viral and anti-carcinogenic actions of retinol suggest that it can suppress vanadium-mediated transformation of BPV DNA-transfected C3H/10T½ cells.

METHOD

Cell culture and transfection

C3H/10T½ fibroblasts from the American Type Culture Collection were grown in Dulbecco's modified medium (Gibco) supplemented with 10% fetal calf serum (Gibco) as previously described (3). Medium also contained 20 mM HEPES [N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid] (Sigma), 60 μg/ml. of penicillin G (Sigma) and 100 μg/ml. of streptomycin sulfate (Sigma). Cells of passages 15-20 were used for transfection. Medium was changed twice weekly.

We transfected C3H/10T½ cells with the pdBPV-1 (142-6) plasmid which contains the complete BPV-1 genome cloned into the plasmid pML2d at the Bam HI site. Cells were transfected by the calcium phosphate precipitation method followed by glycerol shock. Sheared calf thymus DNA was used as the carrier DNA (25). Controls were transfected with only sheared calf thymus DNA (Sigma).

For each experiment, 16-24 hours after transfection, transfected cells on 100 mm plates were subcultured at a ratio of 1:17. The day of subculturing is referred to as day 1. Unless otherwise stated in figure legends, vanadium, mezerein, retinol and ascorbate were added on

day 1 and cells were continuously exposed to the compounds. Plates were then incubated for a further 20 days with changes of medium twice weekly. Ascorbate or retinol was always added before vanadium or mezerein. Foci were stained with 0.025% methylene blue, air dried, and counted by the method described previously (3).

Preparation and addition of compounds

Retinol (Sigma) was dissolved and diluted in dimethylsulfoxide to provide a stock solution of 40 mM, determined spectrophotometrically using a reference absorbance of 1835 for a 1% solution in methanol/1 cm at 325 nm. Stocks were maintained at -70° in the dark. L-ascorbic acid (sodium salt) (Sigma) was dissolved in medium to yield a stock solution of 100 mM, freshly prepared for each medium change. Mezerein (Sigma) was diluted in DMSO to a stock of 0.2 mg/ml, then diluted in medium to the desired concentrations. A 10 mM stock solution of vanadate as Na_3VO_4 (Fisher) was prepared in glass distilled water weekly and kept frozen. A 10 mM stock solution of vanadyl as $VOSO_4 \cdot 2H_2O$ (BDH) was prepared in glass distilled water fresh for each change of medium. Stock solutions were diluted with medium to the desired concentrations.

Cloning of transformed cell lines

Approximately 21 hours after transfection, cells were subcultured to a ratio of 1:17 to 60 mm dishes (day 1). They were incubated as above with the addition of 2 μM vanadate until foci were clearly visible (21 days).

At that time, medium was removed, cultures rinsed twice with 0.05% trypsin containing 0.02% EDTA, and the centres of individual foci, well-isolated from other foci, were extirpated into about 10 μl of 0.05% trypsin containing 0.02% EDTA in 25 mm dishes using an Eppendorf Pipettman (figure 1). Foci were vigorously agitated for 5 minutes to separate cells, then medium added. Ten foci were isolated in this way into medium containing 1 μM vanadate and 10 foci were cloned into medium without vanadate. Clones derived from vanadate foci and cultured in medium containing vanadate were identified as V51, V52, V53...etc. those without vanadate as NV51, NV52... etc..

Cells obtained from trypsinized foci on 25 mm plates were incubated until they reached confluence (about 3 days), then transferred to 60 mm plates and grown to confluence (about 7 days). Cells from each clone were harvested and frozen in medium containing 10% DMSO, grown

on 100 mm plates for DNA extraction and co-cultured with C3H/10T½ cells.

For co-cultures, 2000 untransfected C3H/10T½ cells were coincubated with 200 cells from the clones. For each clone, 2 dishes were incubated in normal medium and 2 other dishes in medium with vanadate. Medium was changed twice weekly. After 21 days of incubation the cultures were stained with 0.025% methylene blue, dried, and the foci were counted.

Subcloning of cell lines

From among the vanadate-derived clones, 4 clones were selected for subcloning: V52, a line maintained in vanadate, and NV51, NV56 and NV57, lines maintained in normal medium. Cells from each clone were seeded on to 100 mm dishes and grown for approximately 10 days until colonies were about 2-3 mm in diameter. From each clone, 10 colonies that were well-separated from each other were extirpated into 25 mm dishes in medium with or without vanadate (according to the original clone). Subclones isolated from clone NV51 were identified as NV51.1, NV51.2, ...etc. Cells from the subclones were processed for DNA extraction and co-cultured as for the clones described above (figure 1).

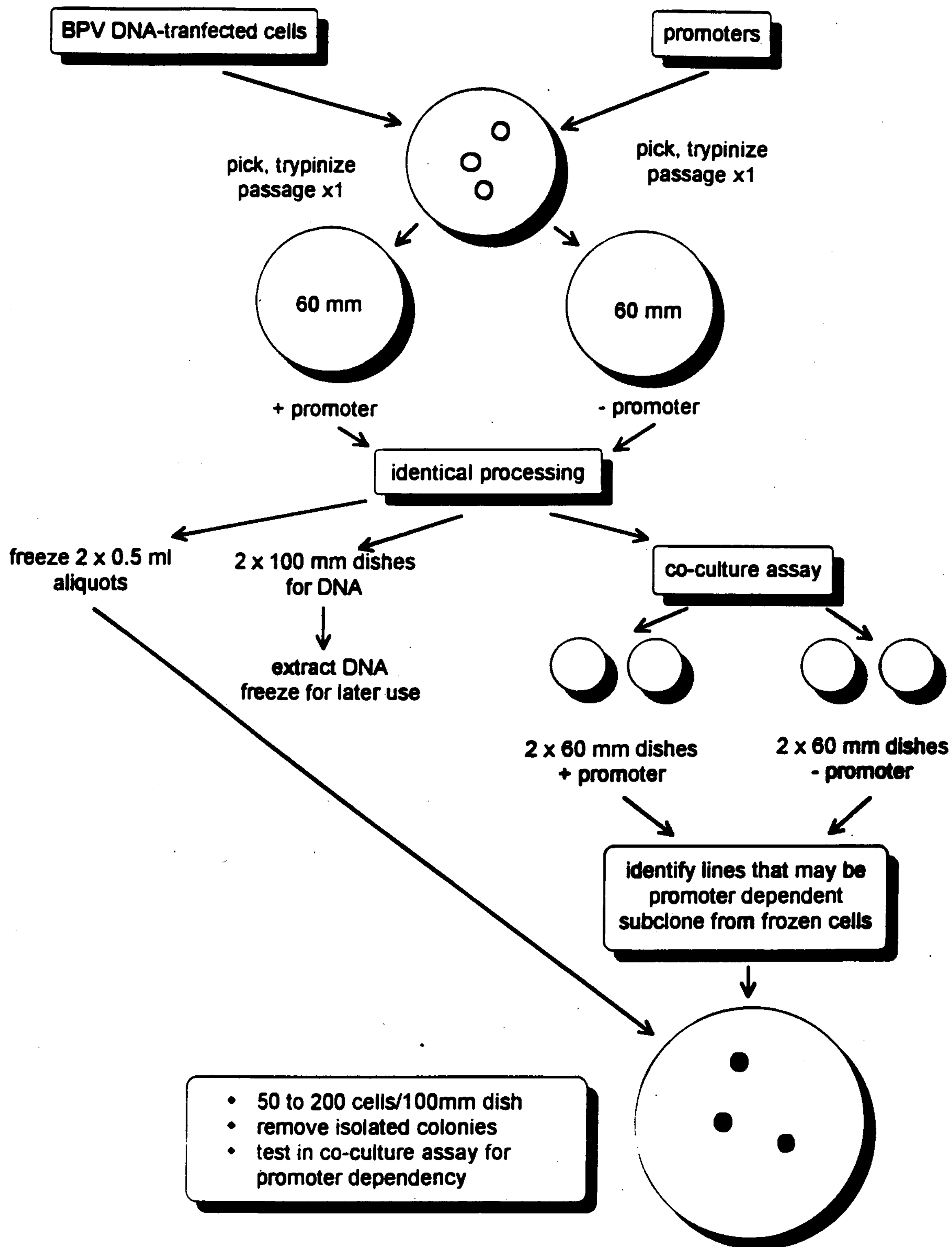


Figure 1: Protocol for isolating clones or subclones from foci in BPV DNA transfected C3H/10T½ cells.

Determination of subculture growth times

To passage subclones, cell suspensions were seeded in 25 cm^2 (50 ml) flasks and incubated, with twice weekly changes of medium, until cultures were approximately 75 % confluent. At this time, defined for these experiments as "passage 1", cells were trypsinized, resuspended in 4 ml of medium, counted, and subcultured as follows: For isolation of DNA, cells were seeded at a density of 2.5 x 10^5 cells/plate in two 100 mm plates and incubated to about 90% confluence for approximately 4 days. At that time, cells were harvested into PBS solution, pelleted by centrifugation, and frozen at -20° for later DNA extraction.

For passaging, cells from each cell line were seeded at a density of 10^5 cells/25 cm^2 flask. Cells from each line were incubated in one flask in medium without retinol, in a second flask with 1.74 μM retinol, in a third flask with 250 μM ascorbate and in a fourth flask with 1 μM vanadate. V52.7 cells were also passaged in medium containing 1.74 μM retinol and 1 μM vanadate.

Cells were passaged at low density, about 75-90% confluence, approximately twice per week. Each passage was initially seeded with 10^5 cells in 25 cm^2 (50 ml) culture flasks. Subculture growth times were calculated

from the cell counts (in duplicate) after each passaging. After incubation, cells were harvested and counted. The number of culture doublings was calculated from this and the subculture doubling times calculated from the number of days which had elapsed.

DNA extraction

Total nucleic acids from cells collected at passages 1, 4, 7, and 10 for all subclones were extracted by a modification of the sodium dodecyl sulfate/proteinase K method (26). Nucleic acids were purified by phenol/chloroform extraction and precipitation in ethanol. RNA was removed by digestion with DNA nuclease-free pancreatic RNAase A. The DNA was subjected to another round of phenol/chloroform extraction and ethanol precipitation. DNA was dissolved in TE buffer and concentrations determined by reading optical density at 260 nm.

Determination of copy numbers

DNA samples of 2 μg were applied to nitrocellulose filters in a Minifold II slot blotting apparatus according to manufacturer's instructions (Schleicher and Schuell, Keene, Maine). ^{32}-P-pdBPV-1 DNA was hybridized to the slot blots in the manner described previously

(21). They were exposed to Kodak X-Omat AR5 film for several different exposures (4, 24, 48, 72 hours) with intensifying screens at -70°.

Copy numbers were quantified by scanning the films with a GS300 densitometer (Hoefer, San Francisco, CA) and integrating the peak areas with an SP4100 integrator (Spectra Physics, Santa Clara, CA.). All copy numbers for clones and subclones were determined at least two times on separate slot blots. Results of the scans were quantified by comparing them with scans of standards on each slot blot reconstructed using the assumed value of one gene copy of BPV DNA equivalent to 14 pg/10 μg of cellular DNA (27).

Co-culture assays

For co-cultures, 200 cells from each subcloned line were seeded with 2000 untransfected C3H/10T½ cells of passages 15-18, in 60 mm dishes, in triplicate. At the same time, plates containing 200 C3H/10T½ cells or 200 cells from cloned lines were set up to monitor numbers of cells seeded. Co-cultures were continuously exposed to test substances for 10-14 days until foci were visible. They were then stained, dried and foci counted. Plates for monitoring numbers of cells were incubated for 7-8

days until colonies contained approximately 40 cells and could be counted. These plates were stained with 0.1% methylene blue, dried and colonies counted.

RESULTS

1. Vanadate and vanadyl enhance BPV DNA-mediated cell transformation

Vanadyl increased numbers of foci in a dose dependent manner to 11-fold at 1.64 μM and 28-fold at 4.92 μM ($p < 0.001$)(figure 2). In the same experiment, vanadate enhanced transformation 20-fold at 2 μM ($p < 0.001$) (figure 2). In the absence of vanadyl or vanadate, an average of 3 foci per plate developed in BPV DNA-transfected cells. BPV DNA is essential to vanadium-mediated enhancement of transformation, since controls transfected with only calf thymus DNA do not develop foci when exposed to vanadium.

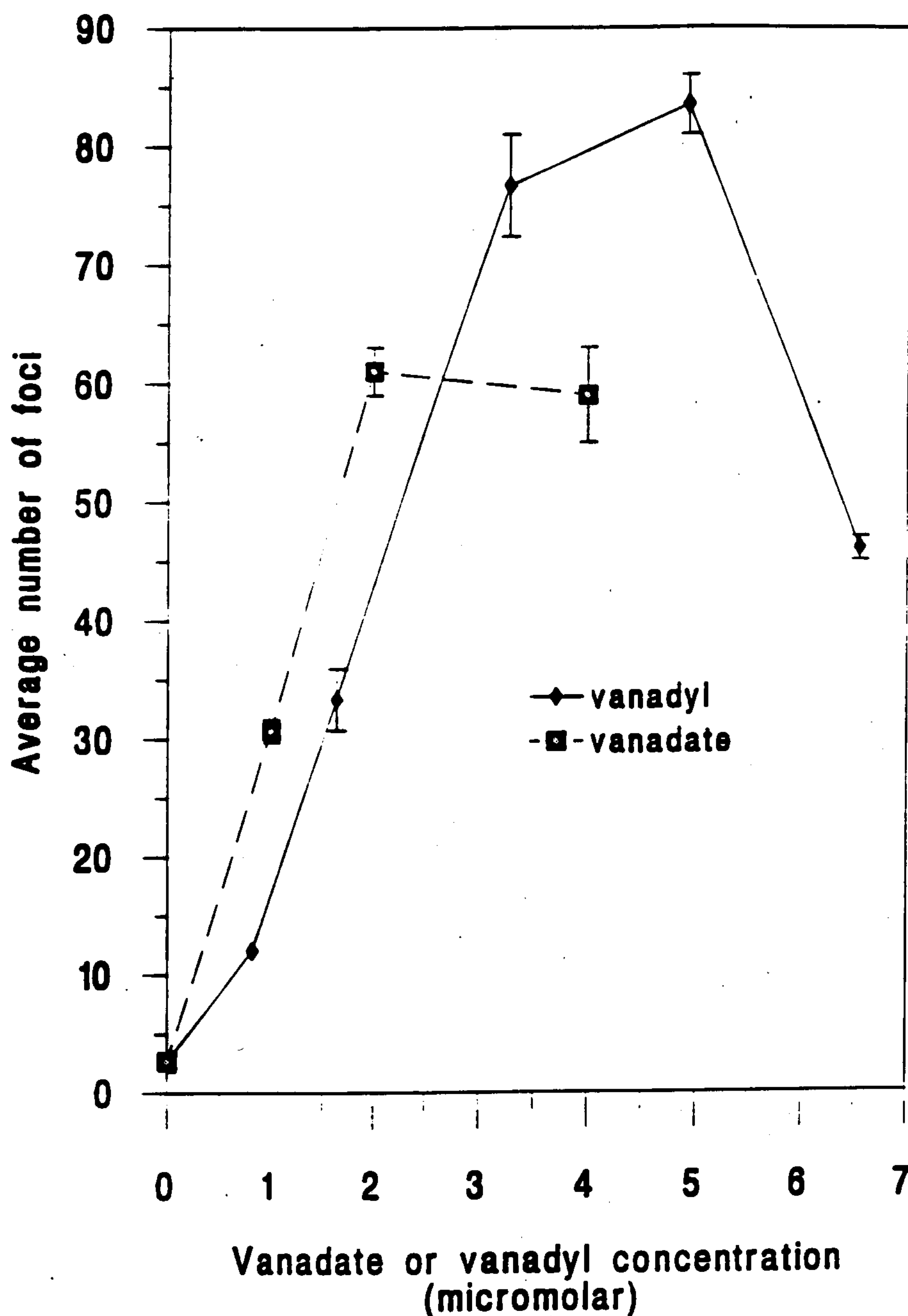


Figure 2: Average number of foci as a function of vanadate or vanadyl concentration

Cells at passage 17 were seeded at 2.2×10^5 cells and transfected with 1 µg of BPV DNA/plate. They were subcultured 21 hours later at 1:17, then exposed continuously to vanadate or vanadyl for 21 days. Cells transfected with BPV DNA but not exposed to vanadium developed an average of 2.67 foci/plate. Cells exposed continuously to 0.5 ng/ml of mezerein developed an average of 91 foci/plate. All data points were determined with triplicate plates. The experiment was repeated three times with similar results. Error bars are standard errors of the mean. For both vanadyl and vanadate values, $p < 0.001$ by analysis of variance.

As reported previously, cells treated with vanadate at concentrations greater than 6 μM reversibly lost adherence to the plates whether transfected or not (4). Detached cells collected from the medium formed colonies if replated without vanadate (4). Vanadate also induced reversible nonadherence or changes in morphology in rat hepatocytes (28), baby hamster kidney cells (29) and normal rat kidney cells (30). In contrast to the effect of vanadate, we observed no loss of adherence in cells exposed to vanadyl in concentrations up to 7 μM. Above 7 μM, no cells were visible either floating in the medium or attached to the plates. If medium was removed, and cells replated in fresh medium in the absence of vanadyl, no colonies formed. Therefore, the most likely conclusion is that very few cells survived above 7 μM. In keeping with our results, there are no reports of vanadyl-induced morphological changes or changes in cell adhesion.

Detachment or impaired adhesion of cells exposed to vanadium opens the possibility that the increase in numbers of foci in vanadium-treated cultures is due to detachment and reattachment of cells. This process could result in many "satellite foci" from an initial very small number of transformed cells. To test this hypothesis, cells were transfected with BPV DNA, exposed to a range of concentrations of vanadate and vanadyl from

0 to 8 μM and grown for 21 days. Cells were collected from the medium on day 7 according to the protocol given in the legend to table 1. Unattached cells would be expected to form colonies.

There was no significant difference in numbers of colonies formed, between the untreated control plates and plates exposed to V(IV) up to 6.55 μM ($p > 0.05$) (Table 1). Above 6.55 μM vanadyl, very few cells were seen on the original plates and no colonies formed when the medium was centrifuged and plated. There was no significant difference in numbers of colonies formed, between untreated control plates and plates exposed to vanadate up to 4 μM ($p > 0.01$) (table 1). Above 6 μM, there was a sharp increase in numbers of colonies formed. Therefore, cells do detach, but there is no significant increase in detachment of cells exposed to 1-4 μM vanadate ($p > 0.01$) or vanadyl ($p > 0.05$) as compared with untreated control cultures. The maximum concentrations used in the ascorbate and retinol experiments were 2 μM vanadate and 3.28 μM vanadyl. These are below the concentrations which caused increased cell detachment.

TABLE 1

Colonies formed by cells in medium removed from transfected cultures exposed to V(V) or V(IV) for 7 days

average/60 mm dish			average/60 mm dish		
V(V) μM	colonies	foci	V(IV) μM	colonies	foci
0	7	3	0	7	3
2	10	62	1.64	10	33
4	10	58	3.28	1	77
6	18	-	4.92	9	83
8	confluent	-	6.55	9	46
			7.65	0	0

C3H/10T½ cells of passage 17 were transfected with 1 μg of BPV DNA on 100 mm plates. Cells were subcultured at a ratio of 1:17 24 hours later into 60 mm plates. One set of plates were treated with V(IV) and one set with V(V). At the end of 7 days, when cultures were nearly confluent, after one medium change at day 4, medium from three plates from each concentration of V(V) or V(IV) was collected. Medium from the three plates (15 ml) was combined and centrifuged at 500 rpm for 5 minutes to pellet cells. Pellets were resuspended in 5 ml of medium without vanadium and plated in 60 mm dishes. Cultures were incubated for 8 days with one change of medium and colonies were stained and counted. Cultures from which medium was collected at day 7 continued to be incubated in V(V) or V(IV) for 21 days after which they were stained and foci counted. Using t-tests (on numbers of colonies formed at each concentration), $p > 0.01$ for vanadate from 0-4 μM and $p > 0.05$ for vanadyl from 0-6.55 μM.

Vanadate at 2 μM or vanadyl at 3.28 μM did not significantly alter colony forming efficiency of transfected cells (table 2) ($p > 0.05$). We confined our studies to these concentrations.

TABLE 2

Colony forming ability of BPV DNA-transfected C3H/10T½ cells exposed to combinations of promoters and suppressors

Promoter	No suppressor	Ascorbate, μM 100	Ascorbate, μM 500	Retinol, μM 1.74
No promoter	100	88.2±0.5	86.6±0.2	123.4±19.0
2 μM V(V)	107.6±7.1	96.6±0.1	112.0±11.4	118.4±0.1
3.28 μM V(IV)	109.3±10.0	89.1±0.1	86.3±3.9	103.9±8.7

Cells at passages 18-21 were transfected with BPV DNA and subcultured at 1:17 into 60 mm plates (approximately 2×10^4 cells/60 mm dish). V(IV), V(V), retinol and ascorbate were added at this time. Cells were incubated for 8 days with 2 changes of medium, at which time they were confluent. All cultures were then subcultured to approximately 200 cells/60 mm dish in triplicate in normal medium containing no vanadate, vanadyl, ascorbate or retinol. Cultures were incubated for 8 days with 2 changes of medium. They were fixed, stained with 0.1% methylene blue and colonies counted. This experiment was done two separate times in triplicate. For each experiment, raw % colony forming ability was calculated as follows: (number of colonies counted/number of cells plated) x 100%. For each experiment, the % colony forming abilities were then calculated as: (raw % colony forming ability/ raw % colony forming ability of untreated controls) x 100%. Results show means and standard deviations for two experiments. Decrease in colony forming ability in cells exposed only to ascorbate was significant at $p < 0.001$ by analysis of variance. All other values were not significant ($p > 0.05$).

2. Ascorbate suppresses transformation by vanadyl, vanadate or mezerein

Ascorbate at 100 μM decreased enhancement of BPV DNA-mediated cell transformation by vanadium by 40-60 % ($p < 0.001$) (tables 3 and 4, figures 3 and 4). Foci that developed were smaller and stained lighter than those formed in the absence of ascorbate (Figure 4). In contrast, an ascorbate concentration of 500 μM was required to suppress enhancement of transformation by mezerein ($p < 0.001$) (figure 5) by 40%. Ascorbate also decreased numbers of foci which developed in cultures transfected with BPV DNA but not exposed to mezerein or vanadium ($p < 0.001$) (figures 3 and 5). It is unclear whether ascorbate decreases transformation in transfected cells not exposed to promoter to the same extent that it decreases promoter-mediated transformation.

TABLE 3

Numbers of foci formed by transfected cells exposed to promoters and to ascorbate

Promoter	Ascorbate (μM)		
Concentration	0	100	500
2 μM V(V)	70±2	27±4	-
1.64 μM V(IV)	32±3	18±2	-
Mezerein[a]	140±10	137±2	89±4

[a] Mezerein applied at 0.5 ng/ml

C3H/10T½ cells were transfected and subdivided as described in methods. Values for untreated controls for each experiment are given in the legends to figures 3 and 5. Values are means of triplicate plates and errors are standard errors of the mean. The experiment was repeated 4 times with similar results. For vanadate, vanadyl and mezerein treatments, $p < 0.001$ by analysis of variance.

TABLE 4

Percent suppression of numbers of foci in transfected cells by ascorbate and retinol

Promoter	Ascorbate (μM)		Continuous retinol (μM)			Promoter, then retinol		
Concentration	100	500	.17	.58	1.74	.17	.58	1.74
No promoter	0	52	39	100	100	-	-	-
2 μM V(V)	62	-	36	56	92	39	57	93
1.64 μM V(IV)	44	-	-	-	-	-	-	-
3.28 μM V(IV)	-	-	49	97	100	22	38	84
Mezerein[a]	0	36	32	88	98	22	30	69

[a] Mezerein applied at 0.5 ng/ml
Percent suppression was calculated from the original data shown in tables 3 and 5 as: 100% -[(Mean number of foci in promoter+ascorbate or retinol treated cultures divided by mean number of foci in cultures treated only with vanadium or mezerein) x 100%]. Cultures were exposed to ascorbate continuously during the experiment. Standard errors are the same as shown in tables 3 and 5 which contain the original data.

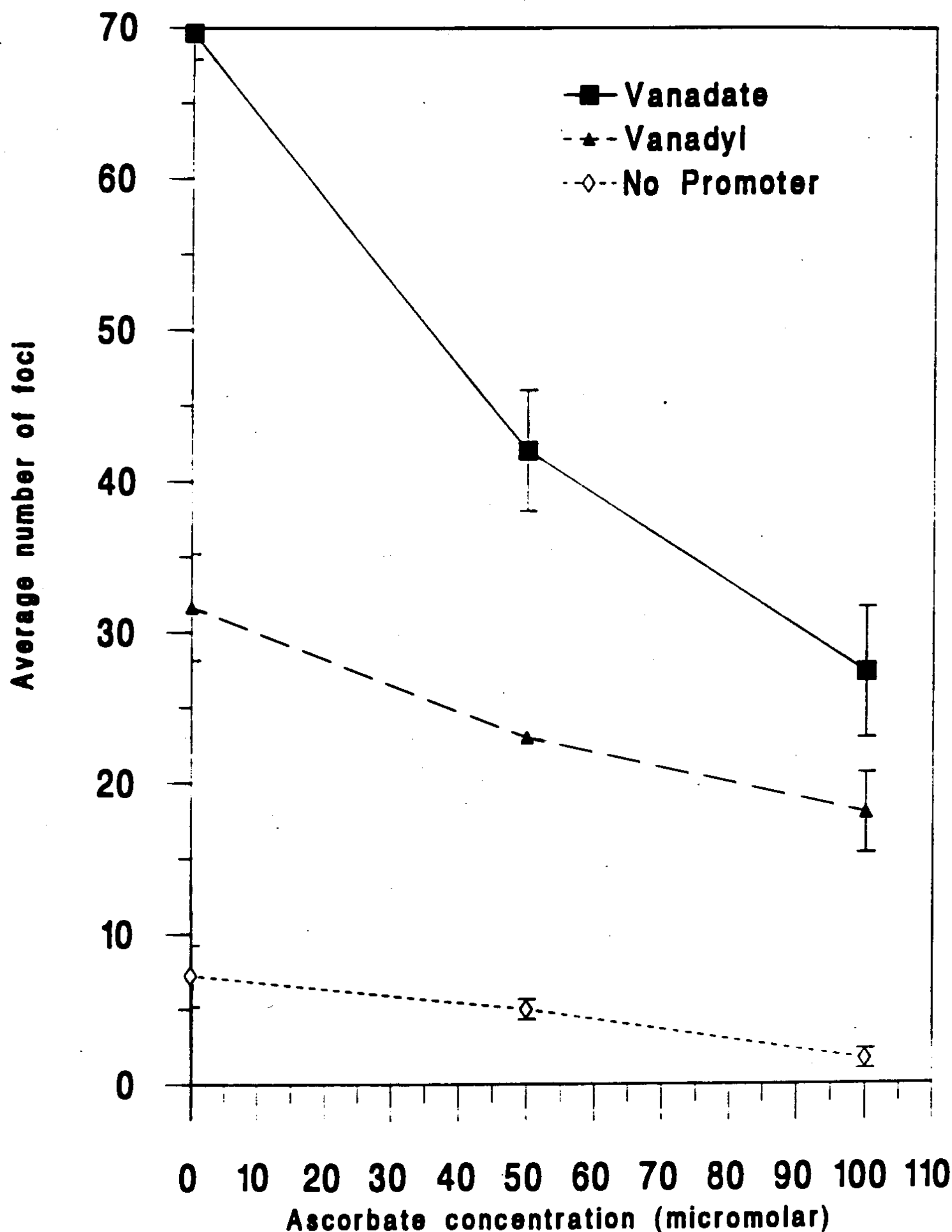
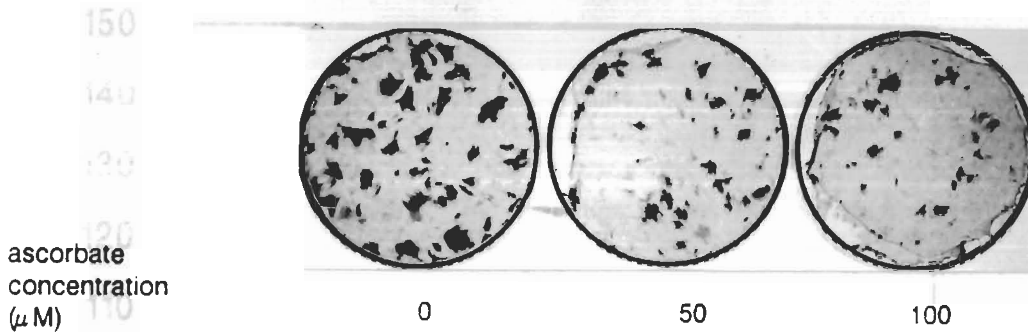
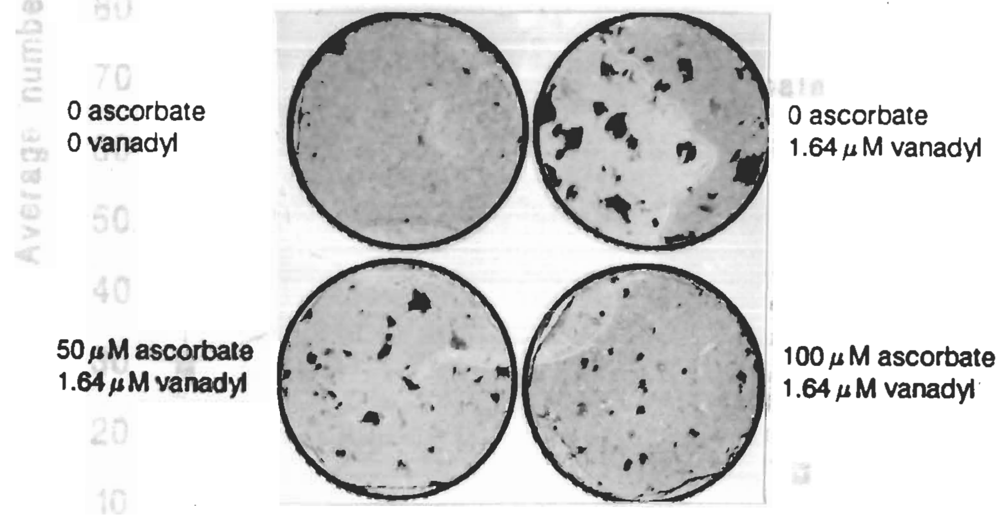


Figure 3: Inhibition of vanadium-mediated transformation by ascorbate

Cells at passage 17 were seeded at 2.2×10^5 cells/plate and transfected with 1 µg of BPV DNA/plate. They were subcultured 21 hours later at 1:17 (day 1), then exposed continuously to 50 µM or 100 µM ascorbate and 2 µM vanadate or 1.64 µM vanadyl for 21 days. Cells transfected with BPV DNA but not exposed to vanadium developed an average of 3 foci/plate. As a positive control for transfection, transfected cells exposed continuously to 0.5 ng/ml of mezerein developed on average 91 foci/plate. All data points were determined with triplicate plates. The experiment was repeated once with similar results. Error bars are standard errors of the mean. For both vanadate and vanadyl values, $p<0.001$ by analysis of variance.



BPV DNA-transfected cells exposed continuously for 21 days to 2 μM vanadate and to various concentrations of ascorbate



BPV DNA-transfected cells exposed continuously for 21 days to 1.64 μM vanadyl and to various concentrations of ascorbate

Figure 4: Suppression of vanadate or vanadyl-induced foci by continuously-applied ascorbate

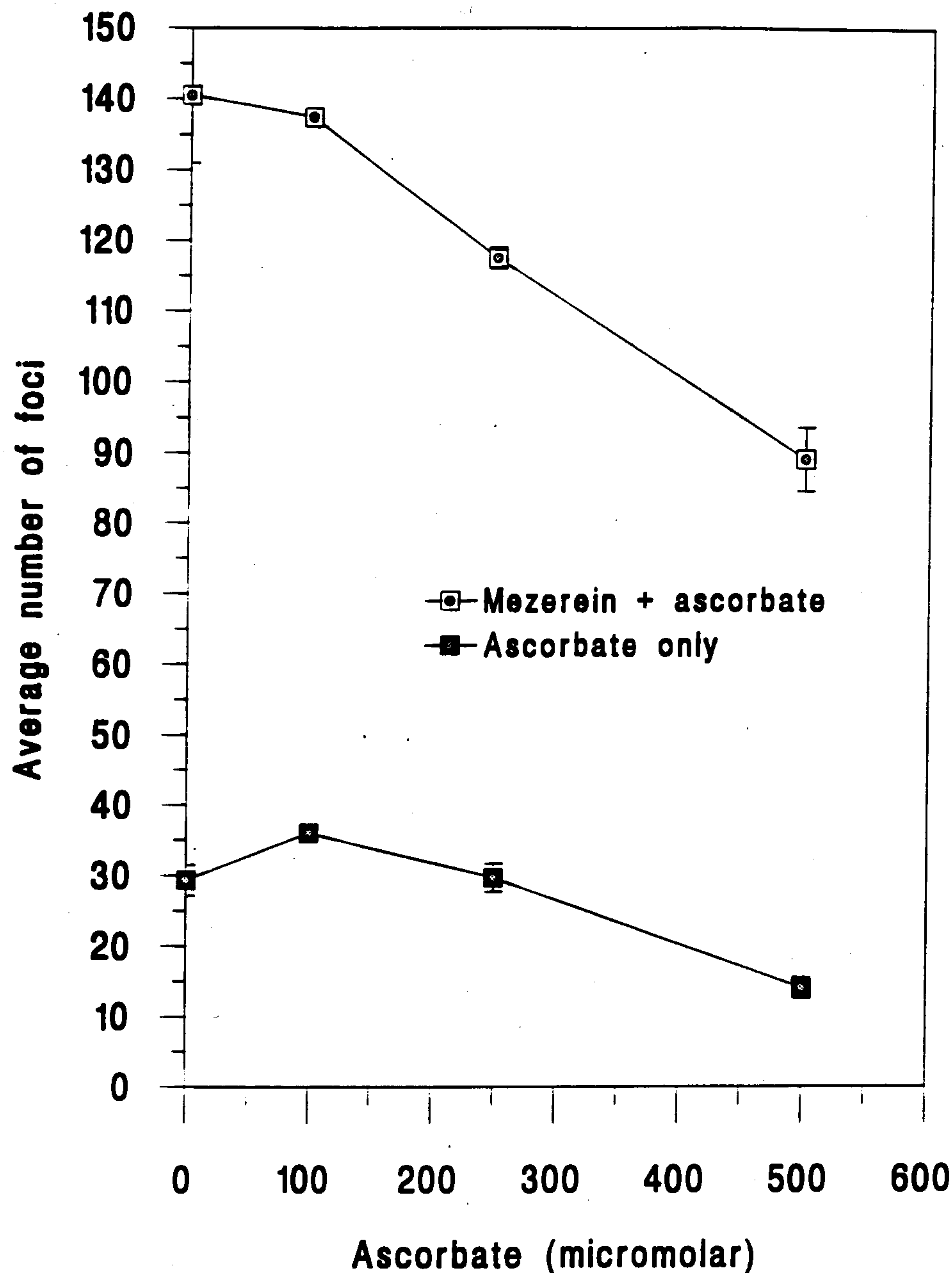


Figure 5: Suppression of mezerein-mediated transformation by ascorbate

Cells at passage 18 were seeded at 2×10^5 cells/plate and transfected with 1 μg of BPV DNA/plate. They were subcultured 21 hours later at 1:17 (day 1), then exposed continuously to ascorbate and 0.5 ng/ml of mezerein for 21 days. Cells transfected with BPV DNA but not exposed to mezerein developed an average of 29.3 foci/plate. All data points were determined with triplicate plates. The experiment was repeated once with similar results. Error bars are standard errors of the mean. For both ascorbate plus mezerein and ascorbate without mezerein values, $p<0.001$ by analysis of variance.

Vanadium-induced changes in cell adhesion are most likely not responsible for the vanadium-promoted increases in numbers of transformed foci. Therefore, effects of ascorbate or retinol on cell adhesion would not account for a decrease in transformed foci.

Ascorbate could appear to suppress neoplastic transformation if it were toxic to cells. Ascorbate alone at a concentration of 100 or 500 μM decreased colony forming efficiency by about 15% ($p < 0.001$) (table 2). Ascorbate in combination with vanadate or vanadyl appeared to decrease colony forming efficiency by a maximum of 14% (table 2), but this was not statistically significant ($p > 0.05$). In contrast, 100 μM ascorbate suppressed the frequency of BPV DNA-mediated vanadate-mediated transformation by 62% in the presence of vanadate and by 44% in the presence of vanadyl (table 4). Clearly the antineoplastic action of ascorbate is by no means fully explicable on the basis of toxicity.

3. Retinol strongly suppresses transformation

Retinol at 1.74 μM applied continuously for 21 days strongly suppressed transformation in the presence of vanadate and vanadyl ($p < 0.001$) (Tables 4 and 5, figure

6). Transformation in the presence of mezerein was suppressed by 96%. Retinol applied continuously also decreased numbers of foci arising in cultures transfected with BPV DNA but not exposed to mezerein or vanadium.

If vanadate, vanadyl or mezerein was applied for only the first 11 days, followed by retinol from days 12 to 21, vanadium or mezerein-mediated enhancement of transformation was also effectively suppressed ($p < 0.001$) (figures 7 and 8, tables 4 and 5).

TABLE 5

Numbers of foci formed by transfected cells exposed to promoters and to retinol

	Suppressor							
Promoter	Continuous retinol (μM)				Promoter, then retinol			
Concentration	0	.17	.58	1.74	0	.17	.58	1.74
2 μM V(V)	25±1	16±4	11±2	2±0	28±2	17±1	12±0	2±0
3.28 μM V(IV)	37±1	19±2	1±1	0±1	37±2	29±3	23±3	6±1
Mezerein[a]	81±9	55±1	10±0	2±1	58±3	45±2	35±2	18±1

[a] Mezerein applied at 0.5 ng/ml

C3H/10T½ cells were transfected and subdivided as described in methods. Values for untreated controls for each experiment are given in the legends to figures 6 and 8. Values are means of triplicate plates and errors are standard errors of the mean. The experiment was repeated 3 times with similar results. For all values, $p < 0.001$ by analysis of variance.

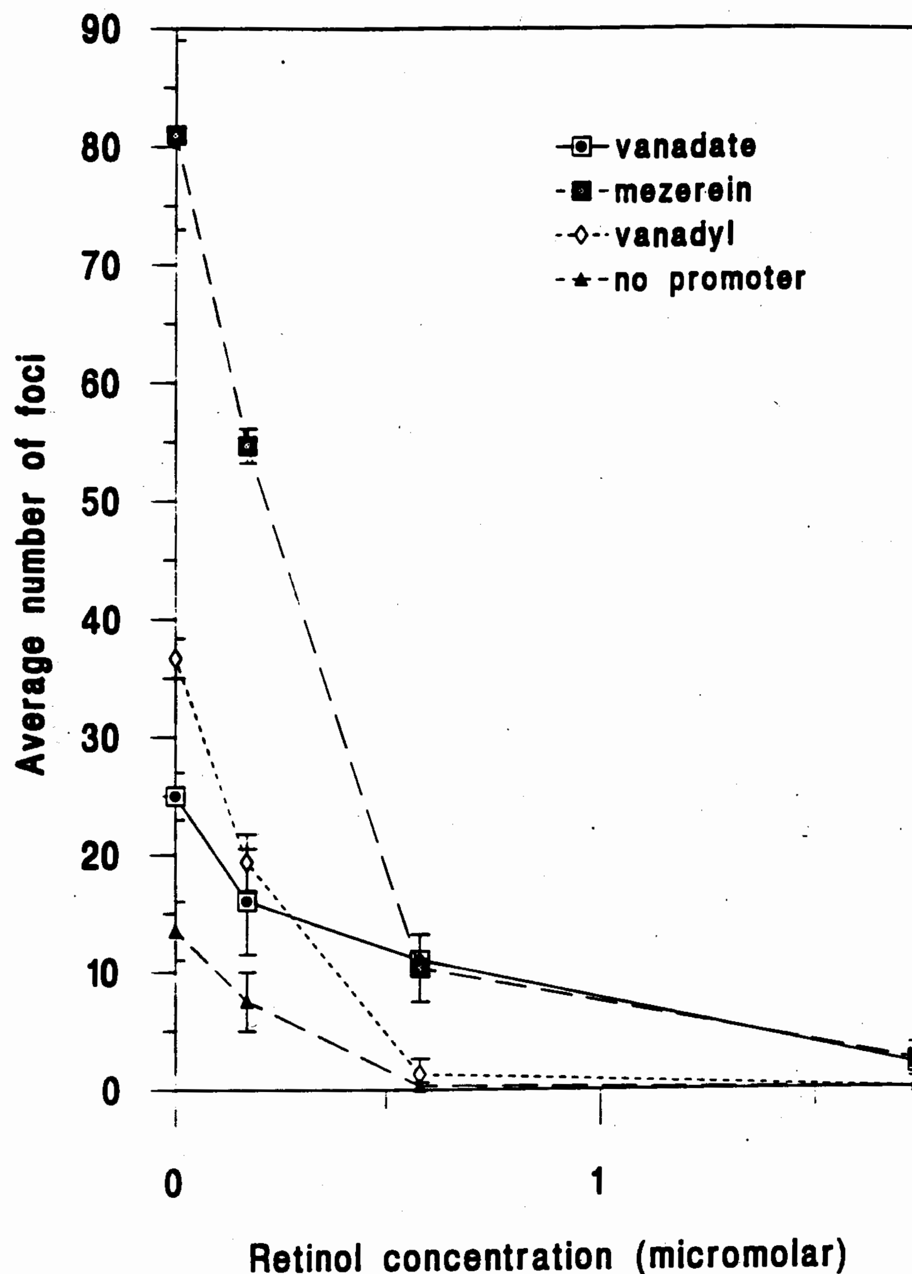


Figure 6: Inhibition of transformation by continuously-applied retinol

Cells at passage 19 were seeded at 3.3×10^5 cells/100mm plate and transfected with 1 µg/plate of BPV DNA. They were subcultured 20 hours later at 1:17 (day 1). At this time, retinol and 2 µM vanadate, 3.28 µM vanadyl or 0.5 ng/ml of mezerein were added. These were applied continuously for 21 days. Transfected cells exposed to medium without promoters or retinol developed an average of 13.5 foci/plate. All data points were determined with triplicate plates. Experiment was repeated twice with similar results. Error bars are standard errors of the mean. For vanadyl, vanadate, mezerein and no promoter values, $p < 0.001$ by analysis of variance.

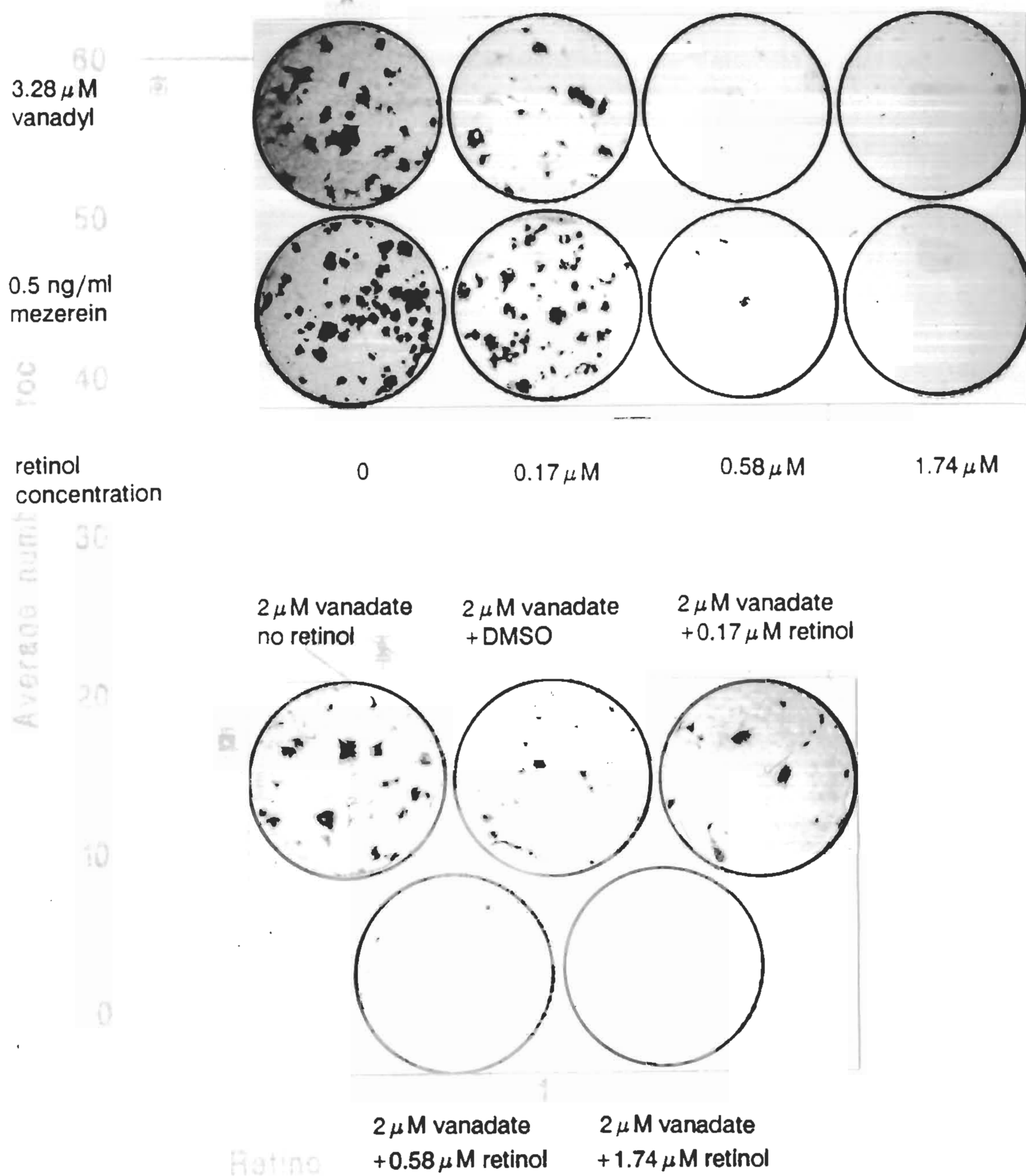


Figure 7: Inhibition of mezerein- or vanadium-mediated transformation by retinol applied 11 days later

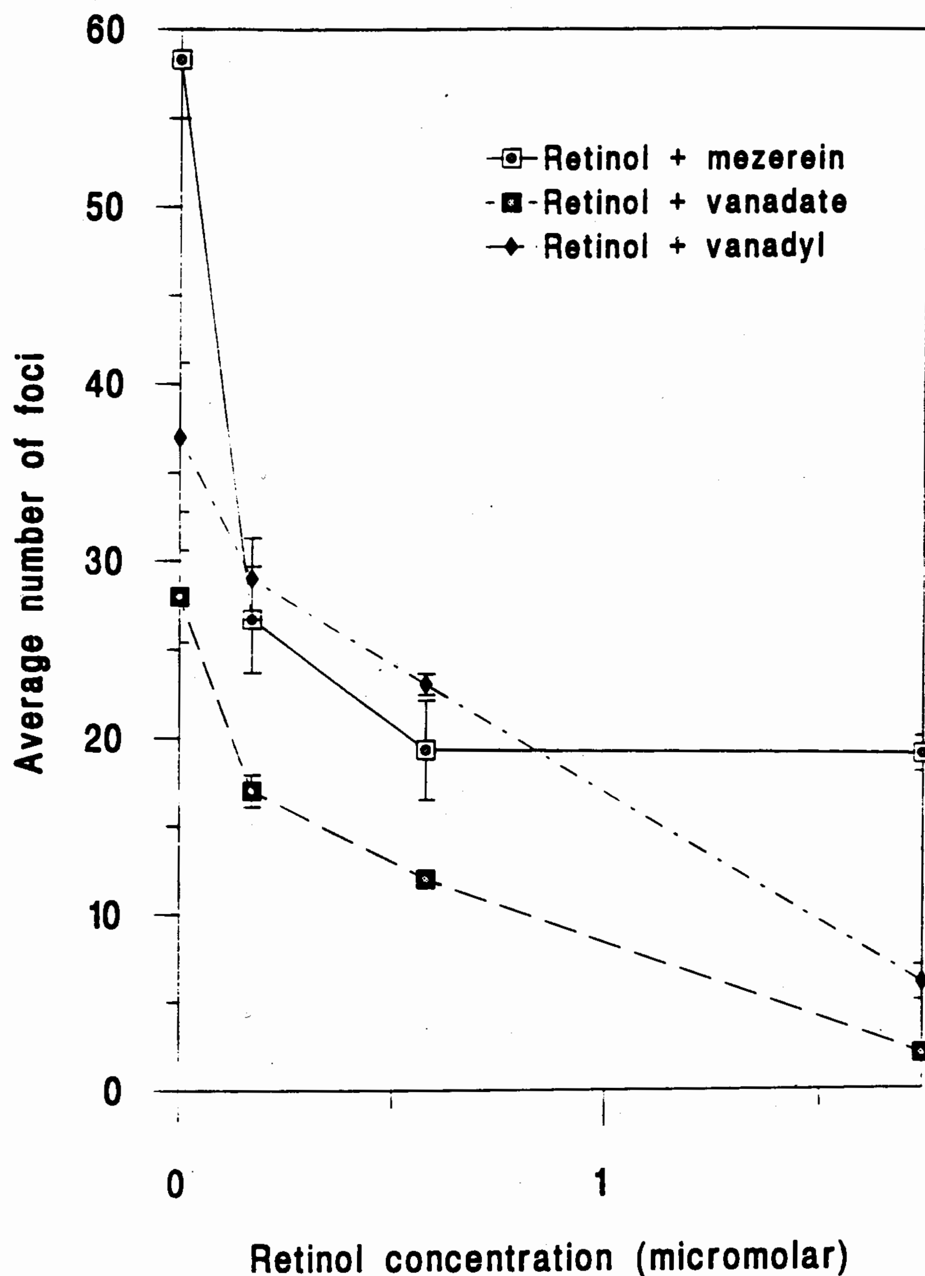


Figure 8: Inhibition of mezerein or vanadium-mediated transformation by retinol applied from day 11 to day 21

Cells at passage 17 were seeded at 2.3×10^5 cells /100mm plate. They were transfected with 1 µg of BPV DNA/plate, then subcultured at 1:17, 21 hours later (day1). Cells received 0.5 ng/ml of mezerein, 2 µM vanadate or 3.28 µM vanadyl from day 1 to day 10. From day 11 to day 21, cells were exposed only to retinol. All data points were determined with triplicate plates. Experiment was repeated with similar results. Error bars are standard errors of the mean. for mezerein, vanadate and vanadyl values, $p < 0.001$ by analysis of variance.

As with ascorbate, toxicity of retinol or combinations of retinol with vanadium might account for suppression of vanadium-mediated transformation by retinol. Retinol at 1.74 μM, the maximum dose used in these experiments, alone or with vanadate or vanadyl did not significantly decrease colony forming efficiency of transfected cells ($p > 0.05$) (table 2). Therefore, toxicity of retinol with V(V) or V(IV) does not account for the ability of retinol to suppress vanadium-promoter transformation.

4. Retinol, but not ascorbate, suppresses focus formation in BPV DNA-carrying transformed cells in co-cultures

We questioned whether ascorbate or retinol could suppress focus formation in transformed cell lines which carried BPV DNA. NV51.2 is a transformed cell line which carries 290 copies of BPV DNA and was derived from a vanadate-induced focus in BPV DNA transfected C3H/10T½ cells. Another line, V52.7, carries 85 copies of BPV DNA and is also derived from a vanadate-induced focus.

Co-cultures were used to evaluate transformation of BPV DNA-carrying cells in the presence of ascorbate, retinol and promoters. Mixtures of untransformed,

untransfected C3H/10T½ cells with cells from subcloned cell lines constitute reconstruction assays in which known numbers of BPV DNA-carrying cells are grown together with untransformed cells. Unlike untransformed cells, BPV DNA-carrying cells do not stop growing when they reach confluence, they continue to grow and produce a multilayered focus. Therefore, co-culture assays identify transformation on the basis of loss of contact inhibition of the transformed cells. Since foci can be counted visually, transformation can be quantified. An alternative method of assessing the effects of ascorbate and retinol on transformation would be to use morphological changes (increasing spindle-shape of cells, cells growing on top of each other) as an indication of transformation in monocultures of subcloned cells. However, it is difficult to quantify these morphological changes.

Co-culture assays do not address other characteristics of transformation such as morphological changes, changes in serum requirements, tumorigenicity in animals or loss of anchorage independence. In addition, increasing numbers of untransformed cells may suppress transformation of some BPV DNA-carrying cells. For this reason, all co-culture assays in this project used a

consistent ratio of 2000 untransformed cells and 200 cells from the subcloned cell lines.

When 200 transformed cells were co-cultured with 2000 C3H/10T½ cells in the absence of ascorbate, retinol or vanadate, V52.7 produced approximately 40 foci and NV51.2 produced 50 (table 6). There are many possible mechanisms to explain the small number of foci. However, the small number of foci argues against nonadherent cells floating free and establishing satellite colonies.

Retinol decreased numbers of foci formed by transformed cells in co-cultures. Continuous treatment of co-cultures with 1.74 μM retinol suppressed focus formation by NV51.2 cells by 33% and by V52.7 cells by 56% ($p < 0.05$) (table 6). Foci that did form stained very pale and appeared to be only 1 or 2 cell layers thick (not the usual multilayered foci).

Co-cultures incubated continuously with 250 μM ascorbate also showed an apparent decrease in foci (table 6). Though this decrease was not statistically significant ($p > 0.05$), it may be an important observation which may warrant further investigation.

TABLE 6

Numbers of foci formed in co-cultures of subcloned cell lines with C3H/10T½ cells exposed to ascorbate and retinol

	Suppressor		
Subclone	none	ascorbate	retinol
NV51.2	48.3±7.6	36.0±2.6	31.7±4.7
V52.7	39.0±4.0	28.0±9.2	17.3±2.3

For co-cultures, 200 cells from the subclones were plated together with 2000 untransfected C3H/10T½ cells. They were incubated continuously in 250 μM ascorbate, 1.74 μM retinol or untreated medium for 21 days. Numbers of foci are means of 3 plates ± standard error of the mean. The experiment was repeated once with similar results. All values differed significantly from untreated controls ($p < 0.05$ using t-tests) except for cultures exposed to ascorbate which did not differ significantly from untreated controls ($p > 0.05$).

Toxicity of retinol to transformed cells is a possible explanation for the suppression of focus formation. This hypothesis is refuted by subculture growth times, (table 7). Subculture growth times varied from 23.8±4.4 to 24.6±3.3 for NV51.2 cells and from 24.1±3.4 to 25.9±2.6 for V52.7 cells regardless of whether they were passaged in normal medium or medium with 1 μM vanadate, 250 μM ascorbate or 1.74 μM retinol. Variations in subculture growth times are not significantly different regardless of treatment ($p > 0.05$). This suggests that growth of cultures is not retarded by retinol, ascorbate and vanadate more than untreated controls. However, it does not rule out the possibility of transient alteration of growth of cultures, especially during the lag phase.

Untransfected C3H/10T½ cells have an average generation time of 15.5 hours (31). Subculture growth times for V52.7 and NV51.2 cell lines are approximately 24 hours. This difference reflects the lag period of approximately 24 hours after seeding during which little cell division would occur.

TABLE 7

Subculture growth times of two transformed cell lines passaged for 10 passages in vanadate, ascorbate or retinol

Treatment	Cell line	
	NV51.2	V52.7
normal medium	23.8±3.7	24.9±4.0
1 μM vanadate	24.6±3.3	24.1±3.4
250 μM ascorbate	23.8±4.4	24.7±2.6
1.74 μM retinol	23.9±2.7	25.9±2.6
vanadate + retinol	ND	24.9±3.9

Subculture growth times are expressed in hours. Cells were counted with duplicate samplings for all 10 passages of each cell line in each treatment according to the protocol described in methods. Values are means of 10 passages ± standard error of the mean. ND: not determined. By t-tests, $p > 0.05$ for all values.

DISCUSSION

In BPV DNA-transfected C3H/10T½ cells, vanadate and vanadyl enhance BPV DNA-mediated cell transformation similarly (table 2) but only vanadate alters cell adhesion. Both vanadate and vanadyl can enter cells, where vanadate is reduced to vanadyl and bound mainly to glutathione (32, 33). Vanadyl may be the active intracellular species which effects transformation, but vanadate and vanadyl may modulate cell adhesion differently at the cell surface.

Ascorbate at 50-100 μM suppressed both vanadate and vanadyl-promoted transformation of BPV DNA-transfected cells by about 50% (tables 3, 4). This observation is consistent with the suppression by 284 μM of ascorbate of 3-methylcholanthrene suppressed transformation of C3H/10T½ cells (34). Ascorbate at 50-100 μM also suppressed transformation of C3H/10T½ cells treated with 3-methylcholanthrene, even if added 14 days later (35). These studies established that ascorbate can suppress chemically-induced transformation. Our experiments demonstrate that ascorbate can also suppress virally-mediated cell transformation.

Numbers of foci formed in co-cultures by BPV DNA-carrying transformed cells were not significantly decreased by treatment with 250 μM ascorbate, as compared with its ability to suppress focus formation by transfected cells (table 6). Neoplastic transformation is a multi-stage process. It is possible that transformed cell lines have undergone further neoplastic change which may render them refractory to suppression by ascorbate. Ascorbate may more effectively suppress less advanced stages of transformation.

Early stages of transformation by vanadium compounds may require active oxygen intermediates. Vanadate and vanadyl are redox active metals that can generate active oxygen (11-13). The ability of ascorbate to inhibit promoter-mediated enhancement of transformation in *transfected* cells is consistent with its ability to scavenge active oxygen intermediates that are essential to transformation. In contrast, focus formation by *transformed* cell lines does not require vanadate and is not promoter-mediated. Ascorbate did not suppress focus formation by transformed cell lines. This argues in favour of a mechanism by which ascorbate suppresses vanadium-mediated transformation of *transfected* cells by scavenging active oxygen intermediates.

Retinol suppresses vanadium and mezerein-mediated transformation of BPV DNA-transfected C3H/10T½ cells more effectively than ascorbate (figures 6 and 7). It is effective even if applied after confluence when foci have begun to develop (figure 8). This eliminates the possibility that retinol suppresses transformation by interfering with uptake or metabolism of promoters. It is consistent with the ability of retinoids to decrease copy numbers of BPV DNA in C127 cells (20) and to inhibit other virally mediated neoplastic processes (18 - 20).

Unlike ascorbate, retinol suppresses focus formation of BPV DNA-carrying cell lines in co-cultures (table 6). Therefore, the antineoplastic actions of retinol may lie in both in its ability to block the actions of tumor promoters and to modulate gene regulation of transformed cells.

We have demonstrated that vanadium compounds enhance transformation of BPV DNA-transfected C3H/10T½ cells. Ascorbate and retinol inhibit this transformation and retinol also inhibits transformation of BPV DNA-carrying cell lines. The next step will be to investigate the role of alteration of gene expression in promoter-mediated enhancement of BPV DNA transformation.

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CHAPTER 4

ISOLATION AND CHARACTERIZATION OF SUBCLONED CELL LINES

ABSTRACT

Mezerein and vanadium enhance transformation of BPV DNA transfected C3H/10T½ cells. Ascorbate and retinol suppress promoter mediated enhancement of transformation. A goal of this chapter was to isolate subcloned lines of cells from foci in transfected cells exposed to promoters which produce more foci in the presence of promoter than in its absence (promoter dependent). Four promoter dependent subcloned lines and many promoter independent cell lines were isolated. Retinol inhibited focus formation of both promoter dependent cell lines and promoter independent cell lines. Ascorbate inhibited focus formation of one promoter independent cell line. Copy number and integration of BPV DNA were investigated in the cloned lines of cells. These preliminary experiments suggest that BPV DNA is integrated, partially deleted or rearranged in some cell lines. Investigation of alteration of copy number in cell lines passaged in promoter, ascorbate and retinol was inconclusive due to instability of copy numbers in untreated controls. The most important outcome of these cloning experiments was the isolation of subcloned cell lines which carry BPV DNA and differ in their expression of the transformed phenotype.

INTRODUCTION

Carcinogenic metals increase numbers of transformed foci formed by BPV DNA-transfected C3H/10T½ cells (chapter 2). We have demonstrated that ascorbate and retinol suppress focus formation (chapter 3). Mechanisms by which tumor promoters may enhance transformation include alteration of viral copy number, alteration of viral integration, modulation of cell-to-cell communication, control of viral gene expression or inactivation of tumor suppressor genes.

Alteration of viral integration has been implicated as a mechanism in neoplastic transformation by papillomaviruses. BPV DNA type 1 exists as monomeric or multimeric episomes, 60-120 copies/genome when transfected as linear BPV DNA into C127 cells (1). If it is circularized at the unique Bam HI site by pML2d or pBR322 and transfected into C127 cells, it exists as monomeric episomes (2, 3). However, it is also carried as both episomal and integrated forms in cloned lines of cells from transfected C127 cells (4). Mouse fibroblast cell lines derived from transfected C57BL/6J cells also carry both episomal and integrated BPV-1 DNA (5). Integrated BPV DNA in these cells is amplified and

contains deletions and rearrangements. Cellular flanking sequences are also amplified (6).

Similarly, BPV DNA type 4 sequences are integrated and rearranged in cloned lines from C127 cells (7, 8). Integration of BPV type 4 DNA in C127 cells favors persistence of BPV DNA (7, 8). Taken together, these studies are consistent with the hypothesis that BPV DNA progresses from monomeric episomes to integrated multimers in cloned cell lines and may contain rearrangements and deletions.

In studies of the condition of BPV DNA in actual tumors, as distinct from cell lines, BPV type-1 DNA is both episomal and integrated in transplanted hamster sarcomas (9). In contrast, BPV DNA was detected only as monomeric episomes in cell lines derived from equine and bovine tumors (10).

Integration of human papillomavirus may be a mechanism in the progression from benign to malignant transformation. Human papillomavirus type 16 is usually episomal in benign tumors but integrated in cervical carcinomas (11), although a few cervical carcinomas show only episomal HPV (12). In cervical dysplasias, HPV 16 progresses from episomal forms in mild dysplasias to integrated forms in higher grade dysplasias (13). HPV 18

is integrated in HeLa cells (14), although circular episomes are also present (14). HPV 18 is integrated and amplified in cervical carcinoma biopsies (14). Overall, it is reasonable to conclude that integration of HPV DNA accompanies progression from benign to malignant lesions.

Integration of papillomavirus sequences into genomic DNA may lead to malignancy by at least three possible mechanisms. HPV DNA integrates in the vicinity of known oncogenes where it may enhance oncogene expression or amplify oncogenes. In HeLa cells, HPV-18 is integrated within 50 kb (kilobases) of c-*myc* on chromosome 8 (15). Alternatively, the circular HPV genome is often opened in the E1/E2 ORF when it is integrated. This disrupts the E2 ORF which controls transcription of the major transforming ORF's (E6 and E7) resulting in unregulated transcription and uncontrolled cell proliferation (16, 17). As a third mechanism, integration of HPV is usually accompanied by amplification of both the viral DNA and cellular flanking regions (12). This may lead to chromosomal instability and activation of oncogenes (12).

The above studies lead to the hypothesis that factors such as metals which enhance focus formation in cell lines cloned from transfected C3H/10T½ cells alter integration or copy number of BPV DNA. To test this

hypothesis, the first objective was to isolate cloned lines of cells which responded phenotypically to promoter by producing increased numbers of foci in co-culture with untransfected C3H/10T½ cells. Isolation of "promoter dependent" cloned lines would enable further investigation of the mechanism by which promoter enhances transformation. A second objective of the cloning experiments was to determine whether there was any evidence for integration, amplification or deletion in these clones and subclones and whether copy number or integration was altered by promoter.

A third objective was to determine whether ascorbate and retinol would suppress focus formation in cloned lines of cells in co-culture, as they do for transfected cells. To investigate this, our goal was to identify and characterize a collection of subcloned cell lines which produced foci in reconstruction assays, contained BPV DNA in widely differing states, possibly integrated, episomal, or containing deletions or rearrangements and which responded to tumor promoters with enhanced production of transformed foci. A final hypothesis states that if modulation of focus formation is a result of alteration of viral copy number or integration, then viral integration and copy number will be altered when

these cells are passaged in mezerein, vanadium, ascorbate and retinol.

METHODS

Cloning of transformed cell lines

Transformed cell lines were cloned and subcloned from foci which developed in BPV DNA transfected C3H/10T½ cells exposed to mezerein, vanadate and vanadyl according to the method described in chapter 3 (figure 1).

Nomenclature for clones and subclones

As noted in chapter 3 (figure 1), clones which were derived from vanadate-induced foci and maintained in medium without vanadate were numbered NV51, NV52, NV53,...etc. Clones derived from vanadate-induced foci and maintained in medium containing 1 μM vanadate were numbered V51, V52,...etc. In a similar fashion, clones derived from vanadyl-induced foci and maintained in medium without vanadyl were numbered NV41, NV42,...etc. Clones derived from vanadyl-induced foci and maintained in medium containing 1 μM vanadyl were designated V41, V42,...etc. Clones derived from mezerein-induced foci

were identified as NM1, NM2,...etc. and M1, M2,...etc. depending upon whether or not they were maintained in medium containing 0.5 ng/ml of mezerein.

Subclones were numbered according to the clone from which they were subcloned. Subclones from NM1 were identified as NM1.1, NM1.2,...etc. Subclones from the clone V52 were designated V52.1, V52.2 ... etc. The discontinuous numbering of some of the clones and subclones is due to loss of clones and subclones because of fungal contamination during the cloning process. For example, the NV4 clones include only NV46, NV48, NV410, NV411 and NV412. The missing clones such as NV41, NV42, etc. were lost to contamination during the cloning process.

Passaging of cell lines, determination of subculture growth times, co-culture assays, DNA extraction and determination of copy numbers

Cells were passaged and subculture growth times were determined as described in chapter 3. DNA was extracted, copy numbers determined and co-culture assays done as described in chapter 3.

Briefly stated, co-culture of transformed cells with untransformed C3H/10T½ cells is necessary to assess the

ability of untransformed cells to overcome contact inhibition. Transformed cells do not respond to contact inhibition to the same extent as untransformed cells. As a result, they pile up into multilayered foci which are clearly visible in a monolayer culture of untransformed cells. Transformed cells growing alone in a monoculture pile up and display spindle-shaped cells which are characteristic of transformed cells. However, it is less subjective to quantify numbers of transformed foci than degrees of alteration of transformed morphology.

Transformed morphology and loss of contact inhibition are two distinct phenotypic expressions which do not necessarily represent the same degree or type of transformation. Nonetheless, C3H/10T½ cells which form foci are tumorigenic in nude mice. Therefore, measurement of the ability of transformed cells to form foci in co-culture is a reasonable assessment of transformation. Measurement of the change in ability to form foci when exposed to promoters or suppressors is an assessment of alteration of the transformed phenotype.

The slot blots used to determine copy numbers used a series of standards from 0 to 500 copies/genome. For this reason, it was not possible to directly determine copy numbers of samples which contained more than 500

copies/genome. A series of dilutions was used to determine copy numbers of cell lines containing more than 500 copies per genome (figure 1). As an example, slot blots of NV51.2 DNA, 2 μg of DNA per well were hybridized to ^{32}P-pdBPV-1 (figure 1a). Representative samples of undiluted NV51.2 DNA (10 μl of a 0.2 μg/μl solution), and the same samples diluted 1:10 and 1:15 with calf thymus DNA are shown. In each case, the slot blots were scanned and compared with a calibration curve developed from the standards (shown on the left side of figure 1a). The integrator reported areas in arbitrary units from the densitometer scans (figure 1b). Raw copy number determinations were based on comparison of these areas with the standard calibration curves. Raw copy numbers were multiplied by the dilution factor to obtain actual copy numbers per genome. There is some variability in copy number determinations for the 1:10 and 1:15 fold dilutions of the same sample. This probably reflects unavoidable, random variations resulting from dilution and aliquoting.

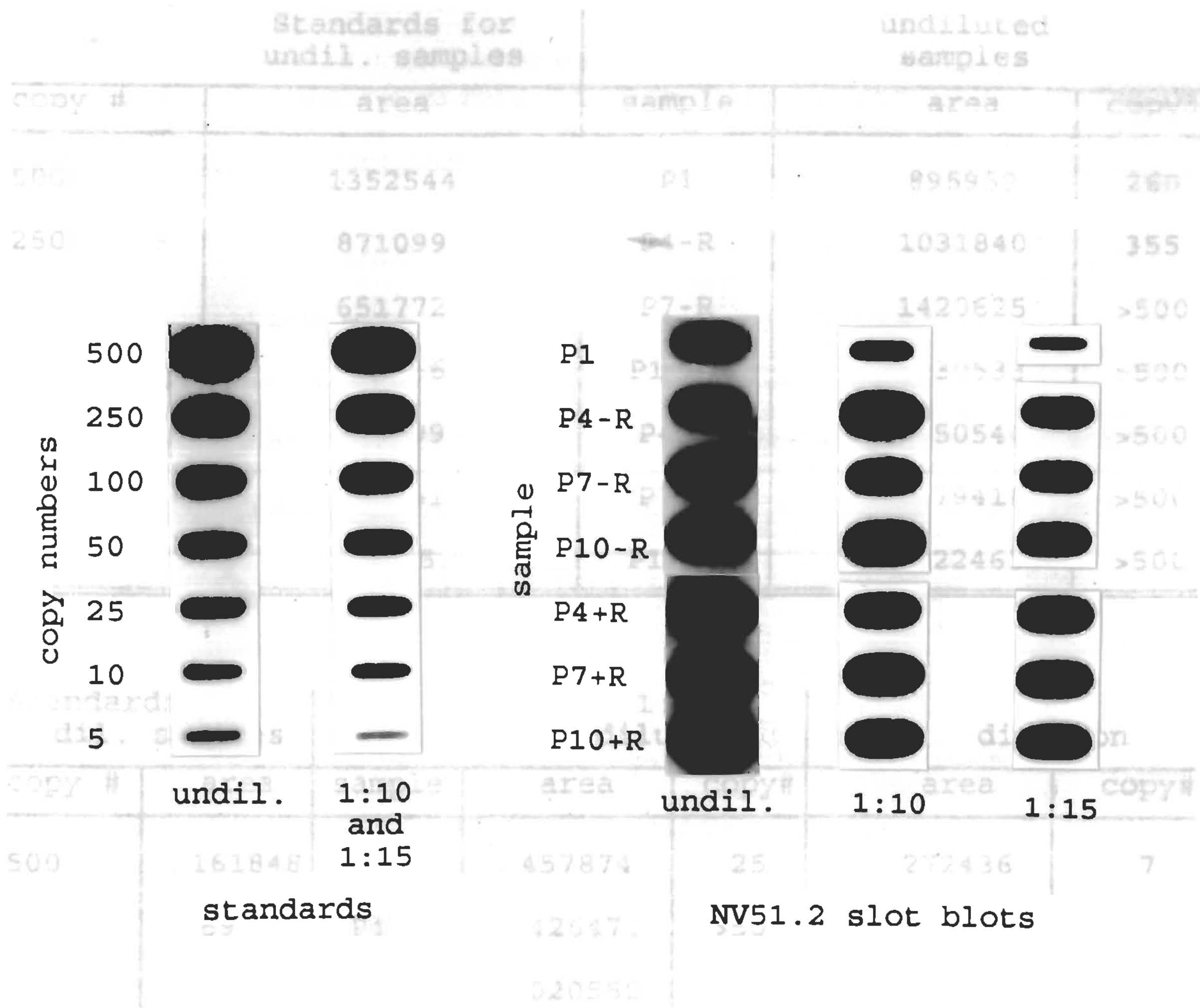


Figure 1a: Determination of copy numbers for cell lines containing more than 500 copies per genome.

Genomic DNA was initially slot blotted at 2 µg of total DNA per well. This could not be read accurately using the calibration curve made from the standards. Total genomic DNA was then diluted at a variety of ratios with calf thymus DNA to yield solutions of 0.2 µg/10µl of total DNA. 10 µl was then spotted on the slot blots. Dilutions eventually yielded samples which could be correlated with copy number using the calibration curves.

Standards for undil. samples		undiluted samples		
copy #	area	sample	area	copy#
500	1352544	P1	895959	260
250	871099	P4-R	1031840	355
100	651772	P7-R	1420625	>500
50	432666	P10-R	1530533	>500
25	298199	P4+R	1850540	>500
10	131351	P7+R	2079418	>500
5	69005	P10+R	1822463	>500

Standards for dil. samples		1:10 dilution			1:15 dilution	
copy #	area	sample	area	copy#	area	copy#
500	1161848	P1	457874	25	272436	7
250	969706	P4-R	1426471	>500	847317	155
100	717737	P7-R	1020550	275	862398	165
50	562346	P10-R	1357767	>500	961997	230
25	425986	P4+R	935329	215	912672	150
10	292954	P7+R	1191989	500	1033035	285
5	117657	P10+R	1078429	325	906073	195

Figure 1b: Calculation of copy numbers in samples containing more than 500 copies copies of BPV DNA per genome using NV51.2 DNA as an example

Copy number column lists the actual copy number read from the calibration curves. These are to be multiplied by the dilution to obtain actual copy numbers. Area is expressed in arbitrary units reported by the integrator for densitometer scans. Abbreviations: +R: exposed to 1.74 μM retinol, -R: not exposed to retinol, P: passage number

Analysis of DNA by digestion with restriction endonucleases (Methods section continued)

Rearrangements or deletions of BPV DNA and its state of integration were determined from restriction endonuclease digestion of total genomic DNA. After extraction and determination of DNA concentrations, each DNA sample was digested both with Bam HI (BRL) and with Sal I (BRL) restriction endonucleases. 2 μg of DNA was incubated with 6 units of Bam HI or Sal I in 20 μl of the supplied buffer (BRL) overnight at 37°. DNA samples from all clones and subclones, undigested, Bam HI digested, and Sal I digested were fractionated on 0.7% agarose gels in Tris-borate-EDTA (TBE) buffer at 40 V for 2-3 hours. These were transferred by the method of Southern (18) to nitrocellulose filters and immobilized by baking. Filters were prehybridized for 1 hour at 42° in 2 x sodium dodecyl sulfate (SDS) /NaCl/sodium citrate (SSC) buffer, 0.1 % SDS, 100 μg/ml of tRNA, and 5 x Denhardt's solution (1% Ficoll (Type 400, Pharmacia), 1% polyvinylpyrrolidone and 1% bovine serum albumin), in 50% formamide. Hybridization, washing of filters and exposure to X-ray film was carried out under the same conditions as above for slot blots.

RESULTS

I. INTEGRATION AND COPY NUMBER IN CLONES AND SUBCLONES

A. Copy numbers

1. Continuous presence of promoter in the medium is not necessary to maintain copies of BPV DNA in clones derived from vanadate or mezerein.

The distribution of copy numbers in clones maintained in promoter and those maintained in the absence of promoter are very similar for clones derived from mezerein, vanadate or vanadyl (table 1 with medians and tables 2,3 and 4 with individual clones). This leads to the conclusion that vanadate or mezerein is not necessary to the maintenance of BPV DNA copy numbers in these clones.

Copy numbers of BPV DNA in clones derived from BPV DNA transfected C3H/10T½ cells are similar to copy numbers of BPV DNA in transfected C127 cells, 50-120 copies per genome (1)

TABLE 1

Summary of median copy numbers with ranges for clones

Clone	Copy numbers	Range	Number of clones
M	63.3	35-90	6
NM	55.0	18-102	9
M+NM	55.0	18-102	15
V5	27.5	12-45	10
NV5	30.0	18-48	10
V5+NV5	30.0	12-48	20
V4	35.0	13-92	6
NV4	22.9	8-22	5
V4+NV4	22.5	8-92	11

Median copy numbers determined for groups of clones. M: clones derived from mezerein-induced foci and maintained continuously in 0.5 ng/ml mezerein, NM: clones derived from mezerein-induced foci but maintained in medium without mezerein, M+NM: grouping includes all mezerein-derived clones, both M and NM. V5: clones derived from vanadate-induced foci and maintained continuously in 1μM vanadate, NV5: clones derived from vanadate-induced foci but maintained in medium without vanadate, V5+NV5: grouping includes all vanadate-derived clones, both V5 and NV5. V4: clones derived from vanadyl-induced foci and maintained continuously in 1 μM vanadyl, NV4: clones derived from vanadyl-induced foci but maintained in medium without vanadyl, V4+NV4: grouping includes all vanadyl-derived clones, both V4 and NV4.

TABLE 2

Copy number and promoter dependency of lines cloned from vanadate-induced foci incubated with (V51-10) or without (NV51-10) 1 μM vanadate. Ranked in descending order by copy number.

clone	copy #	number of foci +vanadate	number of foci -vanadate
NV56*	50	53	45.5
NV58	47.5	0	0
NV57*	42.5	4	0
NV55	40	14.5	2.5
NV51*	35	8.5	3
NV52	30	4	1.5
NV54	22.5	4	2
NV59	22.5	1	0
NV510	22.5	3	1.5
NV53	17.5	3	1.5
V51	45	4.5	2.5
V52*	45	4.5	1.5
V54	45	2.5	1
V53	40	0	0
V510	30	2	0
V55	25	0	1.5
V57	23	3	0
V58	21	1.5	2
V59	17.5	0	1
V56	12.5	0	0

Copy numbers are expressed as number of BPV DNA copies per genome. Values are averages of three determinations. Number of foci indicates the number of foci formed by 200 cloned cells incubated in co-culture with 2000 untransfected C3H/10T½ cells, passage 18-21. Numbers are means of three plates. * indicates that these clones were subcloned.

TABLE 3

Copy number and promoter dependency of lines cloned from mezerein-induced foci incubated with (M1-10) or without (NM1-10) 0.5 ng/ml of mezerein. Ranked in descending order by copy number.

clone	copy #	number of foci +prom.	-prom
NM5	102.5	33.5	17
NM7	96.7	37	29
NM2*	72.5	31	5
NM6	66.7	16.5	ND
NM8	55	36	5.5
NM3	20	34	21.5
NM1*	18.3	60	21.5
NM10	17.5	21.5	6
NM4	16.7	26.5	6.5
NM9	ND	40	17.5
M3	90	156.5	45
M1*	85	42	0
M2*	71.7	67	17.5
M4	55	24.5	3
M5	37.5	41	11
M6	35	19.5	11

Copy numbers are expressed as number of BPV DNA copies per genome. Values represent means of three determinations. Number of foci indicates the number of foci formed by 200 cloned cells incubated in co-culture with 2000 untransfected C3H/10T½ cells, passage 18-21. Numbers are averages of three plates. * indicates that these clones were subcloned. ND indicates that the value was not determined.

TABLE 4

Copy number and promoter dependency of lines cloned from vanadyl-induced foci incubated with (V42-10) or without (NV46-12) 1 μM vanadyl. Ranked in descending order by copy number.

		number of foci	
clone	copy #	+prom.	-prom
NV411	30	3	2.5
NV410	22.5	10.5	8
NV48	18.7	7	3.5
NV412	18.7	6	6
NV46	7.5	3.5	4
V48	91.2	4.5	3
V43	38.7	8.5	37.5
V410	35	5	1.5
V44	23.3	4.5	4
V45	15	4	6.5
V42	12.5	21	10.5

Copy numbers are expressed as number of BPV DNA copies per genome. Values given are the means of three determinations. Number of foci indicates the number of foci formed by 200 cloned cells incubated in co-culture with 2000 untransfected C3H/10T½ cells, passage 18-21. Numbers are averages of three plates.

2. All clones and subclones which form foci in co-culture contain BPV DNA

Foci were not produced in co-culture by subclones which contained less than 10 copies/genome. Certain subclones, NM1.4 and M2.10 (table 5) were exceptions, but they produced only 1-4 foci per plate. This indicates that efficient focus production requires a minimum of 10 copies per genome. It argues against a "hit and run" mechanism such as that postulated for BPV type 4 (7).

Some subclones which carried more than 10 copies/genome, NV51.8, NV51.6, NV51.7 and V52.9 (table 6), did not form foci in co-culture. Therefore, at least 10 copies of BPV DNA is required for neoplastic transformation. Nevertheless, presence of at least 10 copies of BPV DNA is not always sufficient for focus formation. Clearly, factors other than number of viral copies control expression of the transformed phenotype.

Table 5: Copy number and promoter dependency of lines subcloned from mezerein-derived clones. Ranked in descending order by copy numbers.

Copy numbers are expressed as number of BPV DNA copies per genome. Values are averages of three separate determinations. Number of foci indicates the number of foci formed by 200 cloned cells incubated in co-culture with 2000 untransfected C3H/10T½ cells, passage 18-21. It is the mean value of three plates. ND indicates that the value was not determined. Cultures were incubated in 0.5 ng/ml of mezerein for 21 days.

TABLE 5

Copy number and promoter dependency of lines subcloned from mezerein-derived clones

		number of foci	
subclone	copy #	+prom.	-prom
NM1.10	255	ND	57
NM1.7	240	94	60.5
NM1.1	108.7	33	10
NM1.8	85	18.5	13
NM1.9	63.3	70	9
NM1.5	40	17	10
NM1.2	35	25.5	19.5
NM1.4	0	2	4
NM2.6	23.7	75	14.5
NM2.7	8.7	0	ND
NM2.8	ND	28	6
M1.3	20	0	0
M1.2	0	0	0
M1.4	0	0	0
M1.5	ND	0	0
M1.6	0	0	0
M1.7	0	ND	0
M1.8	0	0	0
M1.9	0	0	0
M1.10	ND	0	0
M2.10	5	1	0
M2.1	0	0	0
M2.2	0	0	0
M2.3	0	0	0
M2.4	0	0	0
M2.6	0	0	0
M2.7	0	0	0
M2.8	0	0	0
M2.9	0	0	0

Table 6: Copy number and promoter dependency of lines subcloned from vanadate-derived clones. Ranked in descending order by copy numbers.

Copy numbers are expressed as number of BPV DNA copies per genome. Values given are means of three determinations. Number of foci indicates the number of foci formed by 200 cloned cells incubated in co-culture with 2000 untransfected C3H/10T½ cells, passage 18-21. Numbers indicate the mean of three plates. ND indicates that the value was not determined. Cultures were incubated with 1 μM vanadate for 21 days

TABLE 6

Copy number and promoter dependency of lines subcloned from vanadate-derived clones

subclone	copy #	number of foci +prom.	number of foci -prom
NV51.9	3850	41.5	42.5
NV51.1	495	15.5	24
NV51.2	288.7	56	51
NV51.8	186.2	4	0
NV51.6	81.7	1	0
NV51.7	14.2	0	0
NV51.10	10	28.5	38
NV51.3	2	0	0
NV51.4	>2	0	0
NV51.5	0[c]	0	0
NV56.12	2560	42	ND
NV56.8	860	61	52
NV56.6	600	54	51.5
NV56.14	281.7	84	71.5
NV56.7	0	0	0
NV57.1	1800	34	26
NV57.6	345	20.5	14.5
NV57.3	221.7	52	55.5
NV57.4	208.7	19	29.5
NV57.9	118.3	19	15
NV57.8	195	80.5	90.5
NV57.2	102	ND	58
V52.9	612.5	1	0
V52.7	85	38.5	40
V52.10	8.5	0	0
V52.1	0	ND	0
V52.3	0	0	ND
V52.6	0	3	0

3. Promoter dependency of mezerein-derived subclones compared with promoter dependency of vanadate-derived subclones.

Promoter dependent subclones are defined for this thesis as those which produce twice as many foci in the presence of promoter than in normal medium and produce at least 10 foci in the presence of promoter in early passages. Promoter dependent subclones were isolated only from mezerein-derived clones (NM1.1, NM1.9, NM2.6 and NM2.8) (table 5). Vanadate-derived subclones produced the same number of foci in the presence or absence of vanadate (table 6) (promoter independent).

Promoter dependency seemed to decrease from passage to passage in promoter dependent cell lines. As an example, NM1.9 cells, when co-cultured as 200 NM1.9 cells and 2000 untransformed C3H/10T½ cells after the initial isolation of the cell line produced 70 foci in the presence of mezerein and 9 foci in the absence of mezerein. After five passages, NM1.9 cells produced 30 foci in the absence of mezerein and 59 foci in the presence of mezerein (table 8, page 131), when tested in co-culture. Gradual loss of promoter dependency may parallel increasing levels of neoplastic transformation

as a result of repeated passaging. This requires further investigation.

4. Comparison of copy numbers in parent clones and in subclones

Copy numbers of BPV DNA in subclones which contain BPV DNA are sometimes higher than copy numbers in the original clones (table 7). However, some subclones do not contain BPV DNA. The large variation in copy number of subclones derived from the same clone may reflect random selection during subcloning. The clones may have contained cells which individually carried a random number of copies as well as some untransfected cells. The process of subcloning would select subclones which contain zero copies, less copies, the same number of copies, or more copies than the original clone, resulting both in higher copy numbers and a wider variation. However, it is possible that repeated passaging during subcloning has also been responsible for an increase in copy number arising from amplification of the viral genome, Alternatively, repeated passaging may result in gradual loss of viral copies.

A large majority of subclones derived from V52, M1 and M2 clones contained no BPV DNA or fewer copies than the clone. The reason for this lack of copies is not

immediately apparent from these experiments. Random selection of colonies containing no BPV DNA may have occurred during the subcloning process.

A random process is unlikely to be the explanation for the subclones from clones M1 and M2. Only 2 out of 16 subclones (for which copy number was determined) from M1 and M2 contained BPV DNA. Copies of BPV DNA may have been lost during cell division and passaging. Since these subclones were maintained in medium containing promoter, the presence of promoter may have exerted selection pressure against cells containing BPV DNA. It would be possible to distinguish between these two possible mechanisms (random selection and promoter-mediated selection) by repeating the subcloning process using another sample of M1, M2 or V52 cells and by subcloning a larger number of colonies, some into medium containing promoter and some into medium without promoter. The increased sample size would reduce the influence of random selection. Subcloning into medium not containing promoter would remove promoter-induced selection pressure.

TABLE 7

Median copy numbers of subclones derived from various clones

Clone	original clone	number of subclones	median copy #	range
NV51	35	10	47.9	3850-0
NV56	50	5	600.0	2560-0
NV57	42	7	208.7	1800-102
V52	45	6	4.2	612-0
NM1	18	9	74.1	240-0
NM2	72.5	3	16.2	24-9
M1	85	9	0	20-0
M2	71.7	9	0	5-0

Median copy numbers were determined for all subclones from each clone. NV51 includes all subclones derived from clone NV51, NV56 includes all subclones derived from NV56... etc.

B. Integration

1. Restriction endonuclease digestion of genomic DNA can suggest integration, deletion or rearrangement of BPV DNA

In these experiments C3H/10T½ cells were originally transfected with the plasmid pdBPV-1 (142-6). The complete plasmid, designated pdBPV-1 (142-6), consists of 2 sections (figure 2). One section, the plasmid, pML2d, contains an origin of replication which functions in bacteria and is 2.5 kb (2,500 base pairs) in length. This plasmid is necessary for replication of pdBPV-1 in bacteria for use in transfection. The other section of the complete plasmid, pdBPV-1 (146-2), is the 8 kb linear form of BPV DNA. Therefore, the entire pdBPV-1 is 10,500 bp in length and is double-stranded and circular.

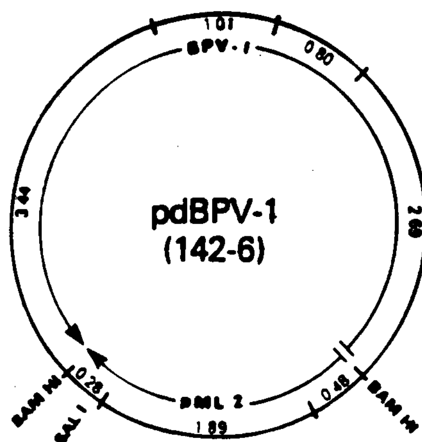


Figure 2: Map of the plasmid pdBPV-1 (142-6) showing the BPV-1 and pML2d sections and Bam HI and Sal I restriction endonuclease sites (modified to show only Bam HI and Sal I sites from (2))

Analysis of undigested, Bam HI- or Sal I-digested DNA from the various clones and subclones, based on the criteria discussed in Appendix 1, can suggest whether pdBPV-1 is integrated or episomal. It also indicates whether pML2d is present or absent and whether both Bam HI sites are intact.

2. BPV DNA in some clones and subclones is rearranged or contains deletions

Restriction endonuclease digestion of DNA from certain subclones suggests that parts of the BPV DNA genomes are deleted or rearranged. Deletion increases progressively from clone to subclone. Bam HI digested DNA from clone NV56 contains the 2.5 kb fragment which corresponds to pML2d (figure 3). Of the subclones derived from it, no 2.5 kb fragment occurred with Bam HI digestion (figure 3). Probing of Bam HI digested DNA from the subclones shows pML2d to hybridize in the same position as pdBPV-1 (figure 3). This suggests that one or two of the Bam HI sites are lost in the subclones. Probing of the original clone, NV56, with ^{32}P-pML2d shows pML2d migrating both with the BPV DNA at about 8 kb and at 2.5 kb (figure 3). This indicates that some of the pML2d in the original clone lacked a Bam HI site as well.

This progressive loss of Bam HI sites, indicates increasing deletion in the subcloning process. Digestion of DNA from the NV56 clone and its subclones with Sal I does not alter the migration pattern of BPV DNA from that observed in undigested DNA (figure 3). Therefore, the Sal I site (in pML2d) is also deleted.

Other clones and subclones also contain deletions. V52.9 (figure 4) lacks the 2.5 kb fragment in Bam HI-digested samples. Probing with ^{32}P-pML2d shows a faint band migrating with the BPV DNA, again suggesting loss of the Bam HI site in a small subpopulation of the cells and probable loss of the entire pML2d plasmid in most of the cells (figure 4). NM1.9 and NM2.6 lack pML2d completely (figure 4).

Several vanadyl-derived clones (figure 5) contained rearranged BPV DNA. The small fragments which migrate between 2.5 kb and 10 kb may result from ligation of parts of BPV DNA. BPV DNA may be profoundly altered by both deletions and rearrangements in clones and subclones derived from foci exposed to vanadate and vanadyl. However, not all BPV DNA is obviously altered from the original clone. BPV DNA in subclones derived from NV57 migrates in a similar pattern to BPV DNA in the original clone (figure 3).

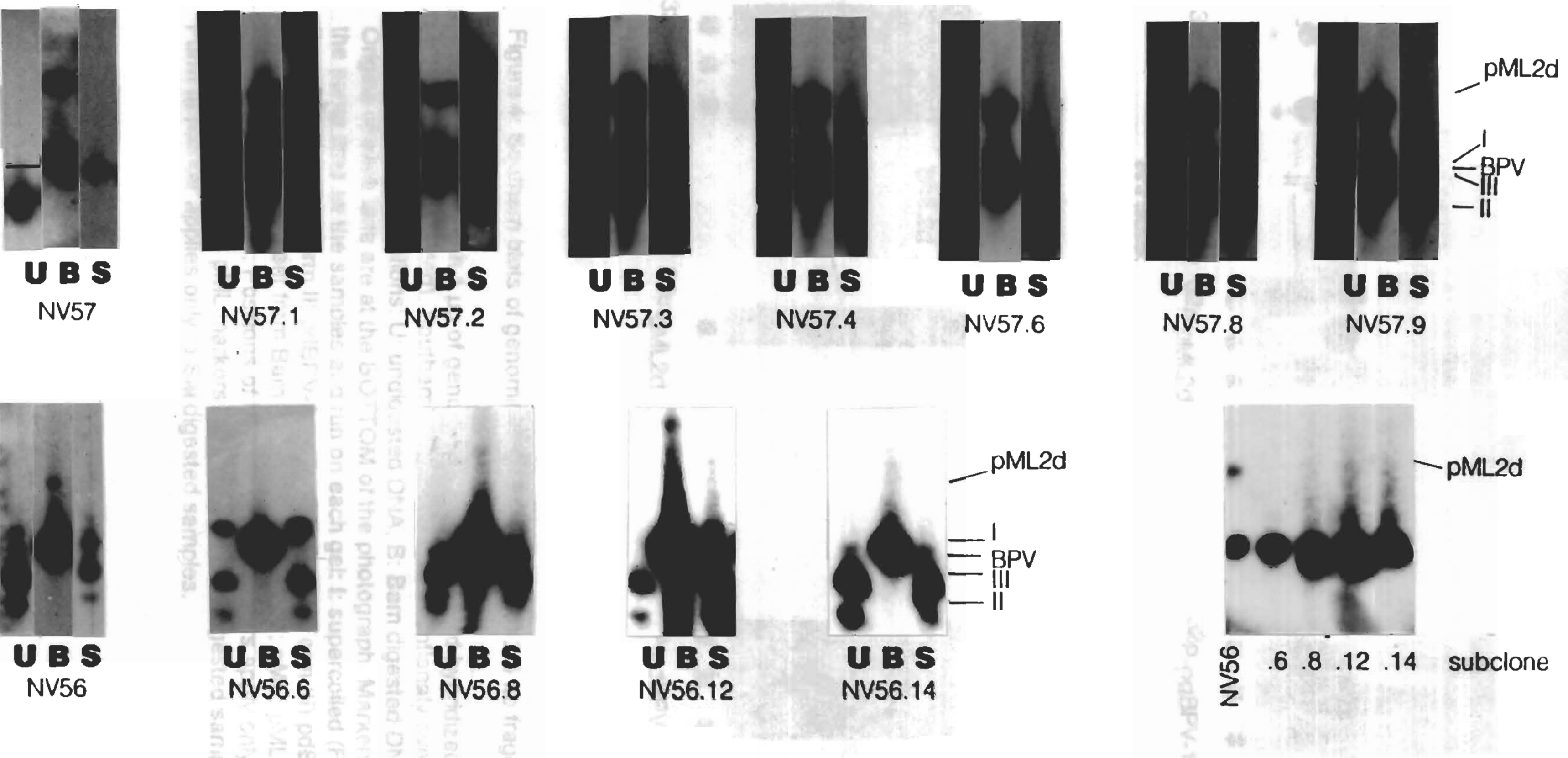


Figure 3: Genomic DNA from the clone NV57 and its subclones and from NV56 and its subclones.

NV57 clones and subclones, 1 µg of genomic DNA per lane, are shown hybridized with ^{32}P-pdBPV-1. NV56 clones and subclones, 1 µg of genomic DNA per lane are shown hybridized with ^{32}P-pdBPV-1 and with ^{32}P-pML2d. Southern blots illustrated are intentionally overexposed to illustrate faint bands. **Abbreviations:** U: undigested DNA, B: Bam digested DNA, S: Sal digested DNA. Origins of each lane are at the BOTTOM of the photograph. Markers which were digested at the same time as the samples and run on each gel: **I:** supercoiled (Form I) pdBPV-1 marker; **II:** nicked circular (Form II) pdBPV-1 marker; **III:** linear (Form III) pdBPV-1 marker; **BPV:** BPV DNA cleavage fragment from Bam digestion of pdBPV-1; **pML:** pML2d fragment from Bam digestion of pdBPV-1. Positions of Forms I and II markers apply only to undigested samples, positions of BPV and pML markers apply only to Bam-digested samples and position of the Form III marker applies only to Sal digested samples.

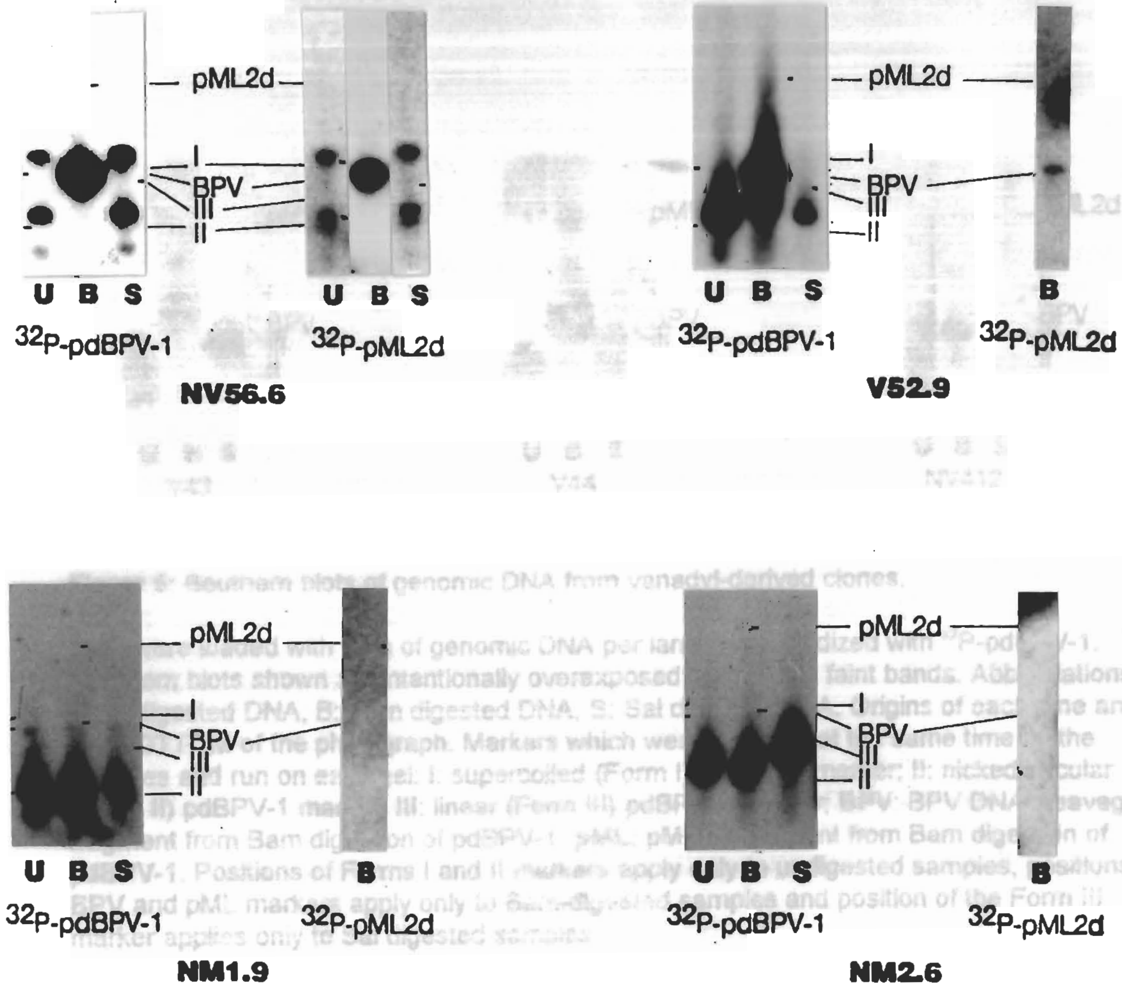


Figure 4: Southern blots of genomic DNA which lack the 2.5 kb fragment in Bam digestions

Gels were loaded with 1 μg of genomic DNA per lane and hybridized with ^{32}P-pdBPV-1 or ^{32}P-pML2d (as indicated). Southern blots shown are intentionaly overexposed to illustrate faint bands. Abbreviations: U: undigested DNA, B: Bam digested DNA, S: Sal digested DNA. Origins of each lane are at the BOTTOM of the photograph. Markers which were digested at the same time as the samples and run on each gel: **I**: supercoiled (Form I) pdBPV-1 marker; **II**: nicked circular (Form II) pdBPV-1 marker; **III**: linear (Form III) pdBPV-1 marker; **BPV**: BPV DNA cleavage fragment from Bam digestion of pdBPV-1; **pML**: pML2d fragment from Bam digestion of pdBPV-1. Positions of Forms I and II markers apply only to undigested samples, positions of BPV and pML markers apply only to Bam-digested samples and position of the Form III marker applies only to Sal digested samples.

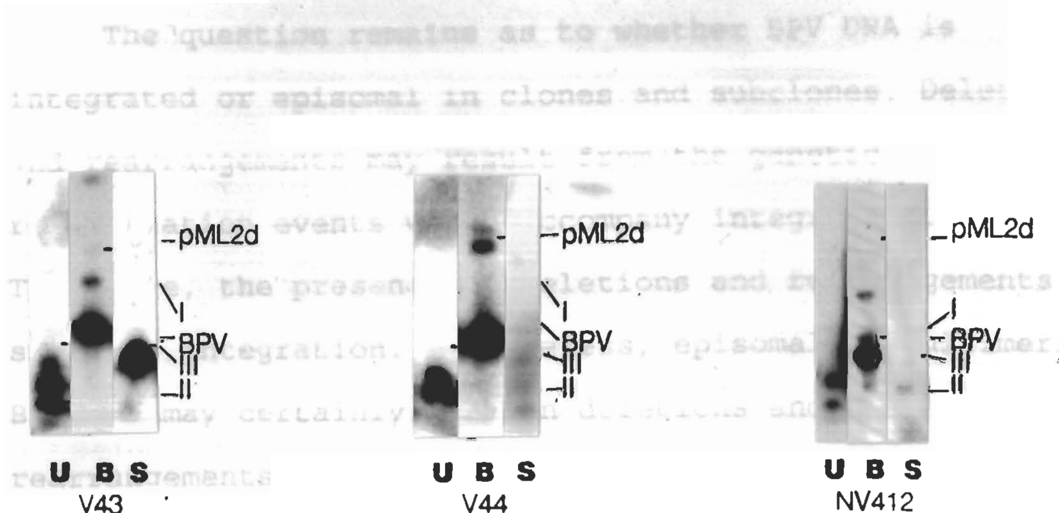


Figure 5: Southern blots of genomic DNA from vanadyl-derived clones.

Gels were loaded with 1 µg of genomic DNA per lane and hybridized with ^{32}P-pdBPV-1. Southern blots shown are intentionally overexposed to illustrate faint bands. Abbreviations: U: undigested DNA, B: Bam digested DNA, S: Sal digested DNA. Origins of each lane are at the BOTTOM of the photograph. Markers which were digested at the same time as the samples and run on each gel: I: supercoiled (Form I) pdBPV-1 marker; II: nicked circular (Form II) pdBPV-1 marker; III: linear (Form III) pdBPV-1 marker; BPV: BPV DNA cleavage fragment from Bam digestion of pdBPV-1; pML: pML2d fragment from Bam digestion of pdBPV-1. Positions of Forms I and II markers apply only to undigested samples, positions of BPV and pML markers apply only to Bam-digested samples and position of the Form III marker applies only to Sal digested samples.

The question remains as to whether BPV DNA is integrated or episomal in clones and subclones. Deletions and rearrangements may result from the genetic recombination events which accompany integration. Therefore, the presence of deletions and rearrangements suggests integration. Nonetheless, episomal or multimeric BPV DNA may certainly contain deletions and rearrangements.

Although the NV56 subclones have deletions, not all are unambiguously integrated. Undigested DNA from NV56.6 and NV56.14 show BPV DNA migrating at the same position (NV56.14) or faster (NV56.6) than the supercoiled standard (figure 3). This suggests that NV56.14 and NV56.6 have episomal BPV DNA. Conversely, NV56.8 and NV56.12 do not contain BPV DNA which co-migrates with the supercoiled standard (figure 3). This leads to the conclusion that they contain integrated BPV DNA. Conversely, this pattern of BPV DNA could also be characteristic of unintegrated multimers.

Taken together, these results indicate that BPV DNA is partially deleted or rearranged and may be integrated in some clones and subclones. These observations are consistent with previous studies which demonstrated

deletions and rearrangements of BPV DNA in cloned lines of cells.

II. PASSAGING OF SUBCLONED LINES IN PROMOTERS, ASCORBATE AND RETINOL

A. Selection of subcloned lines

One objective was to determine whether copy number or integration were altered by passaging of BPV DNA-carrying cell lines in tumor promoters, ascorbate or retinol. We identified five lines of cells which contained BPV DNA in various states of integration and which formed foci in co-culture.

One subclone (NM1.5) contains integrated BPV DNA. BPV DNA in undigested NM1.5 genomic DNA co-migrates with the nicked circular BPV DNA standard (figure 6). There is no evidence of supercoiled BPV DNA. Digestion with Bam HI yields the 8 kb BPV and 2.5 kb pML2d bands and digestion with Sal I yields only DNA co-migrating with the linear BPV DNA standard. The lack of supercoiled BPV DNA in the undigested sample suggests that BPV DNA is integrated. The results of Bam HI and Sal I digestions indicates that pML2d is not deleted and that the BPV DNA is probably not rearranged.

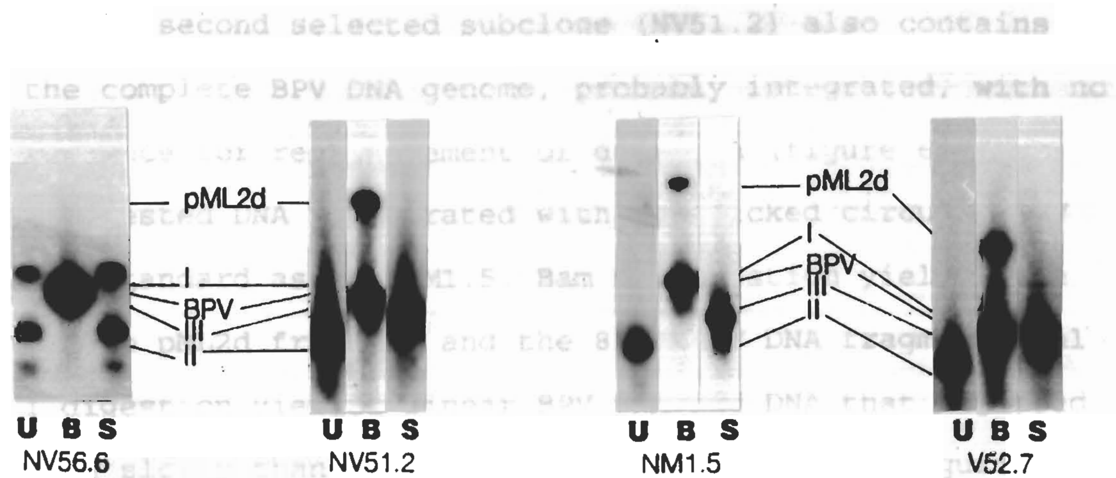


Figure 6: Southern blots of genomic DNA from four selected subclones

Gels were loaded with 1 µg of genomic DNA per lane and hybridized with ^{32}P-pdBPV-1. Southern blots shown are intentionally overexposed to illustrate faint bands. Abbreviations: U: undigested DNA, B: Bam digested DNA, S: Sal digested DNA. Origins of each lane are at the BOTTOM of the photograph. Markers which were digested at the same time as the samples and run on each gel: I: supercoiled (Form I) pdBPV-1 marker; II: nicked circular (Form II) pdBPV-1 marker; III: linear (Form III) pdBPV-1 marker; BPV: BPV DNA cleavage fragment from Bam digestion of pdBPV-1; pML: pML2d fragment from Bam digestion of pdBPV-1. Positions of Forms I and II markers apply only to undigested samples, positions of BPV and pML markers apply only to Bam-digested samples and position of the Form III marker applies only to Sal digested samples.

A second selected subclone (NV51.2) also contains the complete BPV DNA genome, probably integrated, with no evidence for rearrangement or deletion (figure 6). Undigested DNA co-migrated with the nicked circular BPV DNA standard as for NM1.5. Bam HI digestion yielded the 2.5 kb pML2d fragment and the 8 kb BPV DNA fragment. Sal I digestion yielded linear BPV DNA and DNA that migrated more slowly than the linear BPV DNA standard (figure 6). Though this may be due to incomplete Sal I digestion, it may also suggest integration. This would account for the lack of supercoiled BPV DNA in the undigested sample. Therefore two of the selected subclones, NM1.5, NV51.2 contain the complete BPV DNA genome, probably integrated.

A third selected subclone (V52.7) also contained integrated BPV DNA, but integrated at a different site. Genomic DNA contained BPV DNA migrating more slowly than the nicked circular standard (figure 6) and no indication of supercoiled BPV DNA. Bam HI digestion produced the two bands which correspond to BPV and to pML2d and also one band that migrated at about 4 kb. The faint band at 4 kb on Bam HI digestion may result from the "ends" of the chromosome which contain fragments of pdBPV-1. Digestion with Bam HI would remove all of the BPV and pML2d except the fragments attached to the chromosome. They would appear as a faint band. The presence of the faint,

lighter band in the digested samples, coupled with the lack of supercoiled BPV DNA and the material heavier than 23 kb in undigested DNA suggests (figure 6) that BPV DNA is complete and integrated though not at the Bam HI site.

One selected subclone contains episomal BPV DNA with deletions. As discussed previously, undigested DNA from NV56.6 shows only 2 bands migrating slightly faster than the BPV DNA nicked circular and supercoiled forms. Bam HI digestion resolves NV56.6 to one band migrating slightly faster than the linear BPV DNA standard. Sal I digestion does not alter NV56.6 DNA from the undigested state (figure 6). Probing with ^{32}P-pML2d (figure 4) shows pML2d DNA in both bands in undigested NV56.6 DNA and in the Bam HI and Sal I digested DNA. The simplest interpretation is that NV56.6 has only episomal BPV DNA which has one functional Bam HI site and has the Sal I site in pML2d altered or deleted.

An important objective of this project was to isolate subcloned cell lines which were promoter dependent. The analysis of the state of BPV DNA in these lines may suggest a basis for promoter dependency. NM1.9 was one of the two most promoter dependent of the subcloned cell lines. Undigested genomic DNA contained material hybridizing to ^{32}P-pdBPV-1 that corresponded to

supercoiled, nicked circular, and heavier than 23 kb DNA (figure 4). Digestion with Bam HI and Sal I had no effect nor did any DNA hybridize to ^{32}P-pML2d in undigested, Bam HI-digested or Sal I-digested samples (figure 4). Therefore, pML2d was completely deleted. Lack of Sal I digestion corroborates deletion of pML2d, since the Sal I site is in pML2d. The inability of Bam HI to digest the sample suggested that there were no Bam HI sites. Therefore, NM1.9 is a unique cell line which lacks pML2d and lacks Bam HI sites. It suggests a major deletion in pdBPV-1 and that the BPV DNA is episomal. It is tempting to speculate that the loss of part of the BPV DNA genome contributes to promoter dependency in this cell line.

B. Effects of promoter on cloned cell lines

1. Promoters enhanced focus formation in some lines but not in others.

The purpose of co-culturing transformed cells with untransformed C3H/10T½ cells was to determine which cell lines responded to promoter with increased numbers of foci. Not all cell lines responded in co-culture with increased numbers of transformed foci in the presence of promoter (NV56.6, V52.7 and NV51.2 cells, $p > 0.05$,

table 8). On the other hand, mezerein increased numbers of transformed foci in the mezerein-derived lines (NM1.9 and NM1.5 cells ($p < 0.05$) (table 8)). This leads to the hypothesis that if tumor promoters enhance transformation through alteration of copy number or state of BPV DNA, mezerein should alter copy number or state of BPV DNA in NM1.5 and NM1.9 cells but vanadate should not alter copy number or state of BPV DNA in NV51.2, NV56.6 or V52.7 cells.

TABLE 8

Number of foci formed in co-cultures of various cell lines with C2H/10T½ cells exposed to mezerein and vanadate

	Promoter			
subclone	none	+vanadate	+mezerein	
NV51.2	39±8	51±10	48±6	
NV56.6	52±6	54±5	48±3	
V52.7	39±4	35±7	47±3	
NM1.5	27±3	ND	52±7	
NM1.9	30±7	ND	59±6	

Co-cultures were seeded with 200 cells from the subclone and 2000 C3H/10T½ cells, passage 19. Cultures were incubated for 21 days continuously in 0.5 ng/ml of mezerein or 2 μM vanadate. Values given are averages of two experiments, a total of 4 - 6 plates for each value. Errors are standard errors of the mean. ND indicates that the value was not determined. Values for NV51.2, V52.7 and NV56.6 with vanadate were not significantly different from untreated controls ($p > 0.05$, using t-tests). Values for NM1.9 and NM1.9 incubated with mezerein differed significantly from untreated controls ($p < 0.05$, using t-tests).

2. Copy number of BPV DNA in cloned cell lines was unstable

For cell lines passaged in the absence of promoter, copy numbers fluctuated randomly (table 9). This resulted in lack of a baseline control by which to determine whether promoters altered copy number. Copy numbers are apparently unstable even in the absence of promoter. NV51.2 and NV56.6, which carried the largest copy numbers of BPV DNA appeared to be the most unstable (table 9). Therefore, it was not possible to determine whether or not alteration of copy number was correlated with increased formation of transformed foci in promoter dependent cell lines.

TABLE 9

Copy numbers of BPV DNA per genome in subclones passaged with or without promoters

	Passage #			
Subclone	1	4	7	10
NV56.6 -V	600	1025	750	325
NV56.6 +V	600	1450	1850	2250
NV51.2 -V	280	2212	2612	3625
NV51.2 +V	280	2087	-	1000
V52.7 -V	70	100	167	107
V52.7 +V	70	95	82	40
NM1.5 -M	50	52	80	62
NM1.5 +M	50	57	85	310
NM1.9 -M	63	50	30	50
NM1.9 +M	63	50	50	50

DNA was isolated from cells at passages 1, 4, 7 and 10 and copy numbers of BPV DNA determined as given in the methods section. All copy numbers were determined twice in separate slot blot experiments and values reported are means of these two values. For NM1.5 and NM1.9 promoter was 0.5 ng/ml of mezerein (±M). For NV56.6, NV51.2 and V52.7, promoter was 1 μM vanadate (±V).

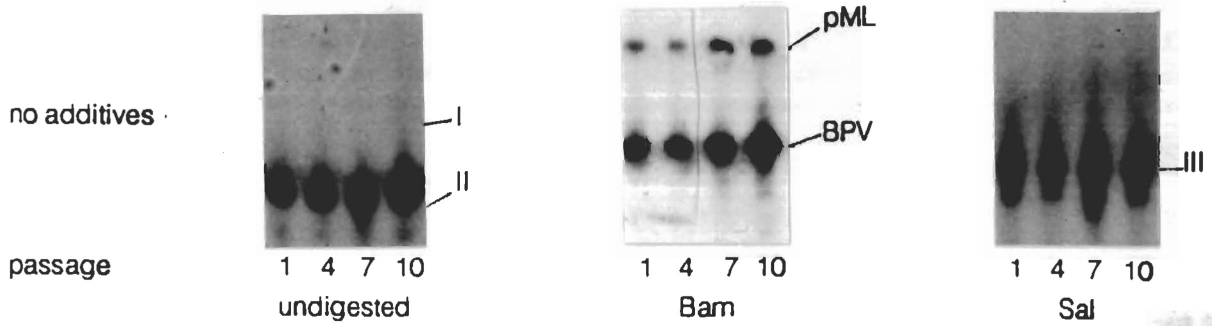
3. State of BPV DNA in subcloned cell lines appeared unaltered after 10 passages in tumour promoter.

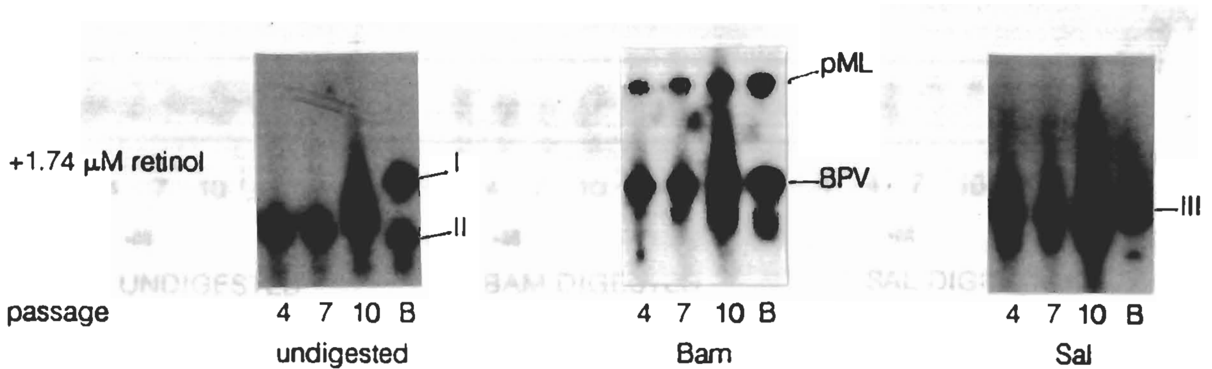
There was no detectable difference in the state of integration of BPV DNA in promoter dependent cell lines passaged in the presence or absence of promoter. Both NM1.5 (figure 7) and NM1.9 (figure 8) are promoter dependent but neither show changes in BPV DNA that is unique to treatment with promoter when passaged with promoter. There is an increase in BPV DNA migrating more slowly than pdBPV-1 in the Sal I digested samples of DNA from cultures of NM1.5 exposed to mezerein. However, an increase in the same class of DNA also occurred in NM1.5 cultures exposed to 250 μM ascorbate or to 1.74 μM retinol (figure 7). Therefore, the alteration is not unique to mezerein treatment.

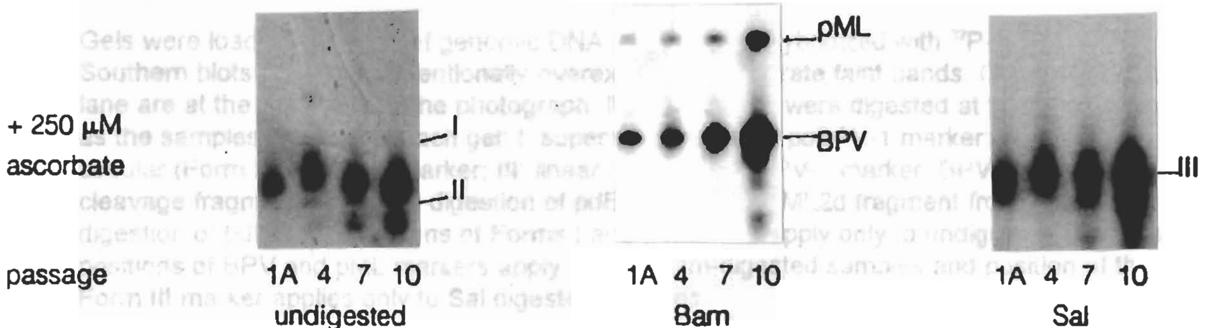
There is no detectable difference in the state of BPV DNA in vanadate-derived subclones passaged in the presence or absence of promoter. V52.7, NV51.2 and NV56.6 showed no detectable difference in the condition of BPV DNA whether passaged with or without vanadate.

Figure 7: Southern blots of genomic DNA from NM1.5 cells passaged for 10 passages in ascorbate, retinol or mezerein

Gels were loaded with 1 μg of genomic DNA per lane and hybridized with ^{32}P-pdBPV-1. Southern blots shown are intentionally overexposed to illustrate faint bands. Abbreviations: U: undigested DNA, B: Bam digested DNA, S: Sal digested DNA. Origins of each lane are at the BOTTOM of the photograph. Markers which were digested at the same time as the samples and run on each gel: **I**: supercoiled (Form I) pdBPV-1 marker; **II**: nicked circular (Form II) pdBPV-1 marker; **III**: linear (Form III) pdBPV-1 marker; **BPV**: BPV DNA cleavage fragment from Bam digestion of pdBPV-1; **pML**: pML2d fragment from Bam digestion of pdBPV-1. Positions of Forms I and II markers apply only to undigested samples, positions of BPV and pML markers apply only to Bam-digested samples and position of the Form III marker applies only to Sal digested samples.







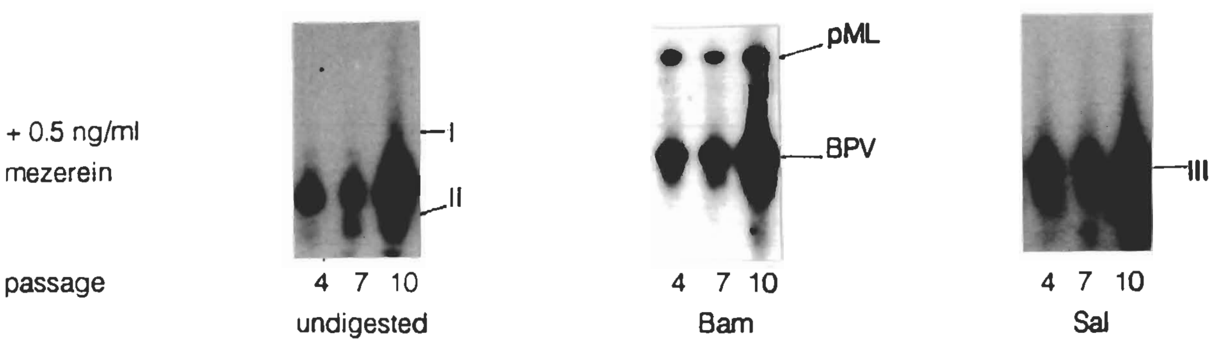


Figure 7: Southern blots of genomic DNA from NM1.5 cells passaged for 10 passages in ascorbate, retinol or mezerein

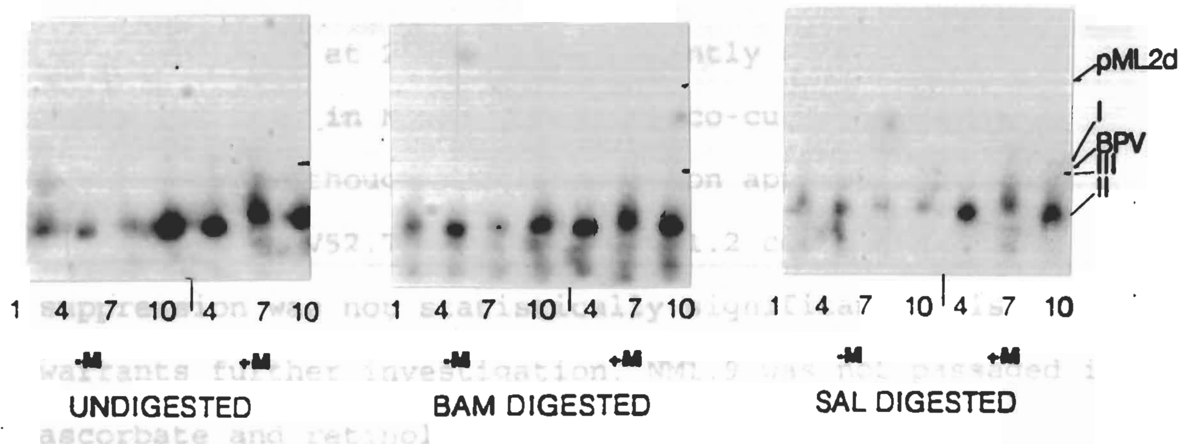


Figure 8: Southern blots of genomic DNA from NM1.9 cells passaged for 10 passages in mezerein.

Gels were loaded with 1 µg of genomic DNA per lane and hybridized with ^{32}P-pdBPV-1. Southern blots shown are intentionally overexposed to illustrate faint bands. Origins of each lane are at the BOTTOM of the photograph. Markers which were digested at the same time as the samples and run on each gel: I: supercoiled (Form I) pdBPV-1 marker; II: nicked circular (Form II) pdBPV-1 marker; III: linear (Form III) pdBPV-1 marker; BPV: BPV DNA cleavage fragment from Bam digestion of pdBPV-1; pML: pML2d fragment from Bam digestion of pdBPV-1. Positions of Forms I and II markers apply only to undigested samples, positions of BPV and pML markers apply only to Bam-digested samples and position of the Form III marker applies only to Sal digested samples.

C. Effect of retinol and ascorbate on cloned cell lines

1. Retinol and ascorbate inhibit focus formation by subcloned cell lines in co-culture

Ascorbate at 250 μM significantly decreased focus formation only in NV56.6 cells in co-culture ($p < 0.05$) (table 10). Although focus formation appeared to be suppressed in V52.7, NM1.5 and NV51.2 cells, this suppression was not statistically significant. This warrants further investigation. NM1.9 was not passaged in ascorbate and retinol.

Retinol at 1.74 μM suppressed focus formation by all four lines, to differing degrees. The most strongly inhibited was NV56.6 at 94% suppression. NV51.2 was the least strongly inhibited at 34% suppression (table 10).

It is clear that suppression of transformation by ascorbate or retinol differs in various cloned cell lines. Transformation by one cell line (NV56.6) is suppressed by retinol by 94%, but transformation of another cell line (NV51.2) is only suppressed by 33%. Suppression of transformation by ascorbate varies from 30% (NV56.6) to 16% (NM1.5). These cell lines will be useful to investigate the difference in mechanisms of action of ascorbate and retinol.

TABLE 10

Numbers of foci formed in co-cultures of subcloned cell lines with C3H/10T½ cells exposed to ascorbate and retinol

	Suppressor		
Subclone	none	ascorbate	retinol
NV56.6	51.6±6.0	36.3±5.5	2.7±2.3
NV51.2	48.3±7.6	36.0±2.6	31.7±4.7
V52.7	39.0±4.0	28.0±9.2	17.3±2.3
NM1.5	27.3±2.9	23.0±0.5	6.0±2.7

For co-cultures, 200 cells from the subclones were plated together with 2000 untransfected C3H/10T½ cells. They were incubated continuously in 250 μM ascorbate, 1.74 μM retinol or untreated medium for 21 days. Numbers of foci are means of 3 plates ± standard error of the mean. The experiment was repeated once with similar results. All values differed significantly from untreated controls ($p < 0.05$ using t-tests) except for NM1.5, NV51.2 and V52.7 exposed to ascorbate which did not differ significantly from untreated controls ($p > 0.05$).

Given the strong suppression of transformation of NV56.6 and NM1.5 cells by retinol, the immediate question is whether retinol is toxic to the cells. Subculture growth times (table 11) did not differ significantly ($p > 0.05$) in all 4 subclones regardless of whether they were passaged in normal medium, ascorbate, retinol, or promoters. This suggests that toxicity to the cells does not account for suppression by retinol of focus formation.

TABLE 11

Subculture growth times (hours) for cell lines passaged 10 times in 0.5 ng/ml mezerein, 2 μM vanadate, 1.74 μM retinol or 250 μM ascorbate

	treatment			
Line	none	promoter	ascorbate	retinol
NV56.6	30.7±6.1	30.1±6.2	31.2±6.3	29.6±3.5
NV51.2	23.8±3.7	24.6±3.3	23.8±4.4	23.9±2.7
V52.7	24.9±3.9	24.1±3.4	24.7±2.6	25.9±2.6
NM1.5	24.5±4.1	24.3±3.9	23.5±2.4	23.9±3.1

Subculture growth times are expressed in hours. Cells were counted with duplicate samplings for all 10 passages of each cell line in each treatment according to the protocol described in methods (chapter 3). Values are means of 10 passages ± standard error of the mean. No values are significantly different from untreated controls ($p > 0.05$ using t-tests).

2. Copy number of cloned cell lines is unstable and state of BPV DNA is not grossly altered in the presence of ascorbate or retinol

Alteration of copy number could not be determined in the presence of ascorbate and retinol for the same reason that it could not be examined in the presence of promoter. Large fluctuations in the copy numbers of untreated, control cultures, precluded determination of copy number alteration in the presence of ascorbate and retinol (table 12).

States of BPV DNA in the four subclones were unchanged by treatment with retinol or ascorbate from the untreated controls. In addition, retinol did not alter integration patterns of BPV DNA in V52.7 cells exposed or unexposed concurrently to vanadate.

In NM1.5 cells, there was an increase in the band migrating more slowly than the 8 kb band in Bam HI-digested DNA and in the band migrating more slowly than 10.5 kb in the Sal I digested DNA (figure 7). However, this same pattern occurred during passaging of NM1.5 in ascorbate and in mezerein but not in passages exposed to only normal medium (Figure 7). Therefore, the pattern is not unique to retinol treatment.

TABLE 12

Average copy numbers for cell lines passaged in the presence or absence of promoter, ascorbate or retinol.

Line	Treatment	Passage 1	4	7	10
NV56.6	none	600	1025	750	325
NV56.6	+retinol	-	850	1250	700
NV56.6	+ascorbate	-	1350	675	4375
NV51.2	none	280	2212	2612	3625
NV51.2	+retinol	-	2950	4637	3087
NV51.2	+ascorbate	-	687	250	275
NM1.5	none	50	52	80	62
NM1.5	+retinol	-	85	72	125
NM1.5	+ascorbate	-	32	42	80
V52.7	none	70	100	167	107
V52.7	+retinol	-	120	192	180
V52.7	+ascorbate	-	70	107	135
V52.7	+V +R	-	77	127	70

DNA was isolated from cells at passages 1, 4, 7 and 10 and copy numbers of BPV DNA determined as given in the methods section. All copy numbers were determined twice in separate slot blot experiments and values reported are means of these two values. Cell lines were passaged in 250 μM ascorbate or 1.74 μM retinol. V52.7 was also passaged in 1.74 μ retinol with 1μM vanadate ("+V +R").

DISCUSSION

One goal of this project was to identify several subcloned lines of cells from promoter-induced foci which were promoter dependent in co-culture with untransfected C3H/10T½ cells. Four (possibly 5) of the subcloned cell lines (NM1.9, NM1.1, NM2.6, NM2.8, and possibly NM1.5) responded to promoters with increased production of foci (table 5). All promoter dependent cell lines were isolated from mezerein-induced foci. All subcloned cell lines from vanadate-induced foci were promoter independent. Loss of promoter dependence may represent a further step in neoplastic transformation.

We attempted to isolate promoter dependent and promoter independent cell lines which were uncontaminated with untransformed cells. It should be noted that, although promoter dependent and promoter independent cell lines were cloned, then subcloned, some lines may still be contaminated with untransformed cells.

As part of the subcloning experiments, we questioned whether there was evidence for deletion, rearrangement or integration of the BPV DNA genome. We have shown that deletions and rearrangements exist in clones and subclones derived from vanadyl, vanadate and mezerein-induced foci (figures 3 - 5). There is evidence

for deletion of the plasmid pML2d in some lines (figure 4), deletion or lack of function of the Sal I or one of the Bam HI sites (figure 3), and rearrangement of the BPV DNA genome (figure 5). This suggests that BPV DNA is profoundly modified in succeeding generations of cells. This is consistent with previous reports of integration of BPV DNA and also integration and rearrangement of the HPV DNA genome in cultured cell lines and in carcinomas.

A related question is whether increasing rearrangement, deletion or integration accompanies progression from clone to subclone. The presence of BPV DNA corresponding to 2.5 kb in Bam HI-digested NV56 cells, and the lack of this fragment in 4 subclones derived from it is evidence for the increasing loss of function of one Bam HI site during subcloning (figure 3). On the other hand, it does not eliminate the possibility that random selection during subcloning simply produced this particular array of subclones.

A third objective was to determine whether focus formation by subcloned cell lines could be suppressed by retinol and ascorbate. Ascorbate suppressed focus formation only by NV56.6 cells in co-culture (table 10). Retinol suppressed transformation of all four lines tested (table 10). This indicated that phenotypic

expression of the subclones could be altered by tumor promoters and suppressors.

We questioned whether phenotypic response to promoters, ascorbate or retinol was correlated with alteration of copy number or integration. Instability and random fluctuation of copy number in the cell lines resulted in the inability to determine whether alteration in copy number was correlated with response to promoters, retinol or ascorbate (tables 9 and 12). However, no detectable alteration of integration of BPV DNA was observed in any of the cell lines after 10 passages in ascorbate, retinol or promoters (figures 7 and 8).

A possible explanation for the ability of retinol to inhibit focus formation would be that retinol acts at the time of transfection to prevent efficient transfection by BPV DNA, uptake of promoter or some genetic or epigenetic processes essential to transformation. However, retinol added as late as 11 days after transfection is still capable of inhibiting transformation (chapter 3). In addition, the ability of retinol to inhibit focus formation of BPV-carrying cell lines argues against the hypothesis that retinol acts at the time of transfection. Therefore, it is unlikely that it prevents early processes in transfection or promotion.

The other side to this argument is that retinol may act at the time of confluence as it does in transfected C127 cells (19). Subcloned cell lines exposed to retinol were always passaged before confluence. However, morphological changes were obvious in subcloned cell lines exposed to retinol. Cells became rounder, flatter, less spindle-shaped, less overgrown and grew in a more orderly, swirl-like pattern characteristic of fibroblasts. This suggests that, though retinol may not alter integration, it can modify the transformed phenotype.

Retinoids inhibit promoter-mediated enhancement of ornithine decarboxylase activity (20), induce expression of keratins by keratinocytes (21), alter gene expression by means of retinoic acid receptors (22) and alter expression of N-*myc* in human neuroblastoma cells (23). These studies lead to the hypothesis that retinol acts through modulation of viral or cellular gene expression to suppress transformation of BPV DNA-carrying C3H/10T½ cells. Investigation of the effect of retinol on viral or cellular oncogene expression in the transformed cell lines can resolve this question.

Ascorbate also suppressed focus formation in one BPV DNA-carrying cell line, but it did not alter integration

of BPV DNA. A likely possibility is that ascorbate acts at the time of transfection or early in the process of promotion leading to transformation and viral integration. Both mezerein and vanadate may promote transformation by means of active oxygen intermediates (24 - 27). Ascorbate could inhibit this process by scavenging active oxygen intermediates (28 - 30). If this is the case, ascorbate as an active oxygen scavenger, would have little effect on integration of preexisting viral genomes.

Nonetheless, ascorbate did inhibit transformation of one transformed cell line in co-culture (table 10). Possibly, some BPV DNA-carrying cell lines (NV56.6) respond to agents which generate active oxygen by becoming more transformed; i.e., BPV DNA-carrying cells become increasingly transformed in the presence of oxidants. However, the counter to this argument is that NV56.6 cells do not form more foci in the presence of vanadate. This unresolved dilemma points out the possibility that ascorbate may suppress transformation by additional mechanisms, such as alteration of collagen gene expression in some transformed cells (31).

The most interesting result of these preliminary experiments is the development of cell lines which carry BPV but which do not form foci in co-culture. These lines

suggest that other factors control phenotypic expression of transformation. The next step will be to explore viral gene expression in these cell lines and the effects of surrounding, untransformed cells on focus formation.

APPENDIX 1: RESTRICTION ENDONUCLEASE ANALYSIS OF BPV DNA

Undigested BPV DNA that is not integrated into cellular DNA exists in two forms: a supercoiled form (Form I) which resembles a double-stranded circle twisted like a rubber band and migrates at 6.4 kb on 0.7% agarose gels. The second form is nicked circular (Form II) which is a double stranded circle with one cut in one of the strands. It migrates at approximately 23 kb. Therefore, if BPV DNA is unintegrated (episomal) and exists in single copies in the cell, undigested DNA hybridized with ^{32}P-pdBPV-1 should yield two bands migrating at 6.4 and 23 kb.

However, in our gels, genomic DNA also migrates at 23 kb or slower. Therefore, a 23 kb band hybridizing to ^{32}P-pdBPV-1 could be integrated into cellular DNA or nicked circular and episomal. For this reason, the best indication of episomal BPV DNA is the presence of the supercoiled form.

Lack of supercoiled BPV DNA and presence of DNA greater than 23 kb hybridizing to ^{32}P-pdBPV-1 does not necessarily indicate that BPV DNA is integrated. BPV DNA may exist as multimers which could be tandem repeats formed into one large circle or many small circles linked

together like pretzels. Neither of these forms is integrated into the cellular genome. To distinguish between episomal multimers and integrated copies, further enzymatic digestion is needed.

Sal I can distinguish between episomal multimers and integrated copies in some cases. Sal I digestion of multimers would cut each unit of BPV DNA once at the Sal I site. This would produce a single band of linear BPV DNA (Form III) migrating at 10.5 kb. In the case of integrated copies, as long as they are not integrated at the Sal I site, Sal I digestion would produce either a smear (integrated randomly), two bands (integrated nonrandomly) corresponding to the two "ends" of the chromosome which contained the BPV DNA, or a band corresponding to Form III BPV DNA and a smear or 2 bands (tandem repeats). In practice, using only Bam HI and Sal I restriction enzyme digestion, it is difficult to determine how BPV DNA is integrated. The best use of Sal I digestion is to confirm lack of integration of BPV DNA migrating more slowly than 23 kb by production of the 10.5 kb band, especially in the absence of supercoiled BPV DNA.

Bam HI digestion is primarily useful to indicate the presence or absence of the plasmid pML2d. This is

important because it confirms deletion of part of the pdBPV-1 plasmid. This can point the way to possible rearrangement and integration of BPV DNA, especially if integration is suggested by analysis of undigested and Sal I digested DNA samples. Bam HI digestion can have three possible outcomes: two bands migrating at 2.5 kb and 8 kb, one band migrating at 8-10 kb, or several bands migrating at other positions.

In the simplest case, production of the 2.5 and 8 kb bands indicates that the entire plasmid is present. Production of one band migrating at 8-10 kb and hybridizing to ^{32}P-pdBPV-1 requires further hybridization with ^{32}P-pML2d to confirm the presence or absence of pML2d. Deletion of part of the pdBPV-1 genome may render one or two Bam HI sites non-functional. In that case, pML2d would be present but unable to be digested into a 2.5 kb fragment by Bam HI. Hybridization with ^{32}P-pML2d would indicate the presence of pML2d migrating at approximately 8-10 kb depending on the size of the deletion. The limits of resolution of 0.7% agarose gels are approximately 2 kb in the 10 kb range. Therefore, only very large deletions, or deletions of restriction sites, could be identified.

Rearrangements are suggested when Bam HI digestion yields several bands of various sizes. Several Bam HI sites may be introduced when parts of the pdBPV-1 genome are spliced together. Again, hybridization with ^{32}P-pML2d can indicate whether pML 2d is present in the various bands.

Bam HI digestion can be ambiguous in terms of whether or not BPV DNA is episomal or integrated. Like Sal I digestion, it can resolve bands greater than 23 kb arising from multimers into the 2.5 and 8 kb fragments but only if the Bam HI sites are present. In addition to the 2.5 kb and 8 kb bands, BPV DNA that is integrated, not at the Bam HI site, will produce two faint bands corresponding to the "ends" of the chromosomes which contain the integrated BPV DNA. Lack of supercoiled BPV DNA, lack of formation of linear BPV DNA by Sal I digestion and presence of faint bands in addition to the 2.5 kb and 8 kb bands on Bam HI digestion, taken together can suggest integration of BPV DNA.

Restriction endonuclease analysis of BPV DNA in genomic DNA is a preliminary technique by which to screen for cell lines which may contain integrated or rearranged BPV DNA. Further analysis that could confirm integration

would include 2-dimensional gel electrophoresis and analytical centrifugation.

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CHAPTER 5

CONCLUSIONS AND FUTURE PERSPECTIVES

CONCLUSIONS

1. Vanadium, arsenic and chromium enhance transformation of BPV DNA-transfected C3H/10T½ cells

An objective of this project was to determine whether carcinogenic metals would enhance transformation of C3H/10T½ cells. In chapter 2 we have shown that exposure to vanadate and vanadyl results in increased numbers of transformed foci. Numbers of transformed foci in BPV DNA transfected C3H/10T½ cells exposed continuously to vanadate for 21 days increased in a dose dependent manner to 50-fold at 4 μM vanadate. This is similar in magnitude of response to mezerein at 0.5 ng/ml. Vanadate can enhance transformation even if added 96 hours after transformation, although it requires treatment for longer than 24 hours to enhance transformation.

Arsenate and arsenite increased numbers of transformed foci by approximately 6-fold. The major actions of arsenic lie in its ability to enhance gene amplification and to inhibit DNA repair mechanisms. Arsenic does not enhance transformation as strongly as vanadium species which enhance transformation 25-50-fold therefore, these are probably not major factors in enhancement of transformation. Chromium (III) enhanced

transformation by 1.4-fold. Chromium binds to DNA, causing strand breaks, and generating active oxygen. By the same reasoning, the weak enhancement of transformation by chromium suggests that these are not major events in enhancement of transformation. Vanadium does inhibit tyrosine phosphatases. This implicates alteration of tyrosine phosphorylation by inhibition of tyrosine phosphatases or stimulation of tyrosine kinases as a major pathway for enhancement of transformation of BPV DNA-transfected cells.

2. Ascorbate and retinol inhibit mezerein- and vanadium-mediated enhancement of transformation

In chapter 3, we explored ascorbate and retinol as possible inhibitors of enhancement of transformation of BPV DNA-transfected C3H/10T½ cells. Ascorbate decreased the effect of vanadium by approximately 50% and the effect of mezerein by 36%. Retinol applied continuously with vanadate, vanadyl or mezerein for 21 days strongly suppressed enhancement of BPV DNA-mediated transformation. Retinol applied after 11 days of treatment with vanadium or mezerein decreased vanadate- and vanadyl-enhanced transformation by 90% and mezerein-enhanced transformation by 66%. Retinol also inhibited

focus formation in co-cultures of subcloned lines of cells which carried BPV DNA.

There are several possible mechanisms by which ascorbate and retinol may inhibit the transformed phenotype. Ascorbate may inhibit transformation in three possible ways: scavenging active oxygen derivatives, modification of the extracellular matrix by collagen production, and alteration of cellular gene expression. The major action of retinol lies in its ability to alter gene expression. It was not possible to exclude any of these hypotheses on the basis of these experiments. Suppression of transformation in BPV DNA carrying cell lines suggests that retinol acts on neoplastic processes which regulate the transformed phenotype; contact inhibition or gene expression.

3. Some subclones responded to promoters, ascorbate and retinol in co-culture assays

An important objective was to isolate subcloned cell lines which responded to promoter with increased numbers of transformed foci and to ascorbate and retinol with decreased transformation. Four of the mezerein-derived subclones responded to mezerein with enhanced

transformation (promoter dependency). The vanadate-derived subclones were equally transformed in the presence or absence of vanadate. Retinol decreased transformation of both promoter dependent and promoter independent cell lines in co-culture with C3H/10T½ cells. The most important conclusion to be drawn from the co-culture assays with subcloned cell lines is that retinol and mezerein, and to some extent ascorbate, can alter phenotypic expression of transformation long after transfection and the neoplastic process that produced the initial focus.

4. There is some evidence that BPV DNA is integrated, deleted or rearranged in some clones and subclones

In chapter 4, we explored integration and copy number of BPV DNA in cell lines subcloned from foci which developed in transfected cells exposed to mezerein and vanadium. An objective was to determine whether there was any evidence for integration of BPV DNA in subcloned cell lines.

DNA isolated from clones and subclones from BPV DNA-transfected C3H/10T½ cells showed evidence of deletion of pML2d, deletion of other parts of the plasmid DNA,

rearrangement and possibly integration of BPV DNA. This is consistent with other studies which have demonstrated integration, deletion and rearrangement of BPV DNA in cell lines subcloned from transfected C127 cells.

A final objective was to investigation alteration of integration and copy number in subcloned cell lines passaged in ascorbate, retinol, vanadate or mezerein. We selected five subclones which were passaged for 10 passages in ascorbate, retinol, mezerein or vanadate. Integration of BPV DNA in subclones passaged in ascorbate, retinol, vanadate or mezerein was unchanged from integration of BPV DNA in untreated controls. There was evidence for instability and random fluctuations in copy number of BPV DNA in subcloned cell lines. Due to instability and random fluctuation of copy number during passaging, it was not possible to determine whether exposure to promoters, ascorbate or retinol altered copy number. The effect of promoters, ascorbate and retinol on copy number merits further investigation.

FUTURE DIRECTIONS

This project has demonstrated that the carcinogenic metals vanadium, arsenic and chromium enhance BPV DNA mediated transformation. Future experiments should investigate the synergistic action between carcinogenic metals and BPV DNA with emphasis on developing an explanatory model of this interaction.

A possible model can be postulated by drawing together the evidence presented in this thesis. Several cell lines subcloned from foci induced in BPV DNA-transfected C3H/10T½ exposed to vanadate or mezerein lack DNA which hybridizes to ^{32}P-pML2d. Several subcloned cell lines clearly lack at least one Bam HI site or the Sal I site. This demonstrates unquestionably that part of the originally-transfected pdBPV-1 plasmid has been deleted.

Several questions are raised by these results. Are the deletions a specific result of treatment with vanadate or mezerein or are they a general result of the subcloning process? Does deletion occur early in the synergistic interaction between BPV DNA and tumor promoters (at the time of transfection) and what role does it play? Are the BPV DNA genomes integrated and does deletion parallel integration?

Although a few foci do develop in cultures which are transfected but not exposed to tumor promoters, exposure of BPV DNA-transfected C3H/10T½ cells to tumor promoter is necessary to produce foci from which clones can be developed. Therefore, it is difficult to control for alterations in BPV DNA that would occur in foci not caused by tumor promoters. For this reason, the question of whether deletions or rearrangements are a result of specific tumor promoters or a result of the subcloning process cannot be answered. Nonetheless, no tumor promoter dependent cell lines developed from vanadate-derived foci and five promoter dependent lines developed from mezerein-derived foci. This suggests that vanadate and mezerein have different actions in the neoplastic process. On the other hand, vanadate and mezerein may have similar actions initially, but vanadate may have additional neoplastic effects.

Neither mezerein nor vanadate in the medium were necessary for the maintenance of viral copies in subcloned cell lines, although viral copy number fluctuated from passage to passage. This again suggests that some irreversible first step, involving tumor promoters, occurred at the time of transfection, or during the 21 days of incubation with tumor promoter, that ensured the persistence of viral copies. Not all

subcloned cell lines contained deletions or unambiguously integrated BPV DNA; therefore, the essential first step does not require deletion or integration.

The initial first step in the synergistic interaction between tumor promoters and BPV DNA may involve active oxygen species. Ascorbate at 100 μM suppressed development of vanadate-mediated foci by approximately 50% *in transfected cells*. Conversely, ascorbate at 250 μM suppressed development of transformed foci in co-cultures using *transformed cells* by a maximum of 31% (NV56.6). Ascorbate did not significantly ($p > 0.05$) decrease numbers of transformed foci in 3 out of 4 lines tested. Both mezerein and vanadate may promote transformation by means of active oxygen intermediates (1). Ascorbate could inhibit this process by scavenging active oxygen intermediates (2-4). If the initial first step involves active oxygen and ascorbate can scavenge this active species, this may prevent development of transformed foci in transfected cells.

On the other hand, retinol, which acts mainly at the level of controlling gene expression (5) effectively suppresses focus formation both in transfected and transformed cells. This points to an important role of gene expression in transformed cells.

What is involved in "establishing" BPV DNA genomes in the transfected cells? Active oxygen generated by tumor promoters or redox-active metals may prepare the cells, possibly by single strand breaks in DNA and recombination repair, for establishment of the viral genome. This is clearly not sufficient since redox-active metals like chromium only weakly enhance the numbers of foci produced by BPV DNA transfected C3H/10T½ cells (chapter 2). Both vanadate and mezerein induce mitosis, probably via the phosphatidylinositol pathway; therefore, mitosis is required for establishment of the viral genome. Once the viral genome is established, viral gene expression would transform the cells morphologically and induce loss of contact inhibition. Clearly, a logical next step in this investigation is to examine viral gene expression in the transformed cell lines, especially the promoter dependent lines.

Further exploration of this model might also include use of inhibitors of active oxygen species. The model postulates that vanadate or mezerein enhance transformation by a combination of mitogenic activity and generation of active oxygen species. Catalase or superoxide dismutase added to the medium did not inhibit vanadate-mediated enhancement of transformation (chapter 2). However, the experiment lacked a positive control. In

addition, catalase and superoxide dismutase added to the medium may not have had access to intracellularly-generated active oxygen species. Other inhibitors, such as mannitol or diethyldithiocarbamate (6), may prove more effective. Other variations on this theme would be to chelate the metal ions using EDTA in the medium, or to inhibit the mitogenic effects of the tumor promoters by inhibitors of phosphatidylinositol metabolism.

Another avenue of exploration of this model would be examination of synergistic effects of metal ions in enhancing transformation. Arsenic may enhance transformation primarily by interfering with DNA repair. Vanadate may enhance transformation by a combination of active oxygen generation and tyrosine phosphatase inhibition. Therefore, various combinations of vanadate and arsenic should synergistically enhance transformation. A similar question is whether small concentrations of vanadate or mezerein can enhance the ability of chromium to increase BPV DNA-mediated transformation. These experiments have important consequences in human carcinogenesis since humans are more commonly exposed to combinations of environmental pollutants over time rather than one pure metal or tumor promoter.

Combinations of metals and tumor promoters may also be investigated using the subcloned cell lines. Vanadate may further enhance transformation in promoter dependent cell lines subcloned from mezerein-mediated foci.

Another area of investigation, using the subcloned cell lines or transfected cells, is the ability of combinations of ascorbate and retinol to inhibit transformation. Although ascorbate alone does not strongly inhibit expression of the transformed phenotype of BPV DNA-carrying cell lines, retinol with ascorbate may prove more effective than retinol alone.

Comparison of the abilities of stage 1 and stage 2 tumor promoters to enhance BPV DNA-mediated transformation may also suggest mechanisms involved in enhancement of tranformation by tumor promoters. In the original mouse skin 2-step model of carcinogenesis (7), complete tumor promoters such as 12-tetradecanoyl-phorbol-acetate (TPA) and stage 1 tumor promoters such as hydrogen peroxide require only 1 application to mouse skin, and are not inhibited by retinoic acid. The effects are partially irreversible. In contrast, stage 2 tumor promoters, such as mezerein, require multiple applications. The effects are initially reversible, then irreversible. Enhanced ornithine decarboxylase activity

and altered levels of polyamines are involved in stage 2 tumor promotion. Stage 2 tumor promoters are inhibited by retinoic acid (7). Based on these criteria, vanadate, like mezerein, is a stage 2 tumor promoter. However, cell lines subcloned from vanadate-induced foci are promoter independent; unlike some cell lines subcloned from mezerein-induced foci. This suggests significant differences in the biochemical actions of vanadate and mezerein. Investigation of some stage 1 promoters, such as enzymatically-generated peroxide would be useful in investigating the biochemical mechanisms involved in the initial stages of establishing the BPV DNA genome in cells. Testing of vanadate in the abovementioned standard mouse skin, 2-step test would resolve this question *in vivo*. Another avenue of investigation would be to expose transgenic mice carrying the BPV DNA genome to vanadate. This could also suggest differential organ-sensitivity to the promoting effects of vanadate.

The difference in promoter dependence of vanadate-derived and mezerein-derived cell lines suggests that vanadate may enhance focus formation in BPV DNA-transfected C3H/10T½ cells by a different route than mezerein. Transfected cells exposed to vanadate, but not to mezerein lose the ability to adhere to the substrate (chapter 2). Taken together with the ability of vanadate

to inhibit tyrosine phosphatases (8) or to enhance tyrosine kinase activity (9), this suggests another area of investigation: alteration of tyrosine phosphorylation either or cell surface receptors or of cytoskeletal proteins. NIH 3T3 cells transfected with *fps/fes*, a src-like oncogene, develop increased numbers of foci in the presence of vanadate (10). This may be due to inhibition of the phosphatase which would normally inhibit action of the oncogene (10). The E5 ORF causes enhanced phosphorylation of the PDGF receptor in C127 cells transfected with BPV DNA (11). Therefore, vanadate may enhance mitosis of BPV DNA-transfected C3H/10T½ cells through an initial step involving the E5 ORF and phosphorylation of the EGF or PDGF receptors.

Another direction to pursue will be the effect of untransformed cells on surrounding cells on transformed cells. The subclones offer a range of phenotypic expression, from promoter independent to promoter dependent. Surrounding cells may suppress transformation of promoter dependent subclones, but not of promoter independent subclones. It would be interesting to explore whether mixtures of promoter dependent and independent cells can enhance or suppress the phenotype of each other.

Site-specific integration and activation of cellular oncogenes are also important areas to investigate, especially in the subclones which are highly transformed but carry only a few copies of BPV DNA. Some human papillomaviruses integrate near cellular oncogenes, resulting in amplification or enhanced oncogene expression (12). This suggests that BPV DNA may be integrated near cellular oncogenes in these cell lines.

The most valuable outcome of this project was the isolation of promoter dependent cell lines. These are an invaluable asset in exploring the synergistic effect of tumor promoters and BPV DNA in neoplastic transformation.

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