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# THE EFFECTS OF RETINOIC ACID ON MOUSE CELLS TRANSFORMED BY BOVINE PAPILLOMAVIRUS TYPE-1

*The University of Arizona* PH.D. 1984

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# THE EFFECTS OF RETINOIC ACID ON MOUSE CELLS TRANSFORMED BY BOVINE PAPILLOMAVIRUS TYPE-1

by

Marilyn Jean Henley

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A Dissertation Submitted to the Faculty of the

DEPARTMENT OF MOLECULAR AND MEDICAL MICROBIOLOGY

In Partial Fulfillment of the Requirements
For the Degree of

DOCTOR OF PHILOSOPHY
WITH A MAJOR IN MOLECULAR BIOLOGY

In the Graduate College

THE UNIVERSITY OF ARIZONA

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As members of the Final Examination Committee, we certify that we have read the dissertation prepared by Marilyn Jean Henley

entitled The effects of retinoic acid on mouse cells transformed by bovine papillomavirus type-1

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SIGNED: Marilyn J. Henley

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## ABSTRACT

The purpose of this research was to determine the effects of retinoic acid on bovine papillomavirus (BPV) transformed cells. Since BPV transformed cells are able to form colonies in agar and their untransformed counterparts are not, of particular interest was the effect of retinoic acid on this marker. Retinoic acid inhibited the growth of colony forming cells, but the extent of inhibition varied among several cloned cell lines. Retinoic acid inhibited the proliferation of BPV transformed cells and the extent of this inhibition also varied.

The copy number of the virus was determined for each of three cell lines by liquid reassociation experiments. The copy number did not change when the cells were treated with RA for a prolonged period of time. Gel electrophoresis and Southern blotting of extrachromosomal DNA from the cell lines revealed the presence of unit length BPV DNA in all three transformed cell lines. The amount of BPV DNA per cell was decreased when the cells were treated with $10^{-5}$ M RA for three days.

# INTRODUCTION

The papillomaviruses along with the polyomaviruses comprise the papovaviridae family. The papillomaviruses contain double-stranded super-coiled DNA with a molecular weight of about $5 \times 10^6$. Little is known about the molecular biology of the papillomaviruses since there is no known *in vitro* system for propagating them. The papillomaviruses are the etiological agent of warts and have been described in eighteen animal species [1, 2]. Five types of bovine papillomaviruses have been described based on restriction endonuclease mapping, nucleic acid homology and histopathology [2, 3, 4, 5, 6]. Generally these viruses produce self-limiting tumors in cattle; however, two viruses, BPV-1 and BPV-2, can produce malignant fibropapillomas [6], and BPV-4 is associated with alimentary canal tumors. BPV-1 identified by restriction endonuclease mapping will be used in this study.

BPV-1 has been implicated in malignant tumor formation in calves, horses, mice and hamsters [2, 7, 8, 9, 10, 11]. Cells from BPV induced tumors in calves and horses contain large numbers of BPV genome equivalents [2, 8, 12]. In productive papilloma infections, mature virions can be detected only in the nuclei of degenerated cells in the

keratinizing layer of the epithelium. When the papilloma undergoes transformation to carcinoma only viral DNA can be detected in the involved cells. The role of the host cell genome in papilloma-induced carcinoma has been noted in rabbit papillomavirus infections. The incidence of these carcinomas is high in wild rabbits but low in domestic rabbits [2]. The in vivo effects of a putative tumor promoter have been reported for BPV-4 papillomavirus-induced carcinomas. The incidence of these carcinomas is increased by repeated contact with the bracken fern [13].

BPV-1 can transform bovine, mouse and hamster cells in culture [2, 9, 14, 15, 16]. Recently transformation of a mouse mammary tumor cell line, C127, by BPV-1 has been reported [17]. These cells do not contain infectious virus, grow in soft agar, and are tumorigenic in nude mice. Molecularly cloned BPV DNA can transform C127 cells [18]. Even a subgenomic fragment of BPV-1 DNA can transform these cells, but the efficiency of transformation is low. BPV DNA isolated from C127 transformed by the intact virus, the cloned BPV genome or the subgenomic fragment is exclusively non-integrated [18, 19]. Multiple copies of supercoiled or open circular monomers representing the entire BPV genome were found in C127 transformed by the inact virus and the molecularly cloned linear BPV genome. Multiple copies of supercoiled or open circular monomers of the subgenomic fragment

were found in C127 transformed by the fragment. Catenated circular forms of BPV DNA were found in C127 transformed by all three methods [18, 19].

Virally transformed cells have been used to study the interactions between a virus and its mammalian cell host and the defective growth control of tumor-like cells. Cellular transformation is defined by a change in cellular morphology, increased saturation density, reduced serum requirement, anchorage-independent growth and the production of tumors in experimental animals. The molecular events which lead to these phenotypic manifestations of viral transformation by papovaviruses have been extensively studied in the cases of polyomavirus and simian virus 40, SV40. In early experiments growth in soft agar was the definitive characteristic of virally transformed cells [20]. The molecular events which lead to these phenotypic manifestations in BPV transformed cells are a complete mystery.

The ability to grow in semisolid medium has been widely used as a reliable indicator of tumorigenicity in a variety of spontaneous and chemically induced tumor cells. According to the stem cell theory of cancer biology [21], these colony-forming cells have the proliferative capacity to maintain a tumor. Nutrients, growth factors and anti-cancer agents have been shown to modulate the growth of colony-forming cells [22, 23]; thus studies utilizing this

marker are relevant to the study of the mechanism of carcinogenesis.

Vitamin A is a fat-soluble substance that is required for growth, the prevention of night blindness, epithelial cell differentiation and normal reproductive capacity in mammals. In recent years the vitamin has been shown to be an effective inhibitor of neoplastic development. It has been known since the 1920s that the incidence of epithelial cancer is increased in vitamin A-deficient individuals. Dietary vitamin A is metabolically converted to one of several analogs for use in numerous vitamin A-requiring functions. Vitamin A analogs are known collectively as retinoids, and retinoic acid (RA) is considered to be the active form of the vitamin in growth functions [24]. The work presented here developed from investigations into the effects of RA on the growth of BPV-1 transformed C127 in agar-containing medium.

The mechanism of action of retinoids is unknown at this time. Two specific retinoid-binding proteins have been implicated in mediating the effect of retinoids on cells in culture: cellular retinoic acid binding protein, CRABP, and cellular retinol binding protein, CRBP. The distribution of CRABP and CRBP is different in adult animal tissues. CRBP has not been detected in serum, but has been detected in all tissues studied except muscle. CRABP has been detected in bladder, brain, eye, mammary gland, ovary, prostate, skin,

testis, trachea and uterus. A survey of fetal organ of the rat showed that both CRBP and CRABP could be detected in all tissues, but neither protein could be detected in fetal rat serum. These proteins are found in spontaneously occurring and experimentally induced tumors. In some malignancies the level of cellular retinoid binding proteins is different than in normal, age- and type-matched tissue [25].

The effects of RA have been studied on a variety of spontaneously, chemically and virally transformed cells in culture [24]. Good correlation has been found between the presence of CRABP and growth inhibition by RA in several cell lines, but the extent of inhibition has not been correlated with the level of binding protein present [24]. It is interesting that the papovaviral transformants tested so far are not affected by RA. The widely studied mouse fibroblast cell line, 3T3, shows a decrease in exponential growth rate and a greatly reduced saturation density in the presence of RA. CRABP was detected in these cells. SV40 transformed 3T3 (SV3T3) are not affected by RA and CRABP was not detected in these cells [26]. This is consistent with the finding in our lab that SV3T3 are not growth-inhibited in agar in the presence of RA [27]. There is conflicting data regarding the presence of CRABP in SV3T3. Other investigators (25) were able to detect CRABP in SV3T3 obtained from another source. Two lines of murine sarcoma virus transformed 3T3 are not

inhibited by RA but a high level of CRABP was detected in these cells. Baby hamster kidney (BHK) cells are growth-inhibited, although slightly by RA; however, polyoma transformed BHK are not significantly inhibited by RA [24].

In contrast, marmoset lymphocytes containing Epstein Barr viral DNA are inhibited by RA [28]. Inhibition is evident only at higher cell densities and is similar in B and T cell lines. The status of CRABP in these lines is unknown. The Epstein Barr virus (EBV) genome, which exists as a linear molecule in the virion, exists as either a supercoiled or open circular multiple copy plasmid in lymphocytes. Catemeric forms of EBV DNA may also exist in lymphocyte cell lines carrying the virus. There is some evidence that EBV genes may be integrated into cellular DNA. The EBV plasmid replicates in synchrony with cellular DNA and EBV episomes are maintained in the same copy number in cell lines which carry them. EBV genomes are induced into replication with difficulty; a doubling of copy number can be achieved by treating the cells with iododeoxyuridine or bromodeoxy uridine [29]. This induction can be reversed by RA [30].

The effects of vitamin A on tumor formation <u>in vivo</u> were first studied on chemically induced tumors in mice. Retinoids were found to be effective in preventing and treating these tumors [31]. These effects are also seen in Rous sarcoma virus induced sarcomas in chickens and hamsters [32]

and on Moloney murine sarcoma virus induced sarcomas in mice [33]. Of particular interest here are tumors induced in domestic rabbits by the Shopes papillomavirus (SPV). RA treated SPV induced tumors are reduced in size, grow slower, and continue to regress even when RA treatment is stopped. The appearance of tumors in SPV-infected rabbits is significantly delayed by the injection of RA [34]. It has also been found that these tumors contain CRABP and that the level of CRABP increases with the age of the tumor [35].

In several experimental models known for the two-stage process of carcinogenesis, RA has been identified as a promotion antagonist [36, 37, 38, 39]. 12-0-tetradecanoyl-phorbol-13-acetate (TPA) is a potent tumor promoter and has been widely used in elucidating the molecular mechanism of tumor promotion. Recent research in the field of carcinogenesis has approached the study of the biochemical mechanism by which retinoids inhibit neoplasia by the consideration that TPA promotes neoplasia. Accordingly, RA may interfere with the biochemical processes induced by TPA.

The effects of TPA on cells in culture is diverse. TPA has been reported to produce both mitogenic and inhibitory influences on DNA synthesis [40]. Specific enhancement of eucaryotic episomal DNA by TPA has been reported, indicating a role for TPA in gene amplification [41]. In several EBV-containing lymphocyte cell lines, TPA has been shown to

increase the copy number of the episome ten to thirty times, and in other EBV-containing cell lines TPA can induce the EBV early antigen and/or mature virions [42, 43]. Similar effects have been shown for the BPV episome. TPA can increase the episomal BPV DNA content of BPV-transformed mouse embryo fibroblasts and cause the establishment of persistent infections in cells which otherwise lose the episome after several passages [44, 45]. This induction of replication is accompanied by induction of BPV transcripts which are identical to those found in BPV-induced tumors [45].

TPA can induce reversible phenotypic changes resembling virus-induced transformation [40]. This suggests a similarity between the mechanism of viral transformation and tumor promotion events. Todaro has suggested that tumor viruses act by inducing cells to produce normally repressed or inactive growth-promoting factors [39]. In this model initiation involves gene damage that facilitates the production of normally repressed growth factors and/or their cellular receptors. Initiated cells might be producing some, but insufficient quantities of these growth-promoting factors to cause phenotypic transformation. Endogenous promoters induced by the expression of the viral genome, or TPA would allow the critical concentration of growth factors for phenotypic transformation to be attained.

In keeping with the Todaro hypothesis and more recent findings pertinent to the establishment and maintenance of viral transformation [46], we will assume that viral transformation is the result of an increase in the expression or functional activity of a normal cellular product. The presence of BPV DNA changes host gene expression, enabling the cells to exhibit phenotypic transformation. If the mechanism of maintenance of transformation is due to a critical concentration of viral gene product which is regulated by gene dosage, we would expect that the copy number of this viral gene is reduced by transformation suppressors--RA in this case. If the mechanism of promotion by TPA is similar to this mechanism of viral transformation, we would expect that treatment of these cells with TPA would increase the concentration of this gene product with a possible concomitant increase in the BPV DNA content of the transformed cells.

# MATERIALS AND METHODS

## Preparation of Virus

Three to five grams of bovine papilloma tissue were suspended in 50 mls of 2 M NaCl, 10 mM sodium citrate pH 7.0 and homogenized in a Virtis homogenizer for 15 minutes at 45,000 rpm in an ice bath. The homogenate was centrifuged at 3000 g for 10 minutes at 4 C. The pellet was resuspended in 50 mls of 2 M NaCl, 10 mM sodium citrate pH 7.0, homogenized and centrifuged twice. The combined supernatant fluid was centrifuged at 32,000 rpm for 90 minutes at 4 C in a Spinco 50.2 rotor. The virus-containing pellet was resuspended in CsCl($\rho$=1.33), 20 mM Tris HCl pH 7.5 and homogenized in the Virtis homogenizer for 5 minutes at 30,000 rpm. The homogenate was centrifuged at 12,000 rpm for 24 hours in a Spinco SW 50.1 rotor. The lowest visible band was collected and dialyzed extensively against 50 mM Tris, 10 mM EDTA pH 7.5.

Serial two-fold dilutions of a virus suspension in PBS were made in Linbro rounded well microtiter wells. The total volume of each virus dilution was 100 µl. Fifty µl of a 1% suspension of mouse red blood cells were added to each well and mixed thoroughly. The reaction was incubated at 4 C and the hemagglutination pattern was recorded at 3 and 16 hours. The red blood cells were collected from a male Balb/CJ

mouse within minutes prior to their addition to the hemagglutination reaction. According to Favre [47], using this technique one hemagglutination unit is equal to $1.5 \times 10^8$ virus particles. This number was used to calculate the number of virus particles in the suspension used to transform mouse mammary tumor cells.

## Cell Culture

The cell line, C127 [17, 18, 19, 48], has been carried in our lab since July 1980. It was kindly provided by C. Heilman, Ph.D., NIH. These cells and other lines described below were grown in DMEM (Gibco) plus 10% FBS (Flow) plus 100 units/ml penicillin, 100 µg/ml streptomycin, 25 µg/ml fungizone (PSF) (Gibco or Flow). They were maintained in humid 5% $CO_2$ at 37 C. Once a week the cells were trypsinized and split using a low split ratio (1:1000). Lines B100, B200, B700, B800, B900 and B1000 were grown from single agar colonies after infection of C127 with BPV-1. B251, B262, B271 and B282 were grown from single agar colonies of B200.

Confluent monolayers of C127 were incubated in medium containing 5 µg/ml iododeoxyuridine (Sigma) for 16 hours. The monolayers were then rinsed with DMEM lacking serum and antibiotics. Adsorption of virus at 37 C was performed by applying one to two mls of DMEM lacking serum and

antibiotics, containing 1 - 2 x $10^{12}$ virus particles/ml, to the monolayers. After a one-to-two hour incubation, the monolayers were overlaid with 10 mls of DMEM containing 10% FBS and PSF. Mock infected cultures were treated the same way; however, the DMEM in the adsorption step did not contain BPV-1. Forth-eight hours after adsorption, the monolayers were trypsinized, the cells were counted and plated in agar-containing medium.

Colony-forming cells were grown in 35 mm Linbro or Costar multiwell plates. One and one-half mls (Linbro) or 2.0 mls (Costar) of underlayer medium, DMEM containing 20% FBS, PSF and 0.5% agar (Difco) were placed in each well and allowed to solidify at room temperature for 30 minutes before the overlayer containing cells was applied. One ml of overlayer medium containing DMEM, 10% FBS, PSF, 0.25% agar and 1.5 x $10^3$ to 5 x $10^4$ cells was applied to each underlayer. The overlayers were allowed to solidify at room temperature before incubation at 37 C. One ml of overlayer was added to each well on days 10 and 20.

Only large (1-2 mm in diameter), well-isolated colonies were considered suitable for isolation. A sterile, 9 inch Pasteur pipette was used to transfer the colony to another 35 mm well on a 6-well plate. A sterile coverslip (3.5 cm x 3.5 cm) was carefully placed on top of the colony, and gentle pressure was applied to disaggregate the cells.

One-half ml of medium was applied to each well, with mild agitation to make sure the medium covered the cells. Fresh medium was added 1 ml at a time at 2-3 day intervals. After two to three weeks, when the cells became confluent, the monolayers were trypsinized and placed in flasks. At this point the cell line was numbered and designated passage 1 (p1).

All-trans-retinoic acid (RA), RO-9359 (Eastman) was stored in the dark at 4 C in powdered form. For use in tissue culture a $10^{-2}$ M stock was made up weekly in DMSO or 100% ethanol and stored at -20 C in the dark. Working dilutions were made from this stock daily. Handling of the retinoid was done in the absence of fluorescent light and experiments containing the retinoid were set up without fluorescent light in the hood. Plates and flasks containing the retinoid were wrapped in foil before being placed in the incubator. TPA (Sigma) was solubilized in 100% ethanol. The stock solution, 1 mg/ml, was stored at -20 C and was diluted to 50 ng/ml daily for use in tissue culture. All control cultures contained 0.1% ethanol.

Cell numbers were determined from experiments set up in the following manner. One x $10^5$ cells were plated in 35 mm Costar dishes with 2 mls of medium containing 0.1% ethanol or RA. Two mls of fresh medium were added on day 3. After 2, 4, 5, 6 and 7 days of incubation, the cells were

trypsinized and viable cells were counted with a hemacytometer in the presence of 0.025% trypan blue. Cell growth experiments which contained TPA were done in 60 mm Linbro dishes. Three x $10^5$ cells were plated in 5 mls of medium containing 0.1% ethanol, $10^{-5}$ M RA, 50 ng/ml TPA and $10^{-5}$ M RA plus 10 ng/ml TPA. Three mls of fresh medium were added on day 2. After 2, 3, 4 and 5 days of incubation the cells were trypsinized and viable cells were counted with a hemacytometer in the presence of .025% trypan blue.

## Assay for CRABP

CRABP was detected as described by Ong and Chytil [49] and modified by Haussler [50]. Cells grown in monolayer culture were collected and washed three times in PBS. The cells were resuspended in 50 mM Tris pH 7.5, 5 mM EDTA at a concentration of 6 x $10^6$/ml. This suspension was homogenized by sonication for 30 seconds. The homogenate was centrifuged at 150,000 g for one hour. A 400 µl aliquot of this cytosol was mixed with 50 nM $^3$H RA (Hoffman-LaRoche, 28.7 Ci/nmol) and incubated in the dark at 4 C for 4 hours. The ligand specificity of binding was tested by adding 200-fold excess, 10 mM, unlabeled RA and unlabeled retinol to an identical reaction mixture. The reactions were layered on top of a 4 ml 5-20% sucrose gradient and centrifuged for 21 hours at 35,000 rpm in a Spinco SW 50.1 rotor at 4 C. The

gradients were fractionated and the radioactivity in each fraction was determined by liquid scintillation spectrophotometry.

## Preparation of Viral DNA

Purified virus was disrupted by treatment of solutions with 1% sodium lauryl sulfate and 1% N-lauryl sarcosine for 15 minutes at 37 C, followed by a 10 minute period at 60 C. These solutions were rapidly cooled and an equal volume of phenol saturated with 0.2 M Tris pH 8.0, 10 mM EDTA was added. This solution was shaken for 15 minutes and the phases separated by low-speed centrifugation. The ethanol-precipitated DNA pellet was resuspended in 0.2 M Tris pH 7.5, 1 mM EDTA. CsCl was added to a density of 1.5818 g/ml and ethidium bromide was added to a final concentration of 1 mg/ml. The solution was centrifuged for 48 hours at 32,000 rpm at 25 C in a Spinco SW 50.1 rotor. The lower band containing supercoiled DNA was collected, the ethidium bromide was removed by the addition of an equal volume of isopropyl alcohol saturated with CsCl, and the DNA dialyzed against 50 mM Tris pH 7.5, 1 mM EDTA. BPV DNA was stored at 4 C.

For use in liquid hybridization experiments, supercoiled BPV was nicked by DNase I DN-EP (Sigma) in 50 mM Tris pH 7.5, 50 mM KCl, 5 mM $MgCl_2$ for 40 minutes at 37 C. The

ratio of DNase/DNA was 1/1000. The reaction was stopped by heating to 65 C for 10 minutes. The reaction mixture was phenol extracted and dialyzed against 70 mM phosphate buffer pH 7.4 at 4 C. Nicked DNA was repaired by DNA polymerase I (Boehringer Mannheim) in 70 mM phosphate buffer pH 7.4, 7 mM $MgCl_2$, 1 mM mercaptoethanol, 0.1 mM each of dATP, dCTP, dGTP (Sigma) using $^3H$ dTTP (NEN, 800 Ci/nmol). The repair reaction mixture contained a μg DNA, 150 μCi dTTP and 20 units of polymerase in a total volume of 1 ml. The specific activity of the labeled BPV DNA was determined by counting the TCA precipitable product formed after 60 minutes at 12.5 C. Unincorporated nucleotides were removed by filtration through a Sephadex G50 Medium (Pharmacia) column which was equilibrated and run with 50 mM Tris pH 8.0, 1 mM EDTA, 0.15% Sarkosyl. Labeled DNA was phenol extracted, ethanol precipitated and resuspended in 10 mM Tris pH 7.5, 10 mM EDTA and stored at -20 C.

For use in filter hybridization experiments, 0.5 ug of BPV DNA was nick translated in a reaction mixture containing 50 mM Tris pH 7.5, 10 mM $MgSO_4$, 1 mM dithiothreitol (DTT), 50 μg/ml bovine serum albumin (BSA), 20 μM dATP, dTTP, dGTP, 50 μCi32p dCTP (NEN, 60 Ci/nmol), 0.8 ng DNase I and 1 unit of DNA polymerase in a total volume of 25 μl. The reaction was carried out at 13 C for 90 minutes at which time 25 μl of 0.02 M EDTA, 2 mg/ml salmon sperm DNA, 0.2%

sodium lauryl sulfate (SDS) was added to stop the reaction. Specific activity of this probe was determined by counting acid precipitable product. Unincorporated nucleotides were separated from labeled DNA on a Sephadex G50 Medium column, which was equilibrated and run in 50 mM Tris pH 7.5, 10 mM EDTA. Labeled DNA was phenol extracted and ethanol precipitated in the presence of 50 µg/ml salmon sperm DNA. The probe was resuspended in hybridization buffer, denaturated by boiling for 15 minutes and used immediately for hybridization to GeneScreen (NEN).

## Isolation of Cellular DNA

For use in liquid hybridization experiments, a cell pellet containing 5 x $10^8$ cells was suspended in 0.2 M phosphate buffer pH 6.8, 8 M urea, 10 mM EDTA and homogenized in a Waring blender for 30 seconds. The homogenate was applied to a 10 g bed of hydroxyapatite (HA) swollen in 0.2 M phosphate buffer pH 6.8, 8 M urea and the bed was rinsed with 0.2 M phosphate buffer pH 6.8 until $A_{260}$ was less than 0.1 unit. Single-stranded DNA was eluted with 0.14 M phosphate buffer pH 6.8 and discarded. Double-stranded DNA was eluted with 0.48 M phosphate buffer pH 6.8 and collected. After phenol extraction, the double-stranded DNA solution was sheared on a Virtis homogenizer at 55,000 rpm for 3 minutes in an ice bath. Single-stranded and double-stranded

DNA were separated on another HA column. The double-stranded DNA solution was dialyzed extensively against $10^{-5}$ M EDTA and lyophilized.

## Liquid Hybridization

Five µl aliquots of mixtures of DNA containing 250 µg cellular DNA and 5 ng of $^{3}H$ BPV DNA were denaturated by heating to 100 C for 20 minutes in 0.48 M phosphate buffer pH 6.8, 10 mM EDTA, 0.5% SDS. DNA was allowed to renature at 65 C. At various time intervals, single-stranded DNA was separated from double-stranded DNA on HA and the eluted fractions were counted in a 2X Omifluor (NEN)-6% Triton (2:1) scintillation cocktail.

## Filter Hybridization

Extrachromosomal DNA was prepared by the method of Hirt [51]. Cells were grown in 100 mm Petri dishes (Flow). The monolayer was rinsed with PBS and the cells were lysed with 0.75 ml of 0.5% SDS, 10 mM EDTA pH 7.5, at room temperature for 15-20 minutes. After gentle agitation the lysate was decanted to a Corex tube and brought to 1.0 M NaCl before being incubated at 4 C overnight. This solution was centrifuged at 15,000 rpm for 30 minutes. The supernatant fluid was phenol extracted and stored at -20 C. A 100-200 µl aliquot of the supernatant plus 20 µl of tracking dye were loaded on a 0.8% vertical agarose gel. The 15 x 18 x .6 cm

gel was electrophoresed for 3 hours at 25 mA in 90 mM Tris base, 90 mM boric acid, 3 mM EDTA, pH 8.3.

The gel was soaked in 250 ml of 0.25 M HCl for 30 minutes. The DNA in the gel was denatured in 250 ml of 0.2M NaOH, 0.6M NaCl with gentle agitation for 30 minutes. The gel was neutralized with 400 ml of 0.5 M Tris pH 7.5, 1.5 M NaCl for 30 minutes. Transfer of the DNA to GeneScreen was accomplished by placing two pieces of blotting paper presoaked in 0.15 M NaCl, 0.015 M sodium citrate (1X SSC) over an elevated glass plate so that the ends formed wicks. The gel was placed on top of the blotting paper and a piece of GeneScreen, cut to the size of the gel and presoaked in distilled water, was placed on top of the gel. Five pieces of blotting paper and a 4-inch stack of paper towels cut to the size of the gel were placed on top of the membrane. Efficient transfer took 12 hours and required 1 liter of SSC. The membrane was dried at room temperature and then baked at 80 C for 4 hours.

The membrane was sealed in a plastic bag and prehybridized overnight with 0.1% ficoll, 0.1% BSA and 0.1% polyvinylpyrrolidone in 2X SSC containing 250 µg/ml sheared, denatured salmon sperm DNA. The prehybridization solution was removed and replaced with 0.3 M NaCl, 60 mM Tris pH 8.0, 2 mM EDTA, 0.02% ficoll, 0.02% BSA, 0.02% polyvinylpyrrolidone, 0.5% SDS, 100 µg/ml sheared, denatured salmon sperm

DNA and an appropriate amount of denatured $^{32}P$ BPV DNA. The solution contained 5 x $10^6$ cpm per filter in a total volume of 5 mls per filter in the bag. The specific activity of the $^{32}P$ BPV DNA probe was 1-2 x $10^8$ cpm/µg. Prehybridization and hybridization were carried out at 60 C with constant agitation. Following 24 hours of hybridization, each filter was rinsed with three 100 ml changes of 0.3 M NaCl, 60 mM Tris pH 8.0, 2 mM EDTA, 0.5% SDS for 30 minutes at 60 C. The filters were allowed to cool to room temperature and were then rinsed again with three 100 ml changes of 3 mM Tris base. The membranes were dried at room temperature, wrapped in plastic wrap and exposed to X-omat AR5 film with a Cronex "Lightning Plus" intensifying screen at -20 C for 3 to 10 days.

# RESULTS

## Description of Cell Lines

C127 mouse mammary tumor cells were transformed to grow in agar by infection with BPV. With a multiplicity of infection of $10^6$:1, transformation to growth in agar occurred at a rate of 4 per 10,000 cells. Colonies which were 1 to 2 mm in diameter appeared after 6 weeks of incubation when 50,000 cells were plated in agar in a 35 mm well. The data in Table 1 was used to determine that colony-forming cells in parental transformed C127 were inhibited in soft agar by all concentrations of RA tested. These data, expressed as the percent of total colonies that grew in agar in the absence of RA, are illustrated in Figure 1. Only 7% of the colony-forming cells were able to grow in the presence of $10^{-5}$ M RA. Fifteen percent of the colony-forming cells grew in the presence of $10^{-6}$ M RA and 50% of the colony-forming cells grew in the presence of $10^{-7}$ M RA.

Several cloned cell lines were selected from colonies growing in agar and after several passages these cells were suspended in agar in the presence or absence of RA. Colonies which were 1 to 2 mm in diameter appeared after three weeks of incubation when 1500 cells per 35 mm dish were suspended in agar. Colony counts from six cloned cell lines at different passage numbers are given in Table 2. These

Table 1. Colony forming cells of BPV transformed C127. -- Five X $10^4$ BPV transformed C127 were plated in agar containing medium with and without RA. The numbers represent colonies which grew to 1 to 2 mm in diameter after 6 weeks of incubation.

| | M Retinoic Acid | | |
|---|---|---|---|
| C | $10^{-5}$ | $10^{-6}$ | $10^{-7}$ |
| 38 | 2 | 4 | 9 |
| 21 | 0 | 3 | 8 |
| 32 | 0 | 1 | 14 |
| 21 | 3 | 5 | 13 |
| 15 | 1 | 1 | 9 |
| 18 | 2 | 3 | 6 |
| 13 | | | |
| 13 | | | |
| 16 | | | |
| 9 | | | |
| 15 | | | |
| 11 | | | |

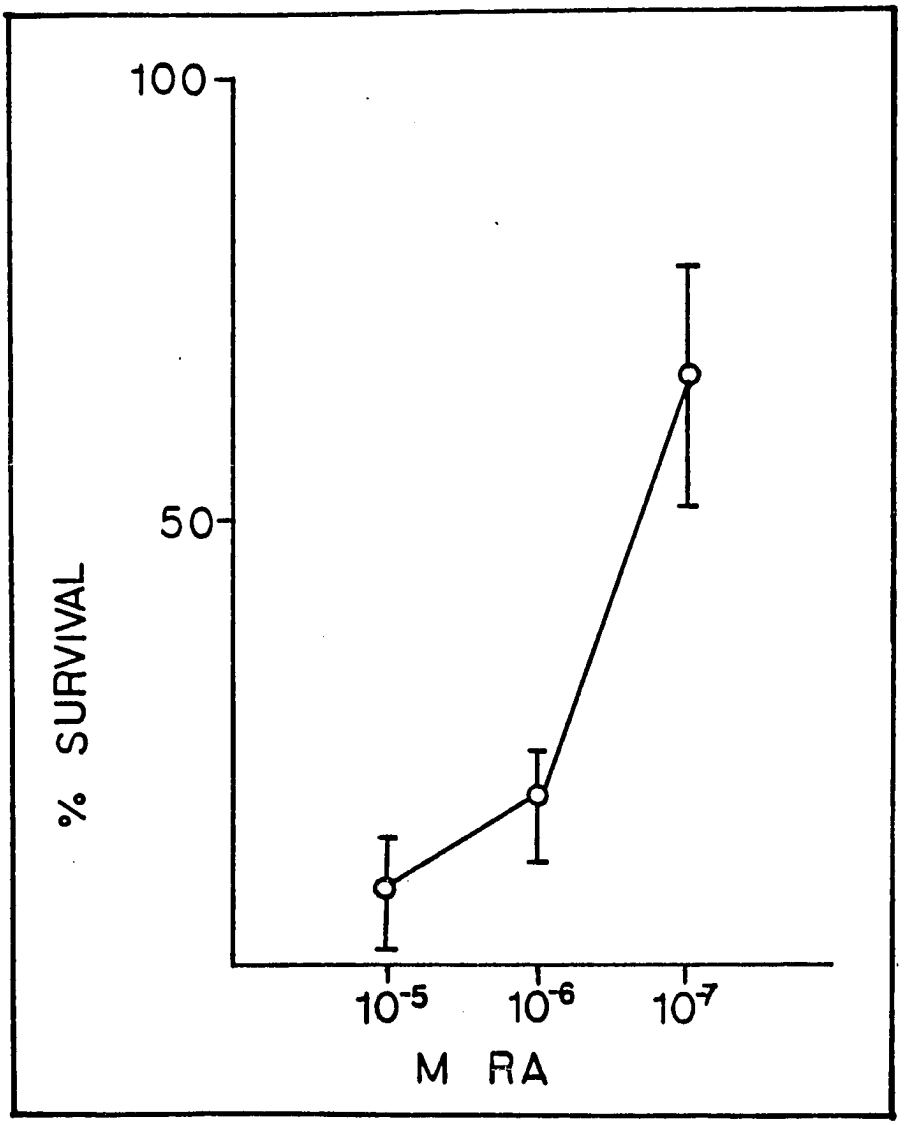



Figure 1. The effects of RA on colony forming cells of BPV transformed C127. -- The means and standard deviation of percent survival were calculated from data in Table 1.

data are illustrated as percent survival of colony-forming cells in Figures 2, 3 and 4. The cell lines exhibited apparent differences in colony-forming ability and sensitivity to RA. The source of this variation was investigated by analysis of variance. The sources considered here are passage number (A), cell line (B) and concentration of RA (C). The percent of survival of colony-forming cells was converted to the mean of the $\log_{10}$ number of colonies in RA-containing medium-the mean of the $\log_{10}$ number of colonies in medium without RA and the ANOVA table, Table 3, was generated.

There is a significant difference between the percent of colony-forming cells at the different passage numbers over all cell lines and concentrations of RA (A). There are significant differences in the percent of colony-forming cells among the cell lines over the different passage numbers and concentrations of RA (B). As expected, there are significant differences in the percent of colony-forming cells at different concentrations of RA over all cell lines at both passages (C).

There is no significant interaction between the concentration of RA and the different cell lines (BC) or between the concentration of RA and passage number (AC). This would tend to rule out differences due to experimental set-up, culture conditions, health of cells, delivery of drug or variation in medium. There is a significant interaction between the variation seen with passage number and the

Table 2. Colony forming cells of BPV transformed, cloned cell lines. -- Single colonies of BPV transformed C127 were isolated and plated in agar containing medium with and without RA. The numbers represent colonies which grew to 1 to 2 mm in diameter after 3 weeks of incubation. The number of passages (p) of each cell line tested is indicated.

| Cell Line | | M Retinoic Acid | | | | Cell Line | | M Retinoic Acid | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | $10^{-5}$ | $10^{-6}$ | $10^{-7}$ | $10^{-8}$ | | | $10^{-5}$ | $10^{-6}$ | $10^{-7}$ | $10^{-8}$ |
| B100 p20 | 51 | 36 | 66 | 85 | 90 | B100 p25 | 80 | 1 | 52 | 96 | 72 |
| | 53 | 27 | 65 | 101 | 96 | | 100 | 2 | 60 | 64 | 84 |
| | 54 | 30 | 51 | 91 | 100 | | 112 | 0 | | | |
| | 60 | | | | | | 98 | | | | |
| | 58 | | | | | | | | | | |
| | 66 | | | | | | | | | | |
| B200 p20 | 65 | 1 | 15 | 39 | 43 | B200 p25 | 37 | 1 | 32 | 65 | 126 |
| | 97 | 2 | 11 | 38 | 39 | | 35 | 0 | 4 | 25 | 84 |
| | 90 | 6 | 5 | 46 | 64 | | 40 | 3 | 31 | 62 | 72 |
| | 75 | | | | | | 50 | | | | |
| | 58 | | | | | | 29 | | | | |
| | 63 | | | | | | 20 | | | | |
| B700 p3 | 22 | 0 | 2 | 1 | 7 | B700 p7 | 20 | 0 | 5 | 7 | 44 |
| | 19 | 0 | 3 | 0 | 6 | | 9 | 0 | 5 | 4 | 34 |
| | 13 | 0 | 4 | 1 | 0 | | 16 | 0 | 6 | 4 | 23 |
| | 44 | | | | | | 11 | | | | |
| | 40 | | | | | | 17 | | | | |
| | 45 | | | | | | 17 | | | | |
| B800 p3 | 800 | 1 | 813 | 829 | 752 | B800 p7 | 82 | 0 | 4 | 22 | 26 |
| | 746 | 0 | | | | | 56 | 0 | 5 | 20 | 17 |
| | 604 | 0 | | | | | 84 | 0 | 13 | 20 | 19 |
| | | | | | | | 80 | | | | |
| | | | | | | | 90 | | | | |
| | | | | | | | 110 | | | | |
| B900 p3 | 803 | 1 | 6 | 5 | 6 | B900 p7 | 41 | 0 | 3 | 23 | 18 |
| | | 0 | 6 | 0 | 15 | | 34 | 0 | 4 | 0 | 8 |
| | | 0 | 4 | | 34 | | 122 | 0 | 0 | 4 | 15 |
| | | | | | | | 47 | | | | |
| | | | | | | | 39 | | | | |
| | | | | | | | 79 | | | | |
| B1000 p3 | 54 | 1 | 6 | 1 | 14 | B1000 p7 | 52 | 0 | 0 | 18 | 28 |
| | 35 | 0 | 0 | 1 | 7 | | 72 | 0 | 3 | 12 | 15 |
| | 34 | 0 | 0 | | 1 | | 60 | 0 | 0 | 12 | 14 |
| | 52 | | | | | | 92 | | | | |
| | 48 | | | | | | 41 | | | | |
| | | | | | | | 28 | | | | |

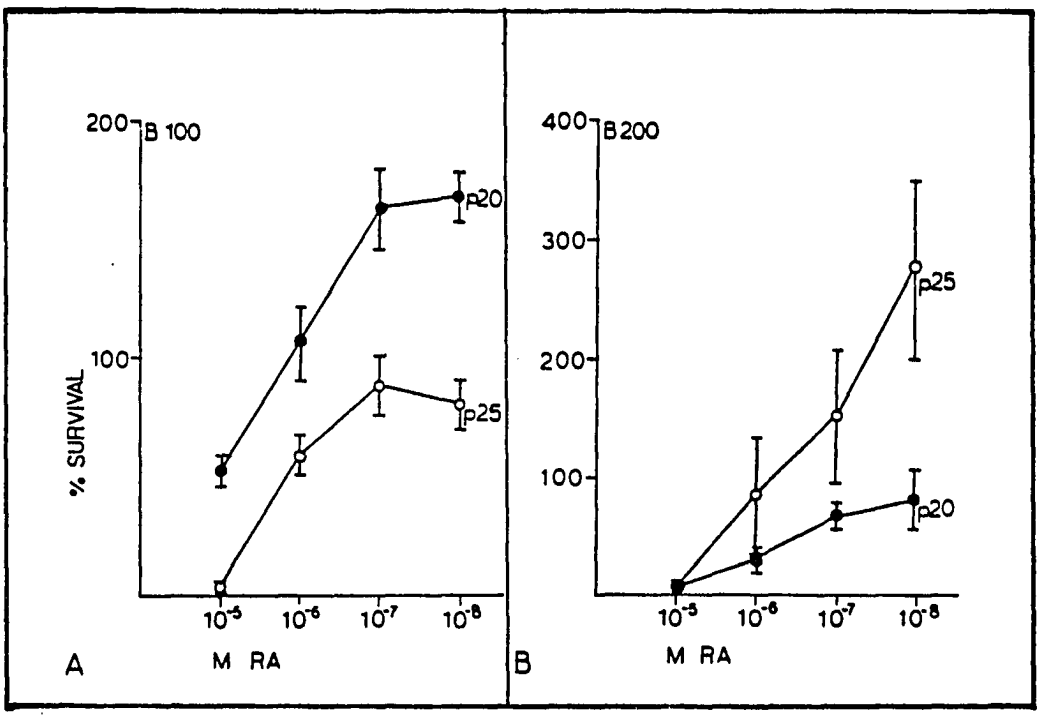



Figure 2. The effects of RA on colony forming cells of BPV transformed cell lines B100 and B200. -- The means and standard deviation of percent survival were calculated from the data in Table 2.

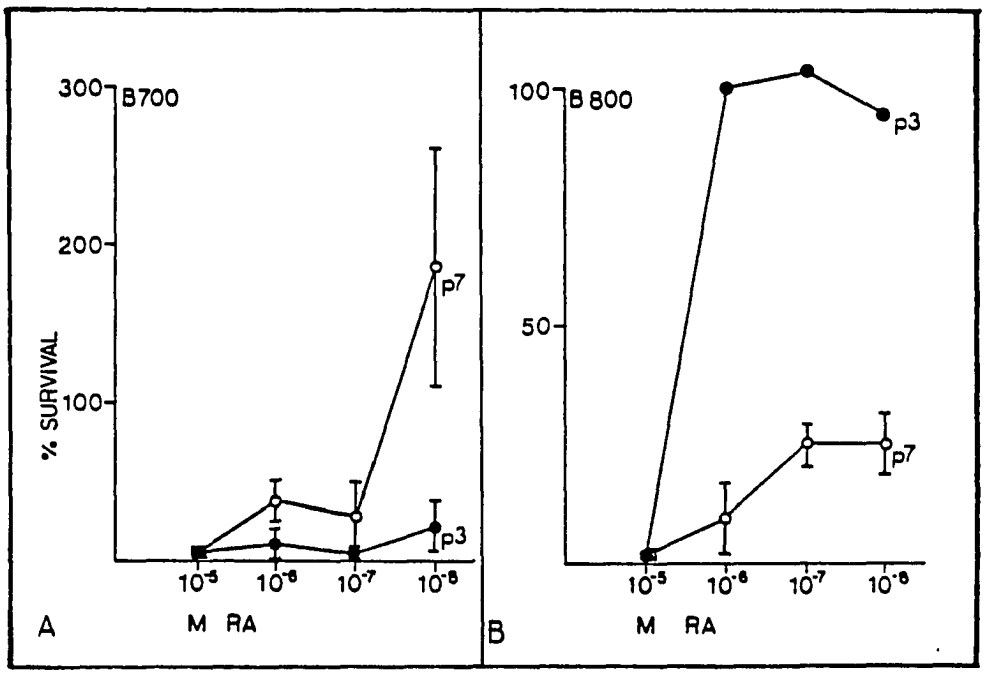



Figure 3. The effects of RA on colony forming cells of BPV transformed cell lines B700 and B800. -- The means and standard deviation of percent survival were calculated from the data in Table 2.

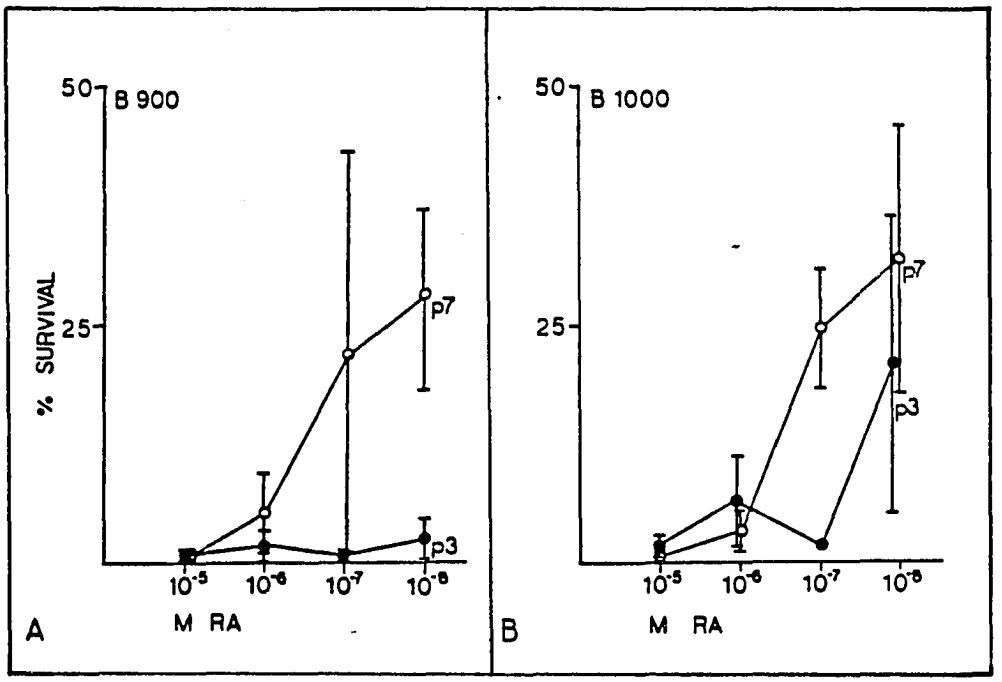



Figure 4. The effects of RA on colony forming cells of BPV transformed cell lines B900 and B1000. -- The means and standard deviation of percent survival were calculated from the data in Table 2.

Table 3. Statistical evaluation of sources of variation in colony forming cells of BPV transformed cell lines. -- The data in Table 2 were used to generate the analysis of variance (ANOVA) summary table.

| Source | SS | DF | MS | F Ratio |
|---|---|---|---|---|
| ABC* | 2.0265 | 15 | 0.1351 | |
| AB | 4.1422 | 5 | 0.8284 | 6.132 $p < 0.005$ |
| AC | 0.3426 | 3 | 0.1142 | n.s. |
| A | 0.6977 | 1 | 0.6977 | 5.167 $p < 0.05$ |
| BC | 2.6775 | 15 | 0.1785 | n.s. |
| B | 11.2791 | 5 | 2.2558 | 16.697 $p < 0.001$ |
| C | 10.9026 | 3 | 3.6342 | 26.900 $p < 0.001$ |

* A = passage number; B = cell line; C = concentration of RA.

SS = sum of squares; df = degree of freedom; MS = mean square.

variation among the cell lines over all concentrations of RA. This is most likely related to the fact that higher concentrations of RA generally show greater inhibition in all cell lines at both passages.

If the variation with respect to RA sensitivity among the clones represented genetic variants, it might be possible to isolate subclones of a particular cell line which demonstrated the same ability to form colonies in agar in the presence and absence of appropriate concentrations of RA.

Colonies of B200 which grew in the presence of $10^{-5}$ M RA (B251), $10^{-6}$ M RA (B262), $10^{-7}$ M RA (B271) and $10^{-8}$ M RA (B282) were isolated and carried for five passages before being suspended in agar in the presence or absence of RA. B271 and B282 contained 5% colony-forming cells similar to the B200 line from which they were cloned. B251 and B262 had an increased number of colony-forming cells of about three-fold. B251 demonstrated a four-fold increase in the percentage of colonies which would grow in the presence of $10^{-5}$ M RA and a two-fold increase in the percentage of colonies which would grow in the presence of $10^{-6}$ M RA. B262 had an increase in the percentage of colonies which would grow in the presence of $10^{-6}$ M RA, but only 0.5% of the colonies would grow in the presence of $10^{-5}$ M RA. The data from these experiments can be found in Table 4.

Table 4. The effects of RA on colony forming cells of B200 and some subclones of B200. -- Single colonies of B2000 which grew in the presence of $10^{-5}$ M RA (B251), $10^{-6}$ M RA (B262), $10^{-7}$ M RA (B271) and $10^{-8}$ M RA (B282) were isolated and plated in agar containing medium with and without RA. The numbers represent colonies which grew to 1 to 2 mm in diameter after three weeks of incubation.

| | | M RA | | | |
|---|---|---|---|---|---|
| Cell Line | C | $10^{-5}$ | $10^{-6}$ | $10^{-7}$ | $10^{-8}$ |
| B251 | 240 | 54 | 85 | 88 | 133 |
| | 234 | 32 | 72 | 72 | 148 |
| | 248 | 58 | 68 | 91 | 178 |
| B262 | 204 | 0 | 35 | 35 | 83 |
| | 154 | 0 | 50 | 40 | 50 |
| | 217 | 1 | 59 | 41 | 48 |
| B271 | 90 | 4 | 42 | 55 | 32 |
| | 103 | 3 | 41 | 55 | 61 |
| | 94 | 9 | | 60 | 74 |
| | 65 | | | | |
| | 65 | | | | |
| | 62 | | | | |
| B282 | 106 | 6 | 38 | 94 | 139 |
| | 103 | 3 | 34 | 73 | 120 |
| | 89 | 3 | 52 | 80 | 135 |
| | 64 | | | | |
| | 57 | | | | |
| | 67 | | | | |

In order to assess whether or not the subclones demonstrated sensitivity to RA which differed from that of the parent clone, B200, regression analysis was used. The percent survival for each concentration of RA was converted to $\log_{10}$. These data are illustrated in Figure 5. The regression coefficients for B262 ($r = -0.79161$), B271 ($r = -0.83523$) and B282 ($r = -0.92996$) are not significant. The variation in percent survival of colony-forming cells from these cell lines cannot be explained by the variation in concentration of RA. The regression coefficients for B200 ($r = -0.97548$) and B251 ($r = -0.96975$) are significant. There is good correlation between the percent survival of colony-forming cells and decreasing concentration of RA. The slopes of the regression lines, $b = -0.15581$ for B251 and $b = -0.37510$ for B200 are significantly different ($p < 0.05$). This is most likely due to the increased survival of colony-forming cells of B251 at the higher concentrations of RA.

Three of the cell lines were chosen for further study. B100 contained 5% colony-forming cells and 55% of these were able to grow in the presence of $10^{-5}$ M RA. B200 also contained 5% colony-forming cells, but only 5% of these were able to grow in the presence of $10^{-5}$ M RA. B251 was one of very few subclones of B200 which grew in the presence of $10^{-5}$ M RA and was selected for its ability to grow in

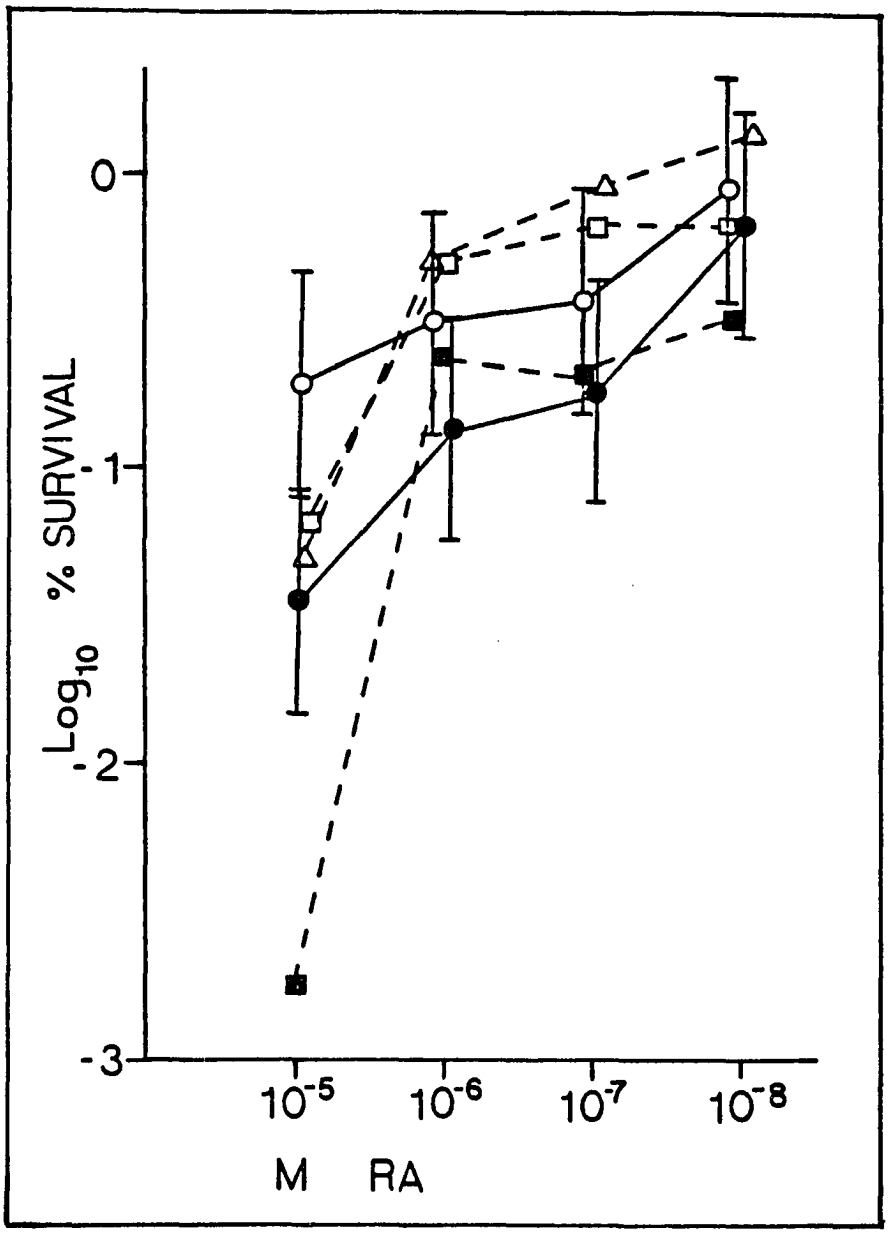



Figure 5. Linear regression analysis of percent survival and concentration of RA for B200 and its subclones. The standard deviation is illustrated for B200 (●) and B251 (○) both of which demonstrated significant comelation coefficients. The correlative coefficients for B262 (■), B271 (□) and B282 (△) were not significant.

agar in the presence of $10^{-5}$ M RA. This cell line contained 15% colony-forming forming cells, and 20% of these were able to grow in agar in the presence of $10^{-5}$ M RA. Figure 6 illustrates the presence of colony-forming cells in these three cell lines under the influence of decreasing concentrations of RA. The number of colonies is expressed as the percent of total cells plated in a 35 mm well; the bars represent the range of colonies counted in triplicate 35 mm dishes.

We wanted to compare the effect of RA on the transformed lines to the effect of RA on the untransformed C127, and to see if the extent of inhibition could be correlated with the level of CRABP. These cell lines would be used to see if the differences in sensitivity to RA could be correlated with differences in copy number of the episome present in the three cell lines.

## Biological Effects of RA

Ruben Lotan, who originally identified the growth-inhibitory effects of RA on transformed cells, expressed those effects as the percent reduction in cell number after 5 days in culture in the presence of $10^{-5}$ M RA [24]. Cell lines which exhibit greater than 10% reduction were considered growth inhibited. Since C127 did not grow in agar, this experimental design enabled a comparison of the growth

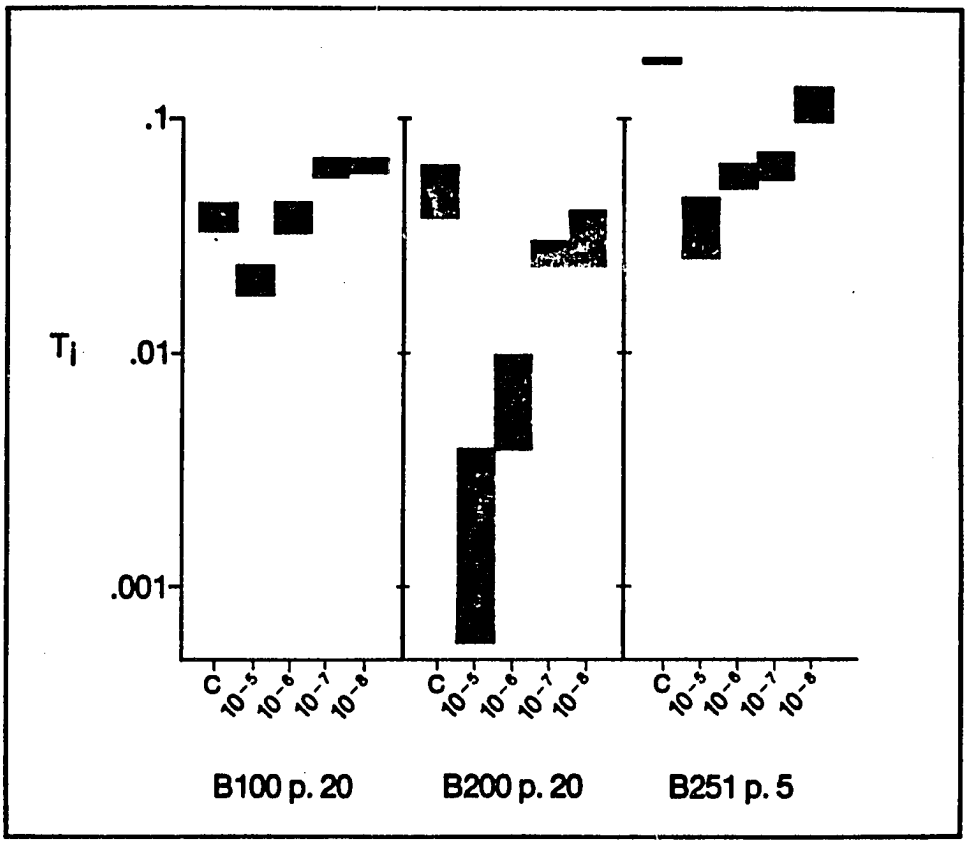



Figure 6. Colony forming cells of B100, B200 and B251 in the presence and absence of decreasing concentrations of RA. -- $T_I$= the number of colonies which grew in agar/the number of cells suspended in agar containing medium. The bars represent the reange of $T_I$ for triplicate cultures. The $T_I$ was calculated from the data in Table 2 for B100 and B200 and the data in Table 4 for B251.

inhibition of transformed cells and untransformed cells to be made. The results of these experiments are presented in Figure 7. Untransformed C127 were inhibited by 48%, B100 were inhibited by 35%, B200 were inhibited by 60%, and B251 were inhibited by 80%. In addition, after prolonged treatment of the three transformed cell lines with $10^{-5}$ M RA, none of the cells were able to form colonies in agar-containing medium.

Preliminary evidence indicated that untransformed C127 did not contain significant levels of CRABP and that B100 and B200 contained increased levels of CRABP [50]. In these experiments specific binding of $^{3}H$ RA to a 2S component of the cytosols of B100 and B200 was higher than in untransformed C127. B251 also demonstrated a high level of $^{3}H$ RA binding but nonspecific binding was high. In order to quantitate and compare levels of CRABP as specific $^{3}H$ RA binding detected on sucrose gradients, uniform conditions had to be maintained. Since the specific activity of $^{3}H$ RA is subject to change due to isomerization between the cis and trans isomers, it was important to perform all binding assays on the same day. In order to evaluate a particular biological response to RA by cell lines which exhibit heterogeneous behavior with respect to RA, it was important to use a passage of cells for binding assays for which the biological response was known.

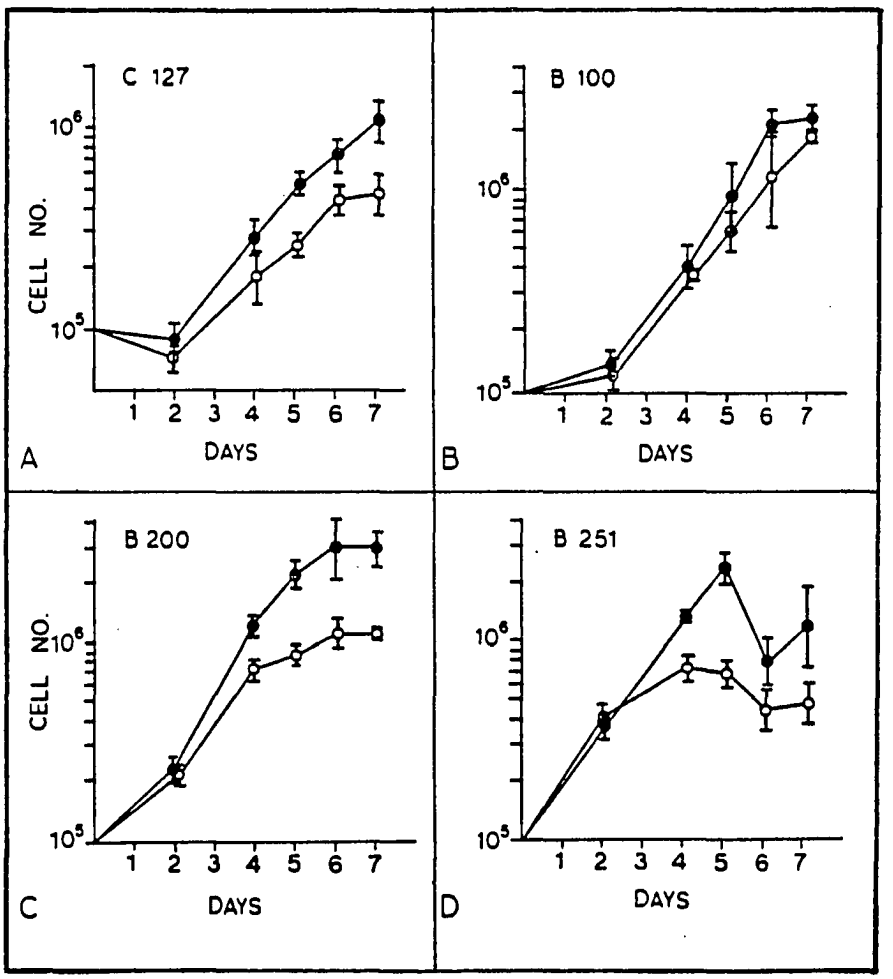



Figure 7. Growth curves of untransformed C127 and transformed clones B100, B200 and B251. -- Cells were grown in 0.1% ethanol (●) or $10^{-5}$ M RA (○). The cell numbers plotted are the manes of triplicate cultures.

The results of sucrose density gradient analysis for $^{3}H$ RA binding on cytosols prepared from p27 of C127, p18 of B100, p20 of B200 and p12 of B251 are shown in Figure 8. The dots represent total binding of $^{3}H$ RA. Ligand specificity is demonstrated by the reduction of $^{3}H$ RA in the presence of unlabeled RA, represented by open circles but not by unlabeled retinol, represented by triangles.

## Liquid Hybridization

To see if there were quantitative differences in BPV genomes present in the three cell lines, B100, B200 and B251, liquid hybridization was performed. The specific activity of the $^{3}H$ BPV DNA probe was $1 \times 10^{7}$ cmp/μg and the piece size of this probe was approximately 500 bases. Under the melting conditions described in Materials and Methods, the probe exhibited 4-6% snapback. $C_{o}/C$ at incubation times between 2 and 8 hours are presented in Figure 9. Reassociation of denatured $^{3}H$ BPV DNA in the presence of denatured DNA from the three clones was accelerated over that of the control reaction containing C127 cellular DNA. The reassociation of the control reaction containing labeled BPV DNA and C127 cellular DNA followed second-order kinetics. Reassociation of $^{3}H$ BPV DNA deviated from second-order kinetics in the presence of cellular DNA from BPV transformants.

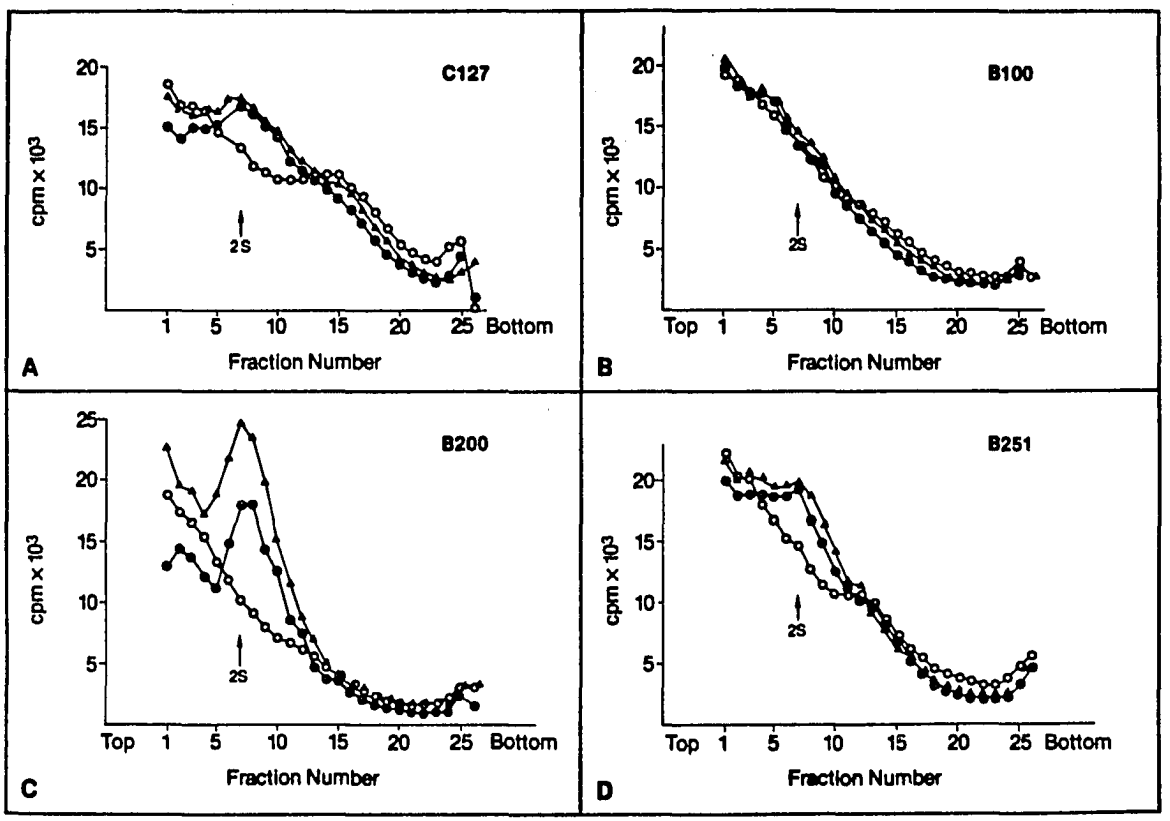


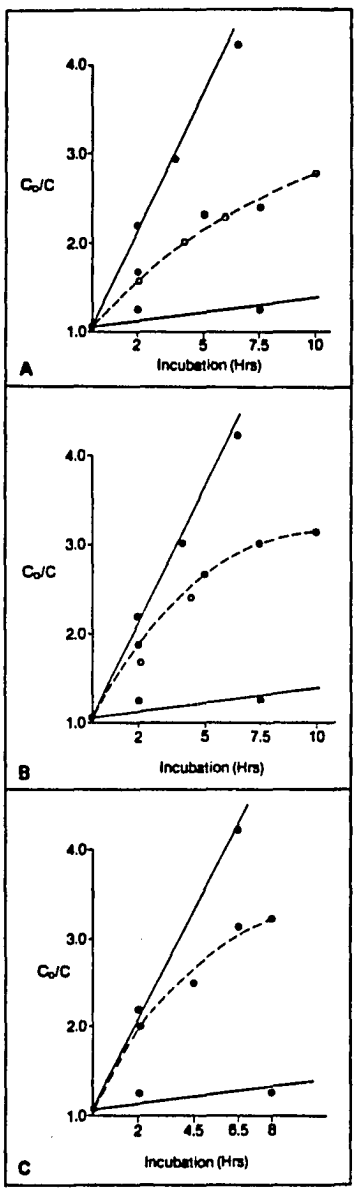

Figure 8. Counts per minute of $^{3}H$ RA in fractions of 5-20% sucrose gradients. -- (A) The gradients contained cytosol from C127 p27; (B) The gradients contained cytosol from B100 p18; (C) The gradients contained cytosol from B200 p20; and (D) The gradients contained cytosol from B257 p12. (●) Represents binding in the presence of 50 nM $^{3}H$ RA, (○) represents binding in the presence of 50 nm $^{3}H$ RA plus 10 nM RA, (▲) represents binding in the presence of 50 nM $^{3}$ RA plus 10 nM retinol.

Figure 9. Liquid hybridization of cellular DNAs and $^{3}H$ BPV DNA. -- Solid lines represent control reactions containing C127 cellular DNA and C127 cellular DNA plus 0.05 µg unlabeled BPV DNA.

A. Dotted line represents reassociation kinetics of B100 cellular DNA (●) and B100 cellular DNA from cells chronically treated with $10^{-5}$ M RA (○).

B. Dotted line represents reassociated kinetics of B200 cellular DNA (●) and B200 cellular DNA from cells chronically treated with $10^{-5}$ M RA (○).

C. Dotted line represents reassociation kinetics of B251 cellular DNA (●).


4.0
3.0
$C_o/C$
2.0
1.0
2
5
7.5
10
Incubation (Hrs)
A
4.0
3.0
$C_o/C$
2.0
1.0
2
5
7.5
10
Incubation (Hrs)
B
4.0
3.0
$C_o/C$
2.0
1.0
2
4.5
6.5
8
Incubation (Hrs)
C

The method of Gelb [52] was used to determine the average number of BPV genome equivalents per diploid amount of cellular DNA. The factor of increased rate of B100 cellular DNA was 3.15, which is equal to 33.54 genome equivalents per diploid amount of cellular DNA. Factors of increased rate for B200 and B251 cellular DNAs were 4.10 and 4.75, respectively, indicating 48.36 and 58.50 genome equivalents per diploid amount of DNA, respectively.

As reported in the previous section, after prolonged treatment of the transformed cells with RA, none of the cells were able to grow in agar-containing medium. This was the marker used to isolate these transformed cells. To address the question of whether RA acts on these cells by reducing the genome equivalents or curing the cells of the episome, a flask of cells of B100 which lacks CRABP, and a flask of B200 which contains a high level of CRABP, were carried in the presence of $10^{-5}$ M RA for one month, for 4 passages. Some of these RA-treated cells were suspended in agar and the rest were used for isolation of DNA. No colony-forming cells were detected. Liquid reassociation showed only subtle changes in kinetics from DNA from untreated cells. Reassociation experiments on cellular DNA from RA-treated cells are illustrated by the open circles in Figure 9.

## Biological Effects of TPA and RA

The effects of TPA on cell number were evaluated at 10, 50 and 100 ng/ml over an incubation period of 5 days. 50 ng/ml was the optimal dose for the effects reported here. There was a 10% reduction in cell number in cultures of untransformed C127 when incubated for three days in the presence of 50 ng/ml TPA. Surprisingly, the transformed lines were inhibited by 50-60% under these conditions. During the first three days of continuous exposure to $10^{-5}$ M RA C127, B100 and B200 exhibited 20% growth inhibition. During this time B251 exhibited 50% growth inhibition in the presence of $10^{-5}$ M RA. A 50-60% reduction in cell number on day 3 after the simultaneous addition of RA and TPA was seen in cultures of transformed lines B100 and B200. A 80% reduction in cell number on day 3 after the simultaneous addition of RA and TPA was seen in cultures of transformed lines B251. Untransformed C127 exhibited inhibition similar to RA alone when treated with the combination. The data, expressed as percent of cell number of untreated controls, are illustrated in Figure 10.

Morphological changes were seen in cultures of all cell lines when treated with 50 or 100 ng/ml TPA fro 4 to 5 days. C127 acquired a transformed phenotype, as seen in Figure 11, panel B. The transformed lines B100, B200 and B251 demonstrated a different type of morphological change

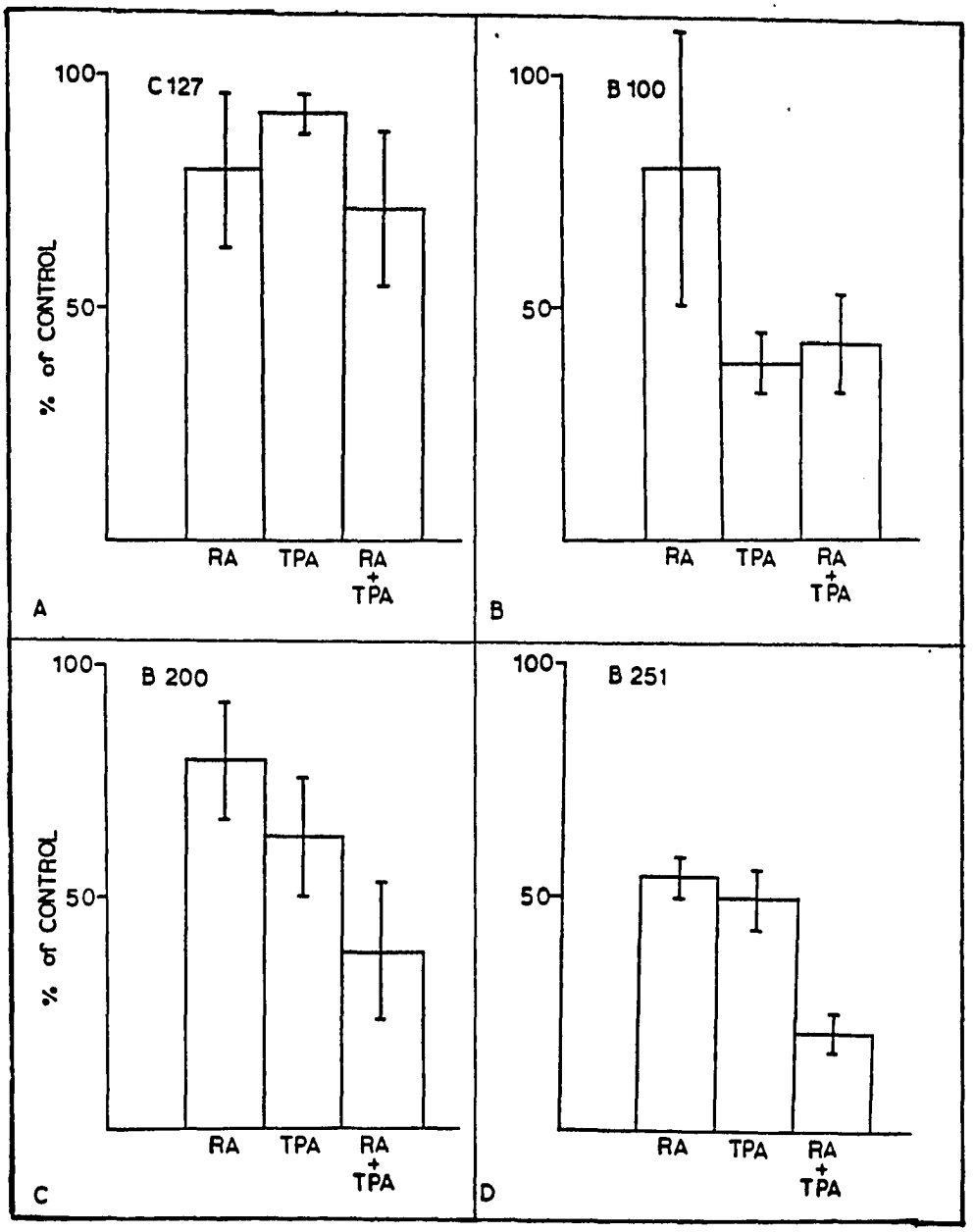



Figure 10. Growth inhibition of untransformed C127 and transformed cell lines B110, B200 and B251 by RA, TPA and RA plus TPA. -- The concentrations of RA and TPA were $10^{-5}$M and 50 ng/ml, respectively. Viable culs were counted on duplicate day 3 cultures. The data are expressed as the percent of control counts from cultures containing 0.1% ethanol.

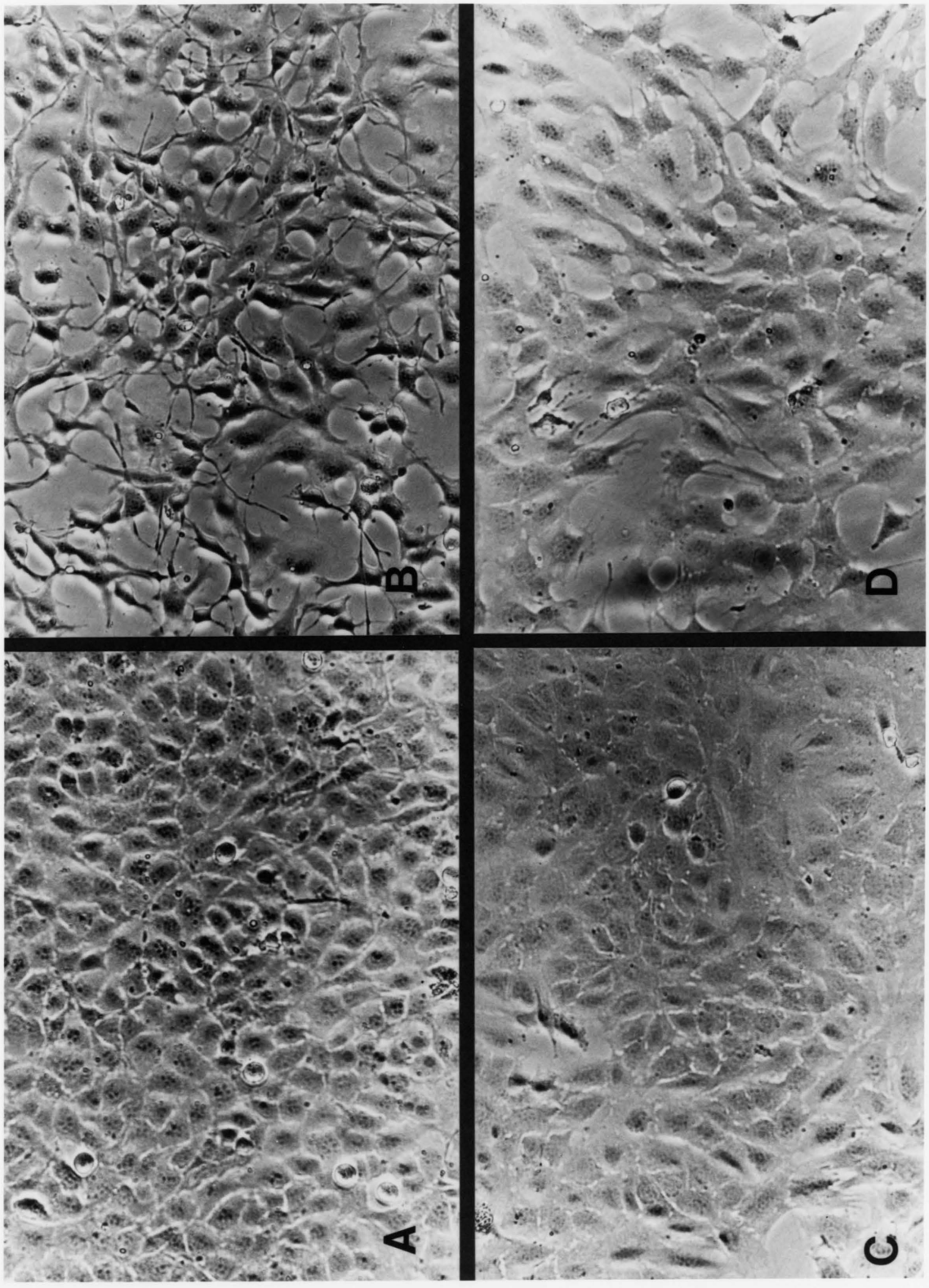
Figure 11. Photomicrographs of monolayers of untransformed C127.

A. Medium contained 0.1% ethanol

B. Medium contained 50 ng/ml TPA.

C. Medium contained $10^{-5}$ M RA.

D. Medium contained 50 ng/ml TPA plus $10^{-5}$ M RA.


A
B
C
D

(Figure 12, panel B), and not all cells were affected. The affected cells were most prevalent in B251. Panel A of Figure 11 shows the cobblestone appearance of a confluent monolayer of C127. The individual cells are cuboidal in shape, and the cell-cell junctions are well defined. Panel B shows the same cells under the influence of TPA. These cells acquired a spindle-shaped morphology and are shown growing in a crisscross fashion which is characteristic of transformed cells. Panel C, which is similar to panel A, shows C127 grown in the presence of RA. Panel D, which is also similar to panel A, shows C127 grown in the presence of RA and TPA.

Panels A, B, C and D of Figure 12 show representative fields of the transformed line B251 grown in the presence of 0.1% ethanol, RA, TPA and RA plus TPA, respectively. The spindle-shaped cells and crisscross pattern of growth in these cells is illustrated in panels A and C. Panel B shows an example of cytoplasmic spreading and acytokinesis induced by the presence of TPA. Other fields of this monolayer showed cells with extensive vacuolization and pseudopodia. These effects could be reversed by the simultaneous addition of TPA and RA, panel D.

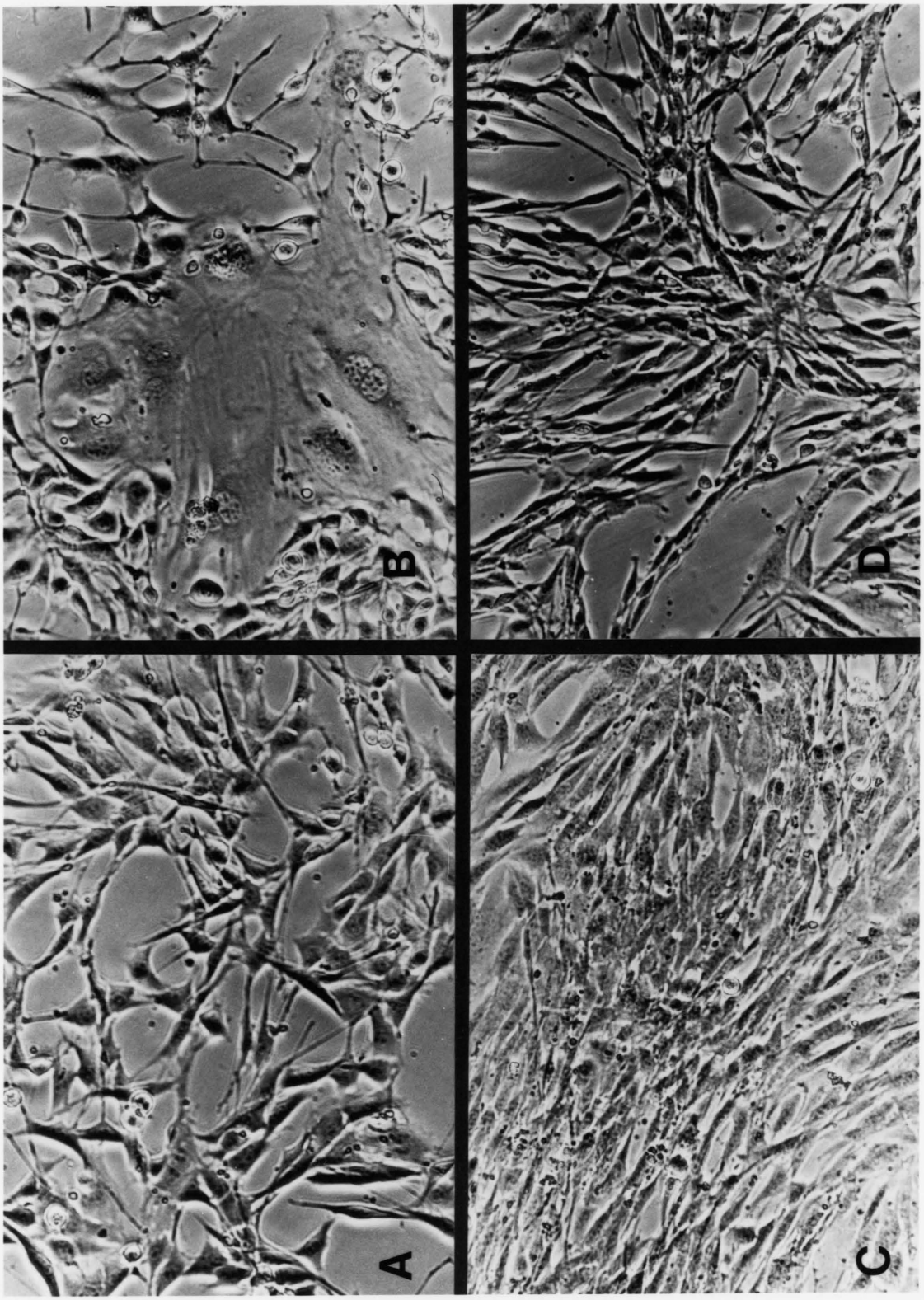
Figure 12. Photomicrographs of monolayers of transformed cell line B251.

A. Medium contained 0.1% ethanol.

B. Medium contained 50 ng/ml TPA.

C. Medium contained $10^{-5}$ M RA.

D. Medium contained 50 ng/ml TPA plus $10^{-5}$ M RA.


A
B
C
D

## Filter Hybridization

Relative changes in the amount of extrachromosomal BPV DNA were evaluated using the Southern blotting technique [53]. Hirt supernatants were prepared from a monolayer of cells which had been grown for 3 days in the presence of 0.1% ethanol, $10^{-5}$ M RA, 50 ng/ml TPA or $10^{-5}$ M RA plus 50 ng/ml TPA. The supernatants were phenol extracted, and the volume of the sample was standardized for $A_{260}$ before being electrophoresed. BPV DNA was added to a similar sample of Hirt supernatant from untransformed C127 as a marker. Lanes B, C. D and E of Figure 13 contained extrachromosomal DNA from B100. DNA from untreated cells (lane B), RA-treated cells (lane C), TPA-treated cells (lane D) and TPA plus RA-treated cells (lane E) exhibit bands which migrate with unit length open circular BPV DNA (lane A). The signal from RA-treated cells (lane C) was reduced from the signal from untreated cells (lane B). The signal from TPA-treated cells (lane D) and the signal from the untreated cells (lane B) were of approximately equal intensity. The signal from RA plus TPA-treated cells (lane E) was reduced from the signal from untreated cells (lane B).

Lanes F, G, H and I of Figure 13 each exhibit a band of extrachromosomal DNA from B200 which migrates with unit length open circular BPV DNA. The signal from RA-treated cells (lane G) was reduced from the signal from the untreated

Figure 13. Southern blotting of extrachromosomal DNA from transformed cell lines B100 and B2000. -- Exposure time for lanes B through I = 10 days.

A. Hirt supernatant from C127 plus 3 ng BPV DNA, exposure time = 3 days.

B. Hirt supernatant from B100 grown in the presence of 0.1% ethanol.

C. Hirt supernatant from B100 grown in the presence of $10^{-5}$ M RA.

D. Hirt supernatant from B100 grown in the presence of 50 ng/ml TPA.

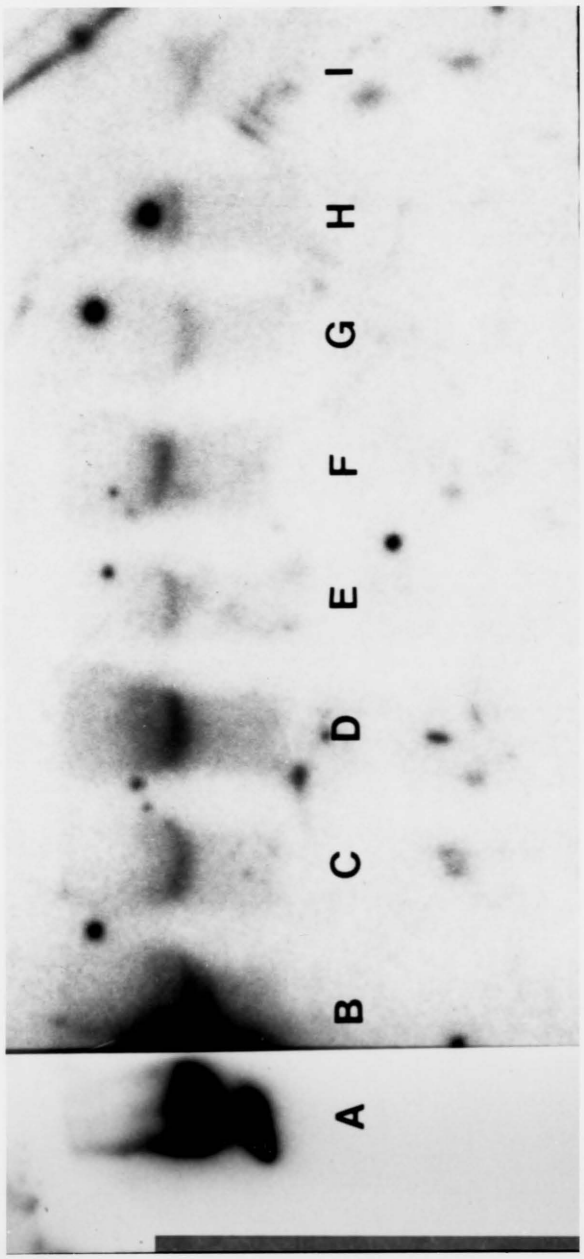
E. Hirt supernatant from B100 grown in the presence of $10^{-5}$ M RA plus 50 ng/ml TPA.

F. Hirt supernatant from B200 grown in the presence of 0.1% ethanol.

G. Hirt supernatant from B200 grown in the presence of $10^{-5}$ M RA.

H. Hirt supernatant from B200 grown in the presence of 50 ng/ml TPA.

I. Hirt supernatant from B200 grown in the presence of $10^{-5}$ M RA plus 50 ng/ml TPA.


A
B
C
D
E
F
G
H
I

cells (lane F). The signal from TPA-treated cells (lane H) was reduced from the signal from untreated cells (lane F). The signal from RA plus TPA-treated cells (lane I) was reduced from the signal from untreated cells (lane F).

Figure 14 shows extrachromosomal DNA from B251. Some of the BPV DNA present in this cell line migrated with unit length open circular DNA and some migrated with unit length supercoiled BPV DNA. Lane A contains these markers. Quantitative differences between the lanes were obvious from examination of the blots. The signal from RA-treated cells (lane B) was markedly reduced from the signal from untreated cells (lane B). The signal from TPA-treated cells (lane C) and the signal from untreated cells (lane B) was of equal intensity. The signal from RA plus TPA-treated cells (lane E) was reduced from the signal from untreated cells. The reduction exhibits specificity with respect to the physical state of the BPV genome. The supercoiled molecules were less affected than the open circular molecules.

Figure 14. Southern blotting of extrachromosomal DNA from transformed cell line B251. -- Exposure time for lanes B through E = 10 days.

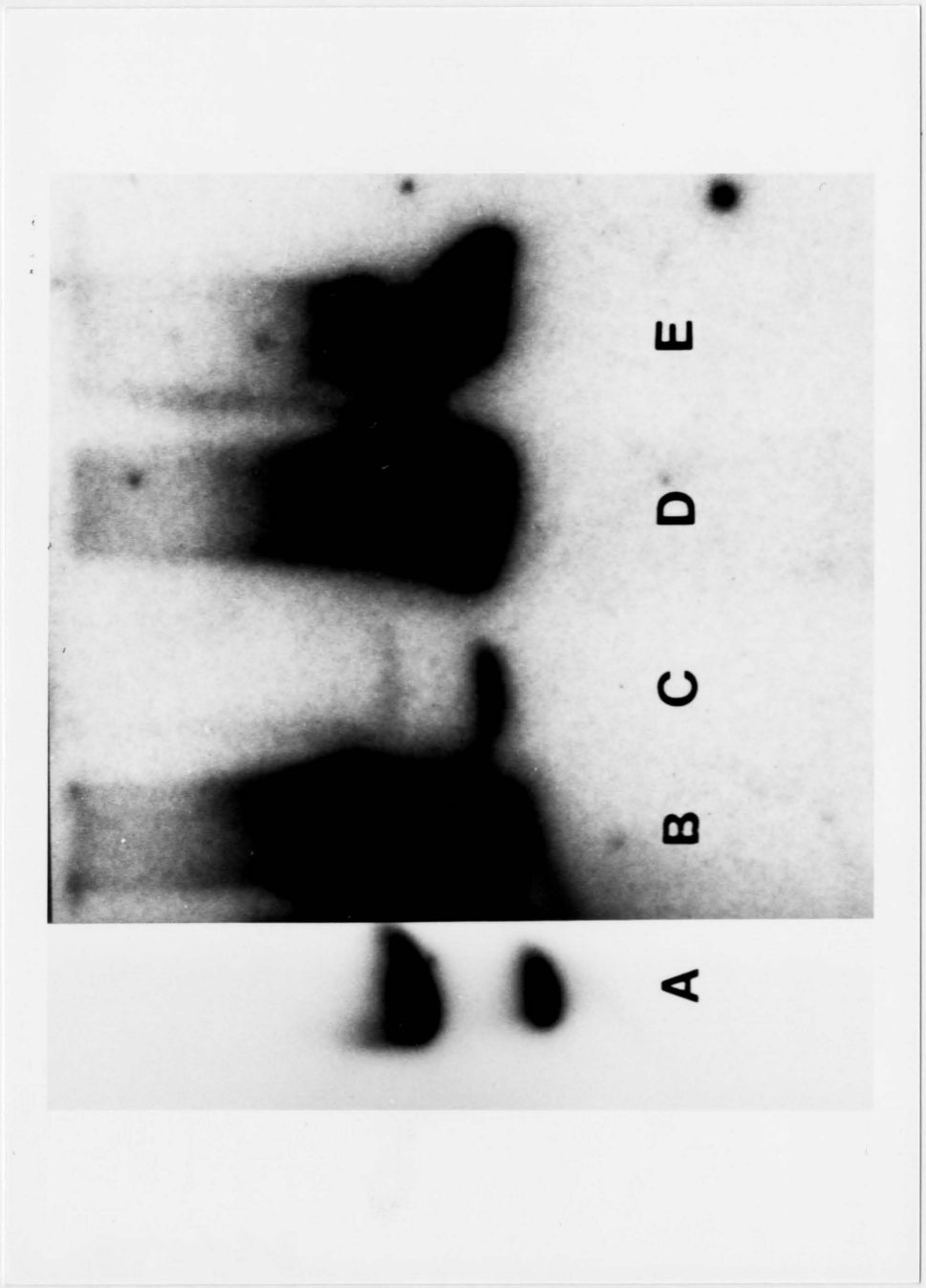
A. Hirt supernatant from C127 plus 3 ng BPV DNA, exposure time - 3 days.

B. Hirt supernatant from B251 grown in the presence of 0.1% ethanol.

C. Hirt supernatant from B251 grown in the presence of $10^{-5}$ M RA.

D. Hirt supernatant from B251 grown in the presence of 50 ng/ml TPA.

E. Hirt supernatant from B251 grown in the presence of $10^{-5}$ M RA plus 50 ng/ml TPA.


A
B
C
D
E

# DISCUSSION

The cell lines developed for and studied in this research demonstrated heterogeneity with respect to sensitivity to RA. Some of the data presented in the previous section are summarized in Table 5. Untransformed C127 were inhibited by about 50% and contained trace CRABP, indicating that the machinery for eliciting a response to $10^{-5}$ M RA was present in the untransformed C127. Two of the transformed cell lines exhibited enhanced sensitivity to the antiproliferative action of RA. However, one of these cell lines contained a very high level of CRABP and the other contained approximately the same level as the untransformed parent. In another transformed line, which contained no detectable CRABP, antiproliferative effects, although reduced from that of the untransformed parent, were observed. In agreement with other laboratories, the data do not support the notion that the amount of CRABP is correlated with growth inhibition by RA.

Since anchorage independent growth has broad acclaim for assessment of the expression of transforming genes, the transformation suppressing activity of RA on colony forming cells was evaluated. Forty-five percent of the colony forming cells of B100 were inhibited by $10^{-5}$ M RA, yet B100

Table 5. Partial summary and comparison of untransfromed C127 and BPV transformed cell lines.

| Cell line | Percent Growth Inhibition by $10^{-5}$ M RA[1] | Percent Colony Forming Cells[2] | Percent Inhibition of Colony Forming Cells by $10^{-5}$ M RA[2] | Detectable CRABP[3] | Genome Equivalents[4] |
|---|---|---|---|---|---|
| C127 | 48 | NA[5] | NA | Low | NA |
| B100 | 35 | 5 | 55 | None | 33 |
| B200 | 60 | 5 | 95 | High | 48 |
| B251 | 80 | 15 | 80 | Low | 58 |

1. $\frac{\text{day 5 cell number + RA}}{\text{day 5 cell number - RA}} \times 100$. Data from Figure 7.
2. Data from Figure 6.
3. Data from Figure 8.
4. Genome equivalents = $\left\{\left[\frac{(C_o/C-1)_{(B100,B200,B251)DNA}}{(C_o/C-1)_{C127\ DNA}}\right] \times 15.6\ ^3\text{H-BPV DNA genome equivalents}\right\} - 15.6\ ^3\text{H-BPV DNA genome equivalents.}$

   $C_o/C$ from Figure 9.
5. Not applicable.

contained no detectable CRABP. B200 contained a high level of CRABP and 95% of the colony forming cells in this cell line were inhibited by $10^{-5}$ M RA. A subclone of this cell line contained only trace CRABP yet exhibited inhibition of colony forming cells at a similarly high percentage. Prolonged treatment with RA caused loss of anchorage independent growth regardless of whether the cells contained detectable CRABP. There appeared to be no correlation between the extent of inhibition of colony forming cells and the amount of CRABP in the clones.

It is important in this regard to note that the CRABP assay employed here was specific binding to a 2S molecule in a whole cell cytosol, and could only detect unbound binding protein. In the case of B200 and its subclone B251, it was expected that B251 would demonstrate a reduced level of $^{3}H$ RA binding since it was one of the very few RA resistant clones in a cell line exhibiting a high level of $^{3}H$ RA binding. Even though a percentage of B251 colony forming cells were refractory to the inhibitory effect of $10^{-5}$ M RA, sensitivity to RA as indicated by inhibition of proliferation and reversal of TPA induced changes similar to the parent B200 was retained. The results presented here do not rule out the possibility of an endogenous molecule being bound to the binding protein, rendering the protein

undetectable by this method or the existence of an altered binding protein.

The lack of correlation between the effects of RA and the presence of CRABP would bring us to the conclusion that either the antitumor effects of RA are not mediated by CRABP or we are not looking at an appropriate effect. There is abundant evidence which indicates that many cellular genes are involved in the multistep process of viral transformation. The involvement of cellular genes in SV40 transformants has been analyzed by suppression of tumorigenecity in cell hybrids between normal cells and their SV40 transformed counterparts. Suppression of these hybrids reflects the recessive nature of cellular mutations underlying tumorigenicity since the resulting phenotype is nontumorigenic. The hybrid nucleus contains the dominant alleles contributed by the normal cells. In these studies it has been shown that different traits induced by SV40 transformation are suppressed to different extents by fusion with nontransformed cells. Thus almost every combination of transformed properties has been found. In fact, a conclusion drawn from this research is that growth in agar is not a reliable indicator of tumorigenicity and the expression of transforming genes [54].

Another putative function of RA, that of promotion antagonist, was tested for correlation with the presence of

CRABP. In keeping with its role as a promotion inhibitor, RA did reverse the morphological changes induced in all cell lines by TPA. The characteristics of the change in morphology showed some specificity with respect to the presence of the viral genome, but reversal of these changes by RA was complete regardless of whether the cells contained CRABP or not. Evidence for the processive nature and interrelatedness of tumor promotion and BPV transformation is demonstrated by the fact that C127 acquired the morphology of BPV transformed cells in the presence of TPA. However, since TPA treated C127 did not grow in agar, did not loose contact inhibition or exhibit enhanced growth rate in the presence of TPA, the possibility exists that more dramatic changes induced by the presence of the BPV episome were required for expression of these transformed properties.

The evidence regarding the relationship between copy number and sensitivity to RA is ambiguous. Liquid reassociation experiments indicated that the amount of BPV DNA in these cell lines varied almost twofold. This suggested that differences in copy number of the episome may account for the different sensitivities to the antiproliferative effects of RA. Fifteen percent of the cells in B251 were able to form colonies in agar while only 5% of the cells in B100 were able to form colonies in agar. Proliferation was inhibited by 35% in B100 which contained 33 copies of the BPV genome

per diploid of DNA while proliferation of B251, which contained 58 copies was inhibited by 60%. Similarly, inhibition of colony forming cells was inhibited by 55% in B100 and by 80% in B251. However, these trends are not borne out when the data on B200, which contains 48 copies of the BPV genome, are considered.

Conflicting results were obtained from experiments designed to demonstrate the effect of RA on the BPV DNA content of the cells. When treated for 4 weeks with $10^{-5}$ M RA the BPV DNA content of the cells was not affected; however, after only three days of treatment the BPV DNA content is reduced. These conflicting results may be reconciled by suggesting that over two weeks of treatment an RA resistant population could become predominant. Alternatively the more sensitive Southern blotting technique detected differences which were undetectable by liquid hybridization.

# CONCLUSIONS

Transformation of C127 mouse mammary tumor cells resulted in a functionally diverse population of cells. The biological effects of RA on BPV transformed C127 were inhibition of proliferation, inhibition of growth of colony forming cells and reversal of TPA induced changes. Consistent with the assumption that cell transformation is the result of an increase in the expression of functional activity of a normal cellular product, only one of these, inhibition of growth of colony forming cells, was unequivocally linked to the presence of the BPV genome, since untransformed C127 do not grow in agar. The fact that the presence of the BPV genome influenced these same parameters--increased the growth rate, induced the ability to grow in agar and was reflected in the type of TPA induced morphological changes--lends support to the theory that RA can suppress the expression of transforming genes.

Some of the data indicated that copy number of the episome played a role in the expression of transforming genes. Retinoic acid reduced the amount of extrachromosomal BPV DNA in the cells. This was accompanied by decreased growth rate and loss of the ability to grow in agar. This occurred in all cell lines regardless of the presence of

CRABP. B100 which contained the least amount of BPV DNA contained a relatively high proportion of cells which were able to grow in agar in the presence of $10^{-5}$ M RA. The antiproliferative effect of RA was reduced from that of the untransformed C127 in this cell line and the presence of CRABP was below detectable levels. B200 and B251 contained greater amounts of BPV DNA and very few cells which were able to grow in agar in the presence of $10^{-5}$ M RA. The antiproliferative effect of RA was enhanced in these cell lines but the levels of CRABP were very different. B200 contained a high level of CRABP and B251 contained a low level.

The role of the episome in regulation of the expression of transforming genes would be strengthened if a compound which experimentally increased episomal BPV DNA could be identified. The amount of the episome was not increased by treatment of the transformed cells with TPA. Apparently, TPA does not induce replication of the BPV episome or enhance transformation in BPV transformed C127. The significance of the antiporliferative effect of TPA on these cells is unclear.

The BPV episome was present in all cell lines as a unit length open circular episome. In B251 the BPV episome existed also as a unit length supercoiled episome. In this cell line the reduction in BPV DNA content by RA was greater for the open circular form than for the supercoiled form.

The biological significance of this finding is open for further experimentation.

The heterogeneous expression of the transformed phenotype seen in BPV transformed cell lines may be due to unequal distribution of the episomes at cell division, thus giving rise to a continually changing population with respect to copy number of the episome. It is still unclear whether a few cells contain large amounts of the episome or whether each cell in a population maintains a constant copy number. The rationale for a viral gene dosage theory for transformation by BPV would be strengthened by identification of a BPV specific transforming protein. Years of searching for unique proteins in BPV transformed cells and BPV tumor cells have been unproductive and an alternative mechanism for maintenance of the transformed state is being considered [2].

With the exception of a few outstanding examples in the area of drug resistance genes, regulation of gene expression in procaryotic and eucaryotic systems is thought to occur at the level of transcription. Four transcripts from BPV transformed mouse and hamster cells have been identified. Three of these transcripts have been identified from BPV induced warts. Apparently a 1.6 kb transcript which has been mapped to the transforming region of the BPV genome is unique to transformed cells [55]. It will be interesting to

see if B100, B200 and B251 also contain this transcript and if identical transcription units can be identified when the cells are grown in the presence of RA.

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