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The University of Arizona PH.D. 1984

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THE EFFECTS OF RETINOIC ACID ON CELLULAR TRANSFORMATION AND TUMORIGENESIS INVOLVING CELLS WITH KNOWN ONCOGENES

by

Neill Alan Giese

A Dissertation Submitted to the Faculty of the

PROGRAM IN PHARMACOLOGY AND TOXICOLOGY

In Partial Fulfillment of the Requirements
For the Degree of

DOCTOR OF PHILOSOPHY

In the Graduate College

THE UNIVERSITY OF ARIZONA

1984

THE UNIVERSITY OF ARIZONA
GRADUATE COLLEGE

As members of the Final Examination Committee, we certify that we have read the dissertation prepared by Neill Alan Giese

entitled The Effects of Retinoic Acid on Cellular Transformation and Tumorigenesis Involving Cells With Known Oncogenes

and recommend that it be accepted as fulfilling the dissertation requirement for the Degree of Doctor of Philosophy.

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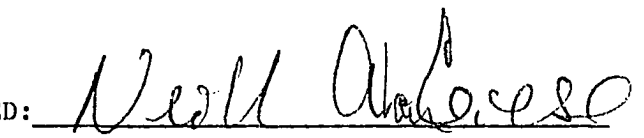
SIGNED: 

DEDICATION

I would like to dedicate this dissertation to my parents, Clarence and LaVerne Giese. Throughout all of my academic endeavors they have been unconditional in their support and understanding which has made the achievement of my goals easier and more meaningful.

ACKNOWLEDGEMENTS

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ABSTRACT

Vitamin A is known to have an important role in cellular differentiation and proliferation. In addition to regulating normal cellular processes vitamin A has also been shown to possess potent antineoplastic activity. The work in this dissertation characterizes the role of retinoic acid (RA) in cellular transformation and tumorigenesis with known oncogene involvement.

These studies were initiated by examining the effects of RA on human carcinoma cell lines which express an activated c-ras gene. The bladder carcinoma, EJ/T24 ($c\text{-}ras^{H}$) and the two lung carcinoma cell lines, LX1 ($c\text{-}ras^{K}$) and A2182 ($c\text{-}ras^{K}$), were not sensitive to RA. No inhibition of anchorage- or density-dependent growth was observed. Therefore, since these *in vitro* markers of transformation indicated a lack of effectiveness of RA on carcinomas containing a c-ras gene, retrovirally transformed cells were tested for RA sensitivity.

Kirsten murine sarcoma, Balb/c murine sarcoma virus, and Simian sarcoma virus transformed NIH/3T3 and NRK cells were used in these studies. In contrast to the human carcinoma cell lines, anchorage-independent growth of some of the virally transformed cells was very sensitive to inhibition by RA. Anchorage-independent growth of KNRK and SSVNRK cells was sensitive to high concentrations (5 μM) of RA; whereas, Balb/cMSV3T3 and SSV3T3 were sensitive to 1-20 nM RA. BALB/cMSVNRK anchorage-independent growth was stimulated 3.5 fold by

1 μM RA. KNRK displayed a 60% reduction in anchorage-dependent growth at 10 μM RA while little inhibition was observed with the other retrovirally transformed cells. A high level of sensitivity to RA inhibition of anchorage-independent growth was correlated with the presence of cytoplasmic retinoic acid binding protein (CRABP). This indicated that CRABP may have some role in the inhibition of retrovirally induced cellular transformation.

RA was shown to significantly reduce the incidence and size of Balb/cMSV3T3 cell tumors in nude mice. The inhibition of tumorigenesis in vivo therefore confirmed the results observed in vitro.

To investigate the mechanism by which RA was acting to inhibit retroviral transformation, v-onc mRNA levels were examined. RA had no effect on v-onc mRNA levels in cell lines sensitive to the inhibition of transformation. The effect of RA on the relative rate of synthesis of p21, the transforming protein of KMSV and Balb/cMSV, was investigated. No effect of RA was observed in any of the cell lines. Also, GDP binding by p21 in KNRK cell was unchanged by RA treatment indicating that the functional activity of this transforming protein was not modified.

RA does appear to be effective in inhibiting retrovirally induced cellular transformation and tumorigenesis. Evidence presented here indicates that this inhibition is not due to a direct effect of RA on the expression of the v-onc gene and/or gene product. Therefore, some other essential cooperating event(s) occurring within the cell are being acted upon by RA.

INTRODUCTION

Historical Aspects

It was first demonstrated by Stepp in 1909 that a fat soluble principle was present in the diet that was essential for life. Mice maintained on a lipid free diet could not survive unless an extract from egg yolk was used as an additional source of nutrition (Ganguly et al. 1980). The missing factor termed fat soluble A was also shown to be present in animal fat and cod liver oil (Ganguly et al. 1980). Drummond in 1920 suggested that this fat soluble factor be called "vitamin A".

Inquiries about the chemical nature of vitamin A were aided by the discovery in 1928 that β carotene possessed high vitamin A activity (Isler et al. 1928). Carotenoids had been discovered earlier in the 19th Century with the isolation of β carotene first being reported in 1831. In 1930 it was first demonstrated that β carotene was metabolized to vitamin A and subsequently stored in the liver (Moore 1957). The chemical structure of carotene (Karrer et al. 1930) and retinol (Karrer et al. 1931) were elucidated shortly thereafter.

The physiological role of vitamin A will be discussed in the next section but it is of historical interest to point out here that in 1935 Wald demonstrated the importance of vitamin A derivatives in the visual process. It was later shown, in 1944, that retinol and retinal were involved in vision (Morton 1944; Morton and Goodwin

1944). In 1946, retinoic acid (RA)* was synthesized and shown to be important for growth (Van Dorp and Arens 1946).

From these pioneering studies much was learned about the chemical nature of vitamin A and clues to its physiological role were beginning to unravel thereby providing the necessary scientific foundation for more detailed physiological and biochemical studies.

The Role of Vitamin A in Normal Physiology

Vitamin A (retinol) and the closely related naturally occurring analogues, retinal and RA, are known to have an important role in general growth, the maintenance and differentiation of epithelial and meschymal tissue, visual function and reproduction (Lotan 1980) (Figs. 1 and 2). Retinyl esters and carotene represent the major source of vitamin A which are present in the diet. Retinyl esters are hydrolyzed in the gut to give retinol which is then taken up into the mucosal cells and esterified with long chain fatty acids. These newly formed retinyl esters are transported via the lymphatic system and released into the blood as chylomicrons which are picked up by the liver. The liver provides a storage depot for vitamin A in the form of retinyl esters (Huang and Goodman 1965; Goodman et al. 1966). carotene is taken up by the mucosal cells where it is enzymatically cleaved to give two molecules of retinal (Goodman and Olson 1969)

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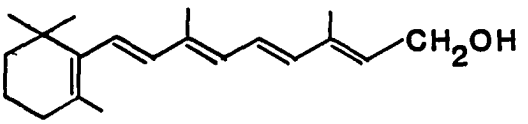
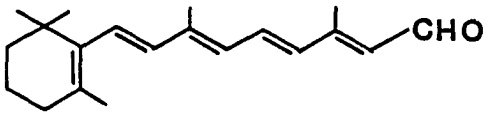
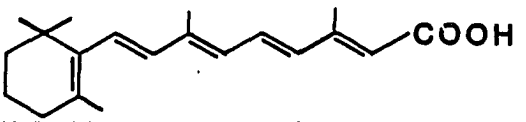
*A list of the abbreviations used throughout the text is included on page 87.



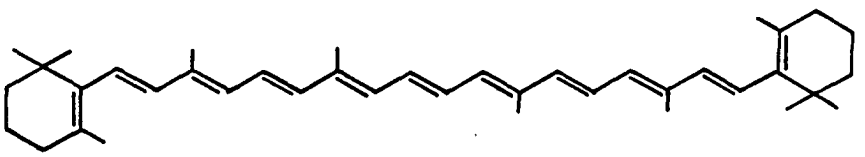
VITAMIN A (RETINOL)



VITAMIN A ALDEHYDE (RETINAL)



VITAMIN A ACID (RETINOIC ACID)



β-CAROTENE

FIGURE 1. CHEMICAL STRUCTURES OF VITAMIN A AND RELATED MOLECULES

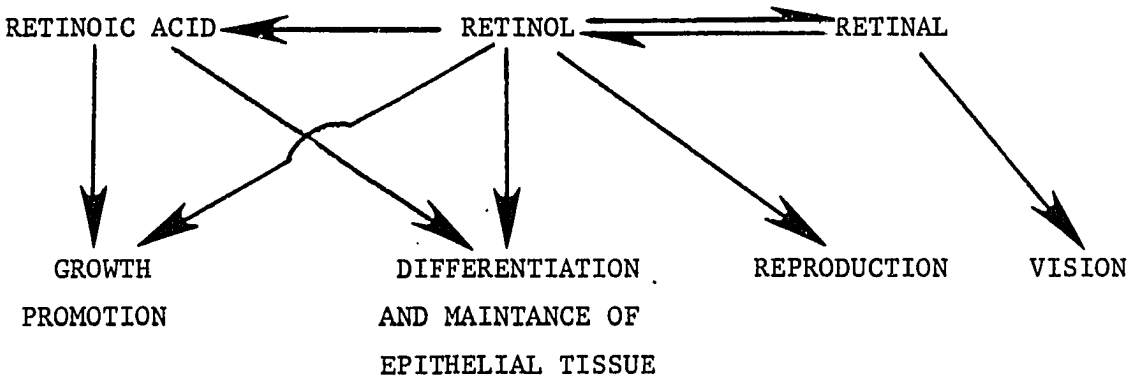


FIGURE 2. BIOLOGICAL ROLE OF VITAMIN A

which are further converted to retinol by a non-specific aldehyde reductase (Fidge and Goodman 1968). The retinol thus formed follows the same pathway to the liver as previously described. When the physiological demand for vitamin A increases, retinyl esters which are stored in the liver, are hydrolysised to retinol which then binds to RBP which is made in the liver. The retinol-RBP complex is released into the circulation where it binds prealbumin and is transported to its site of action (Goodman 1974; Peterson et al. 1974). RA is absorbed via the portal system bound to serum albumin and does not accumulate in the liver or other tissues in any appreciable amounts (Fidge et al. 1968). RA is not reduced to retinol or retinal (Dowling and Wald 1960), but is rapidly metabolized mainly to more polar compounds and excreted via the urine and bile (Smith et al. 1973).

The role of vitamin A in normal mammalian physiology as summarized in Fig. 2, was initially explored by maintaining animals on a diet lacking vitamin A. The deficiency state that was induced led to the observations that, as endogenous stores of vitamin A became depleted, the animals would stop growing and as the deficiency became more pronounced weight loss would occur and eventually the animals would die (Moore 1957). The first detailed description of the effects of vitamin A deficiency at the cellular level was provided by Wolbach and Howe (1925). In this classic paper it was clearly noted that alterations had occurred in the proliferation and differentiation of epithelial tissue. Abnormal differentiation was expressed as

hyperkeratinization and squamous metaplasia. These early observations have been confirmed using an in vitro system in which compounds possessing vitamin A activity are capable of reversing deficiency induced squamous metaplasia in hamster tracheal explants (Sporn et al. 1975).

It has also been well established that vitamin A deficiency has pronounced effects on the reproductive processes in both males and females in a variety of animal species (Ganguly et al. 1980). In males, testicular atrophy occurs, while the germinal epithelium undergoes degenerative changes and sperm fail to develop past the spermatid stage (Ganguly et al. 1980). Female animals which are acutely deficient do not conceive. When the deficiency is mild, conception occurs but the offspring are reabsorbed or born deformed (Moore 1957).

The area of normal physiology in which the role of vitamin A is most clearly understood is that of the visual process. The cis and trans isomers of retinal participate in Wald's visual cycle through their interaction with opsin to form the visual pigment rhodopsin (Wald 1968). Vitamin A deficiency first leads to the reversible degeneration of rods which causes night blindness. If the deficiency is allowed to continue, a degeneration of the cones will also occur and recovery is no longer possible resulting in total blindness (Bramer and White 1969).

Animals that are maintained on a vitamin A deficient diet which has been supplemented with RA undergo normal growth and epithelial

differentiation. These animals appear normal but are blind and sterile. Therefore, RA is capable of supporting growth and inducing cellular differentiation but cannot replace retinol in vision (Dowling and Wald 1969) or reproduction (Ganguly et al. 1980).

The physiological role of vitamin A is varied and complex. Although the various effects of vitamin A were described in the early part of the century, with the exception of its role in vision, the biology of vitamin A remains largely descriptive.

Vitamin A and Carcinogenesis

The suggestion of a possible link between vitamin A and cancer was evident in the pioneering studies conducted by Wolbach and Howe (1925) and Mori (1922). It was noted that the differentiation of stem cells into mature epithelium failed to occur and that excessive proliferation was present in many of the deficient epithelial tissues. Cancer has been defined as a fundamental defect in cellular differentiation which results in abnormal proliferation (Mintz and Fleischman 1981; Pierce et al. 1978). Because of the role of vitamin A in cellular proliferation and differentiation it was logical to predict that retinoids may have potential in the treatment and prevention of cancer.

Animal deficient in vitamin A have been shown to have an increased susceptibility to chemical carcinogenesis (Cohen et al. 1976, Rogers et al. 1973). A greater incidence of tumors and putative tumor precursors were observed in the vitamin A deficient animals.

Epidemiological studies have also noted an inverse relationship between vitamin A intake and the development of human cancer (Bjelke 1975; Mettlin et al. 1979; Shekelle et al 1981).

Besides an apparent increase in the susceptibility to cancer in the vitamin A deficient state, retinoid treatment is known to have a direct role in the inhibition of cellular transformation. A large body of literature exists which has demonstrated that retinoids are effective in the inhibition of tumor formation in animals which have been caused by a variety of agents including chemical carcinogens, ultraviolet radiation and viruses (Lotan 1980). Tumors arising at many sites are capable of being suppressed by retinoids including skin, bladder, lung, mammary gland and colon (Lotan 1980). The effect of retinoids on fully transformed, invasive, neoplastic cells is in some cases due to an inhibition of cellular proliferation (Lotan 1980) and in other cases terminal differentiation occured resulting in a more benign nonneoplastic phenotype (Flynn et al. 1983; Speers 1982).

There also exists an extensive amount of literature on the suppression of the malignant phenotype <u>in vitro</u>. Many transformed cells in culture, when exposed to retinoids, display alterations in cell growth. These changes include a decrease in the rate of proliferation and/or saturation density of cells grown on a substratum as well as an inhibition of anchorage-independant growth of cells suspended in soft agar (Lotan 1980). These effects can be due to a reversible inhibition of cellular proliferation (Lotan 1980) or the

induction of differentiation leading to a more benign phenotype (Strickland and Mahdavi 1978; Breitman et al. 1981; Flynn et al. 1983; Sidell 1982).

Retinoids are also capable of suppressing the expression of the transformed phenotype of cells treated with chemical carcinogens in vitro. Merriman and Bertram (1979) have shown that after the initiation of mouse fibroblasts with 3-methylcholanthrene, retinoids could inhibit the formation of transformed foci. This effect was reversible upon withdrawal of the retinoid.

There is also mounting clinical evidence that retinoids may be effective in the prevention and treatment of human cancer. As previously stated, there have been reports of an inverse relationship between vitamin A intake and the incidence of cancer. Also, retinoids have proven effective in reversing precancerous bronchial metaplasia in heavy smokers (Gouveia et al. 1982; Mathe et al. 1982). Preneoplastic and neoplastic lesions of the cervix have proven to be responsive to topical retinoid treatment (Meyskens et al. 1983; Surwit et al. 1982). Other malignant solid tumors which have responded to retinoid therapy include squamous cell carcinoma (Meyskens 1982), eccrine paroma (Roach 1983), and cutaneous metastatic melanoma (Levine and Meyskens 1980). Retinoid therapy has also given impressive results in the treatment of neoplasias of hematopoietic origin. A number of reports have demonstrated a dramatic remission of cutaneous T cell lymphoma (mycosis fungoides) (Claudy et al. 1983; Hunziker et al.

1983; Kessler et al. 1983). A response to retinoids has also been observed in patients with acute nonlymphocytic leukemia (Flynn et al. 1983).

At the present there is an overwhelming amount of evidence indicating that retinoids are effective in either preventing or inhibiting carcinogensis. These antineoplastic effects are observed in a wide variety of neoplastic lesions of varied etiology indicating that the mechanisms involved may be diverse.

Mechanisms of Antineoplastic Activity of Vitamin A

Although the initial observations implicating a role for vitamin A in differentiation and inhibition of carcinogenesis were made over 50 years ago, little progress has been made in uncovering the particular cellular or molecular mechanisms involved. Table 1 contains a list of potential mechanisms that have been suggested to have a role in the antineoplastic effects of vitamin A (Lotan 1980; Sporn and Roberts 1983). The effects of vitamin A on the differentiation of neoplastic cells has been discussed in the preceding section. It is clear that terminal differentiation to a benign phenotype does occur under the influence of retinoids both *in vitro* and *in vivo* (Strickland and Mahdavi 1978; Speers 1982; Flynn 1983). This effect, although important, probably only occurs in a limited number of cases because the antineoplastic effects of retinoids on most cancer cells are reversible upon withdrawal of the retinoid (Lotan 1980).

TABLE 1

PROPOSED MECHANISM OF ANTINEOPLASTIC ACTIVITY OF VITAMIN A

1. Induction of differentiation.
2. Inhibition of cell proliferation and DNA synthesis induced by carcinogens.
3. Suppression of the promotion phase of carcinogenesis.
4. Enhancement of host immune responses.
5. Changes in the cell membrane due to the induction of glycoprotein synthesis mediated by mannosyl retinyl phosphate.
6. Inhibition of peptide hormone mitogenesis either through receptor modifications or hormone production.
7. Effects on calcium- and phospholipid-dependent, cyclic AMP-independent protein kinase activity.
8. Alteration in the expression of genes involved in the regulation of cellular proliferation (e.g., oncogenes).

Retinoids have been shown to block the promotion stage in the two stage model of carcinogenesis (Boutwell 1983). In this model, skin tumors are induced by first applying a chemical carcinogen to initiate and then promoted by repeated dosing with the 12-O-tetradecanoylphorbol-13-acetate. The ability of retinoids to inhibit promotion is highly correlated with their ability to block the induction of ornithine decarboxylase which is a known biochemical event of promotion (Boutwell 1983).

The immune system is known to have an important role in the natural resistance to carcinogenesis. An enhancement of cell mediated immunity has been demonstrated in vivo in which T cell production was shown to be stimulated by retinol administration during the host vs. graft reaction (Malkovsky 1983). A stimulatory effect on T cells has also been observed in vitro (Sidell 1981). Vitamin A deficiency has also been associated with decreased immunological responsiveness (Mark et al. 1981). It should be noted that some reports on the effects of retinoids on in vitro immune responses have indicated that inhibition occurs. RA has been shown to inhibit the mixed lymphocyte reaction (Mastro et al. 1982) and the induction of interferon in human cells, in response to a variety of agents is suppressed by RA (Abb et al. 1982). The enhancement of immune responses is an important consideration but it is clear that other mechanisms must be involved because retinoids are effective in suppressing cellular transformation in a wide variety of in vitro systems which are devoid of immunological influences.

The participation of retinoids in sugar transfer reactions by means of the intermediate mannosyl retinyl phosphate is one of the few biochemical reactions in which a direct role of retinoids has been demonstrated (Deluca et al. 1979; Wolf et al. 1979). It has been suggested that retinoids can promote the synthesis of new glycoproteins which could modify membrane structure and function. These changes in the cell surface may account for the antineoplastic activity of vitamin A (Lotan et al. 1982). It has been recently pointed out by Sporn and Roberts (1983) that the evidence for the participation of RA in sugar transfers is not very convincing and that new retinoids have been synthesized (retinoidal benzoic acid derivatives) which are even more potent than RA in both *in vivo* and *in vitro* assays (Loeliger et al. 1980). Since these compounds are 100-1000 times more active than retinol it is unlikely that a direct involvement in protein glycosylation is the mechanism by which they inhibit carcinogenesis.

A very new area of investigation into the mechanism of retinoid activity has suggested that RA induction in the F9 embryonic carcinoma system may involve calcium and phospholipid-dependent, cyclic AMP-dependent protein kinase activity (Anderson et al. 1983). This may provide important insight for understanding retinoid control of cellular proliferation and differentiation.

The role of peptide growth factors are assuming a fundamental importance in the regulation of cell growth and differentiation

(Bradshaw and Sporn 1983). Retinoids have been shown to block phenotypic cell transformation produced by sarcoma growth factor (Todaro et al. 1978). A recent study by Welsch et al. (1984) demonstrated that retinoids could suppress peptide hormone-induced stimulation of mammary carcinoma cells in vivo and in vitro. Another example of retinoid involvement in peptide growth factor activity is the inhibition of mitogenesis induced in 3T6 cells by epidermal growth factor (Jetten 1982). It is interesting to note that this inhibition of mitogenesis is accompanied by a 6 fold increase in epidermal growth factor receptor number but it is unknown if these additional receptors are functionally coupled. The role of retinoids in the regulation of cell proliferation by growth factors is not clearly understood at present. This is pointed out by the reports that retinoids enhance mitogenesis of peptide growth factors (Dicker and Rozengurt 1979) and increase the phenotypic expression of cellular transformation induced by sarcoma growth factor (Jetten 1983).

At the molecular level the simplest hypothesis that would explain the antineoplastic effects of vitamin A would be an effect on gene expression. Retinoids have been shown to modify the level of a number of proteins including keratins (Fuchs and Green 1981), collagen (Beach and Kenney 1983), collagenase (Brinckerhoff and Harris 1980), plasminogen activator (Strickland and Mahdavi 1978), alkaline phosphotase (Reese et al. 1981), the receptor for epidermal growth factor (Jetten 1980), and transglutaminase (Yuspa et al. 1982). It is

not known whether the mechanism involved in the regulation is direct or indirect. Only in the case of the keratins has the regulation of mRNA levels been demonstrated (Fuchs and Green 1981). It has been proposed that retinol and RA may regulate gene expression through a direct interaction with the nucleus analagous to steroid hormone action (Chytil and Ong 1978; Mehta et al. 1982; Lian et al. 1981). The presence of specific binding proteins for retinol and RA in the cytoplasm of many cells has been used to support the above hypothesis (Chytil and Ong 1979). These binding proteins are termed cytoplasmic retinol binding protein, CRBP, and cytoplasmic RA binding protein, CRABP. At present the role of CRBP and CRABP in the action of retinoids is far from clear. A number of observations support their role in vitamin A action. Namely, specific and saturatable binding sites for the retinol-CRBP complex have been demonstrated in liver nuclei and chromatin (Lian et al. 1981) and a correlation between binding activity and biological activity has been shown to occur for a wide range of synthetic retinoids (Sherman et al. 1983; Sani et al. 1984; Trown et al. 1980). Also, mouse embryonal carcinoma cell mutants which fail to differentiate in response to RA lack CRABP (McCue et al. 1983; Schindler et al. 1981). Evidence against a role for CRBP and CRABP in vitamin A action has also accumulated. No correlation between growth inhibition (Lotan et al. 1980) or inhibition of chemically induced cellular transformation (Libby and Bertram 1982) and the presence of the binding proteins has been found.

Lotan et al. (1983) have reported the isolation of mutant melanoma cells which have lost their sensitivity to RA but still have high levels of CRABP bringing the role of CRABP into question. It is possible that the defect could involve the interaction of the drug-receptor complex with nuclear receptor sites. The role of CRABP in the induction of differentiation has also been questioned due to the fact that HL60 cells (promyelocytic leukemia) which are very sensitive to RA do not have detectable levels of CRABP (Dover and Koeffler 1982). Therefore, it appears that retinoids can regulate cell growth and differentiation in the absence of retinoid-binding proteins. The role of CRBP and CRABP in retinoid action, if any, will only be understood with further investigation.

From this discussion on the mechanism of action of vitamin A it is evident that the effects are quite varied but that the plasma membrane and the nucleus may be major targets of retinoid activity. Whether the effects are direct or indirect, it is likely that vitamin A modifies the expression of genes that control cellular proliferation and differentiation.

Vitamin A and Viral Carcinogenesis

The first evidence that vitamin A may have a role in the formation of virus particles was presented in a report by Bough (1963). He was able to demonstrate that vitamin A produced filamentous forms of the predominately spherical myxovirus. This study shows that retinoids can alter viral structure and potentially viral function.

March and Biely (1967) conducted the first study on the influence of vitamin A and retrovirally induced cancer. Chickens were fed diets either high or low in vitamin A and the incidence of leukemia was determined after administration of avian leukosis virus. The animals in the high vitamin A group had a two fold increase in tumor incidence. This observation is hard to interpret because the overall incidence in either group was <5%. An increase in sarcoma formation due to Rous sarcoma virus infection has been observed (Polliack et al. 1972; Polliack and Ben-Sasson 1971). These stuies were done by injecting virus into the wings of chickens and then treating the site of injection topically with a 20% solution of retinyl palmitate. This represents a dose greater than 1000 times higher than doses currently being employed for the inhibition of carcinogenesis.

In a mammalian system, vitamin A has been shown to inhibit retrovirally induced sarcoma formation (Seifter et al. 1973; Seifter et al. 1976). Mice were injected with the acute transforming virus, Moloney murine sarcoma virus, and maintained on a normal diet or a diet which contained 5-10 times the normal vitamin A content. There was a 50% reduction of tumor incidence, an increased latency period to tumor formation and an increased rate of tumor regression. The authors suggested that vitamin A may be enhancing the host's immune system.

Studies done with retrovirally transformed cells in culture

have shown that RA can block phenotypic expression of cellular transformation without immunological influences (Mukherjee et al. 1982; Mukerjee et al. 1983). RA was shown to inhibit anchorage-dependent and -independent growth of cells transformed by Rous sarcoma virus and KMSV. This is in contrast to a report by Todaro (1978) which failed to identify an effect of retinol or RA on the initiation or maintenance of transformation induced by Moloney murine sarcoma virus in either NRK or 3T3 cells.

Vitamin A has also proven to be active against papilloma viruses. Papillomas induced by Shope rabbit papilloma virus were delayed in their appearance and decreased in size with continued systemic administration of retinyl palmitate (McMichael 1965). A very interesting clinical study was conducted on a patient with epidermodysplasia verruciformus induced by human papilloma virus type 5 (Lutzner 1980). The early stages of this disease consist of benign scaly plaques and flat wart-like lesions. All patients with severe forms of the disease later develop multiple squamous cell carcinomas of the skin (Orth 1981). With the patient in this study, oral retinoid treatment for two months resulted in the disappearance of the wart-like lesions and a marked reduction in the size of two tumors that were missed during surgery. Biopsy of the few remaining benign lesions gave no evidence of papilloma virus histologically or with specific immunofluorescence. Also, the amount of viral DNA in the samples was decreased 100 fold.

Herpes virus represents the third family of oncogenic viruses that are influenced by retinoids. Epstein-Barr virus induction of early antigen expression in lymphatic cells is blocked by RA (Yamamoto et al. 1979; Bauer 1983). These studies indicate that retinoids are able to inhibit the expression of viral genes which could be important in inhibiting viral carcinogenesis.

In summary, three families of oncogenic viruses; retroviruses, herpes viruses and papovaviruses, are known to be influenced by retinoid treatment. Although these studies are preliminary they indicate that retinoids may modify viral infectivity, replication and/or expression, any one of which could be important in the inhibition of viral carcinogenesis. The aim of work presented in this dissertation was to extend the existing knowledge in this area.

Oncogenes and Cancer

In 1969, Huebner and Todaro published their now classical paper which put forth the oncogene hypothesis. They proposed that oncogenes exist in all normal cells and that activation of these genes by retroviruses or chemical carcinogens would lead to cancer. The oncogene hypothesis was based on the observations that infectious leukemia viruses and saroma viruses appeared in tumors of mice that had been treated with either radiation or chemicals (Tooze 1973; Gross 1970). The leukemia and sarcoma viruses observed in these studies represent two classes of retrovirus, the rare, highly oncogenic class

with oncogenes and the ubiquitous class, without oncogenes (Weiss et al. 1982). The first class, acute transforming retroviruses, are usually not transmitted either in the germ line or by infection and are, therefore, very transient in their existence (Weiss et al., 1982). Timely examination of tumors resulted in the isolation and characterization of viruses that would have otherwise become oblivious.

Retroviruses have been intensely studied because of their interesting biological characteristics, including, replication by reverse transcription, high-efficiency integration into the host genome and the transduction of cellular genes. The fact that acute transforming retroviruses had acquired cellular information was brought to bear through studies of Rous sarcoma virus. It was discovered that this virus was comprised of two distinct portions (Stehelin et al. 1976). The first part of the viral genome contained genes which were essential for viral replication, integration and particle formation. In addition to these viral genes, the second part of the genome contained sequences that were not required for normal viral functions and were unrelated to essential virion genes. The src gene thus defined, although not necessary for normal viral functions, was shown to be responsible for the transforming functions (Weiss et al. 1982). It was soon discovered that src had originated from a normal cellular chicken gene, proto-oncogene, which is an integral part of the host genome (Hughes et al. 1979). These studies demonstrated that cellular genes were capable of becoming oncogenes.

Cellular genes are capable of being activated by retroviruses, therefore, it is possible that these or similr genes may be activated by nonviral means which could lead to the formation of cancer. It had been previously shown that viral oncogenes were able to transform cells in culture by transfection. If viral DNA was put into the medium with NIH/3T3 fibroblasts, some of the cells would take up the DNA, integrate it into their genome and become transformed (Cooper 1982). Therefore, if human cancer cells contained activated oncogenes, it may be possible to transform NIH/3T3 cells with DNA from the tumor cells. The early studies of Weinberg and Cooper clearly demonstrated that tumor cells contained activated oncogenes that had arisen by nonviral mechanisms.

Many tumors have been shown to contain an oncogene whose activation occurred at some point during the carcinogenic process. The types of neoplasias which are represented in this group including, fibro- and rhabdomyosarcoma, glioblastoma, neuroblastoma, leukemia and lymphomas. Also, tumors of the bladder, skin, pancreas, bowel, lung and breast have been shown to express activated oncogenes (Land et al. 1983).

The proposed mechanisms by which oncogenes become activated are summarized in Table 2. The first mechanism, transduction by a retrovirus, has already been discussed, but it should be pointed out that this is an unnatural occurrence for the virus which always, with the exception of Rous sarcoma virus, results in the generation of a

TABLE 2

MECHANISMS PROPOSED FOR ONCOGENE ACTIVATION

1. Transduction by a retrovirus through illigitimate recombination.

2. Promotor insertion leading to transcriptional activation.

3. Transcriptional activation due to the influence of an enhancer element.

4. Overexpression due to gene amplification.

5. Chromosomal translocations resulting in transcriptional activation and/or somatic mutation.

6. Somatic mutations leading to alterations in the structure of the oncogene protein.

replication defective virus that would soon become extinct (Duesberg 1983). When the virus acquires the cellular oncogene transcriptional activation and somatic mutations occur which result in the activation of the gene (Duesberg 1983). Retroviruses have been extremely important for our understanding of cancer because they have identified and provided the means for isolating genes that have a major role in controling cell growth.

The next two mechanisms of oncogene activation also involve retroviruses. Long terminal repeats (LTR) are present at both ends of the retroviral genome. An LTR can increase transcriptional activity of a cellular gene by acting either as a promotor itself or by acting as an enhancer which increases the transcriptional activity, when positioned either upstream or downstream, of the existing promotor.

Tumors that arise during avian leukosis virus infection (this virus does not have an oncogene), have an activated cellular myc gene (Hayward et al. 1981; Payne et al. 1982; Fung et al. 1983). The activation is due to the insertion of an LTR adjacent to the myc gene which results in it providing promotor or enhancer functions.

Another mechanism of activation involves overexpression due to amplification of the proto-oncogene. In some cases, as many as 100 copies of the proto-oncogene have been shown to be present in a tumor cell. Proto-oncogenes that have been shown to be amplified include, c-myc, n-myc, c-ras^{K} and c-abl (Land et al. 1983). At present the precise role of proto-oncogene activation in human cancer remains to be elucidated.

A very active area of research on the mechanism of proto-oncogene activation involves specific chromosomal translocations. It appears that in some cases of Burkitts lymphoma, the myc gene becomes juxtaposed to the immunoglobin domains. This results in the loss of normal myc regulation and the gene comes under the influence of immunoglobin regulatory sequences (Leder et al. 1983).

The last mechanism involves a somatic mutation occurring in the coding region of the proto-oncogene which results in a structural alteration of the protein. This mechanism has been clearly defined for members of the ras gene family. The first activated oncogene to be fully characterized was found to be present in the human bladder carcinoma cell line, EJ/T24 (Parada et al. 1982; Pulciani et al. 1982b; Reddy et al. 1982). The only alteration to be found that was necessary for the activation of the EJ/T24 c-ras^{H} gene, was a point mutation in 12th amino acid codon, resulting in a glycine to valine change. The initial criticism that c-ras^{H} activation may be an artifact of cell culture is no longer valid. Many primary tumors have been subsequently shown to have an activated ras gene where the surrounding normal tissue did not (Pulciani et al. 1982; Santos et al. 1984; Feig et al. 1984). Also, animal models of skin and mammary carcinogenesis have demonstrated that ras gene activation is an integral part of tumor formation (Balmain et al. 1984; Sukumar et al. 1983). These studies will be brought up again in the Discussion but it is worth pointing out here that retinoids are effective in inhibiting tumor formation in the above animal models.

In conclusion, there is now, 15 years later, substantial experimental evidence to confirm Huebner and Todaro's oncogene hypothesis.

Objectives

It was the purpose of the research presented here to test the hypothesis that RA would be effective in inhibiting oncogene induced cellular transformation and tumorigenesis. Given that RA proved effective, the work would proceed with the intent of uncovering the molecular mechanisms involved. This would be possible due to the fact that molecular probes were available for studying the transcription, translation and functional activities of the oncogenes and their gene produces which are known to have a role in carcinogenesis.

MATERIALS AND METHODS

Preparation and Storage of Retinoic Acid

For treatment of cells a stock of RA (Sigma) was prepared in absolute ethanol or DMSO and stored in liquid nitrogen prior to use. The [^{3}H]RA (30 Ci/mmol; gift from Hoffman-LaRoche, supplied by Dr. Mark Haussler) was stored at -20°C in toluene at 0.5 pmol/ml. Purity of the retinoids was verified by high pressure liquid chromatography (verified by the lab of Dr. Haussler). All operations involving retinoids were carried out in the dark or in amber-filtered light.

Cell Lines

A description of the cell lines used is summarized in Table 3. The human carcinoma cell lines LX1, A2182, and EJ/T24 were grown in DMEM with 10% heat-inactivated (56°, 20 min) fetal bovine serum. The NIH/3T3 transformants (44-9-1-1 and 118-4-3-1) were grown in DMEM with 10% heat-inactivated newborn calf serum. KNRK, Balb/cMSVNRK and SSVNRK are nonproducer cell lines derived from NRK cells transformed with KMSV, Balb/cMSV and SSV, respectively. The NIH/3T3 viral transformants, K3T3, Balb/cMSV3T3 and SSV3T3 were also nonproducing cell lines transformed by the same viruses as described above for the NRK cells. Both the parent cell lines, NRK and NIH/3T3, as well as all of the viral transformants were a generous gift from Dr. S.

TABLE 3

Description of Cell Lines

Cell Line	Description	Oncogene	Source or Reference
EJ/T24[1]	Human bladder carcinoma	c-ras^{H}	L.B. Chen (Bubenick et al., 1973; Marshall et al, 1977; O'Toole et al, 1983; Parada et al, 1982; Reddy et al, 1982; Pulciani et al., 1982b; Tabin et al., 1982; Taparowsky et al, 1982)
44-9-1-1	NIH/3T3 transformed with EJ/T24 DNA	c-ras^{H}	M. Barbacid
A2181	Human lung carcinoma	c-ras^{K}	M. Barbacid (Pulciani et al., 1982a; Pulciani et al, 1982b)
118-4-3-1	NIH/3T3 transformed with A2182 DNA	c-ras^{K}	M. Barbacid
LX-1	Human lung carcinoma	c-ras^{K}	L. B. Chen (Der et al., 1982)
NIH/3T3	Mouse embryo fibroblast	-	S. A. Aaronson

[1]EJ and T24 cell lines were apparently derived from the same source (O'Toole et al., 1983).

Aaronson's laboratory at the National Cancer Institute, Bethesda, MD. The parent NRK and NIH/3T3 cells as well as their viral transformants were grown in DMEM with 10% heat-inactivated calf serum.

Assay for Inhibition of Anchorage-dependent Growth

Growth curves were obtained using 35 mm Petri dishes which were seeded at $5 \times 10^4 - 2 \times 10^5$ cells per dish. This allowed saturation density to be reached by day 6. Cells were removed by treatment with 0.25% trypsin for daily cell counts determined using a hemocytometer for 7 days to establish normal growth. The percentage of change in growth based on cell count was calculated at the end of log phase growth. The retinoid treated cells were incubated in the presence of 1 μM or 10 μM RA while the control group contained 0.1% ethanol or DMSO.

Assay for Inhibition of Anchorage-independent Growth

This assay was performed essentially as described by Hamburger and Salmon (1977). Briefly, colony formation in soft agar was carried out using 35 mm Petri dishes containing a 1 ml bottom layer of 0.5% agar (Difco) in DMEM with 20% fetal bovine serum. A 1 ml top layer of 0.33% agar (in the same medium) containing 10^3-10^5 cells was added. The plates were then incubated at 37°C in a humidified atmosphere of 5% CO_2 and 95% air for 7 to 14 days. For cell lines containing activated c-onc genes, retinoid treated plates contained 1 M RA in both layers while control plates contained vehicle alone. An ED_{50} for RA induced inhibition of colony formation of the virally transformed

cells was interpolated from the dose response curves by seeding cells in a concentration range of 0.1 nM to 10 μM RA. Colonies were quantitated using a Bausch and Lomb Omnicon Feature Analysis System Model II. Colonies greater than 60 μm in diameter were scored as positive.

Determination of Cytoplasmic Retinoic Acid-binding Protein

The level of CRABP in each of the cell lines was determined as previously described by Haussler, et al. (1983). Briefly, at least 10^8 cells were harvested, washed 3 times with phosphate buffered saline, and resuspended in a buffer containing 10 mM Tris-HCl, pH 7.4 and 1 mM EDTA. The cells were disrupted by sonication (Kontes micro-ultrasonic cell disrupter) and a cleared cytosolic extract was prepared by centrifugation at 160,000 x g for 45 min in a Beckman TY65 rotor. For sucrose gradient analysis, 0.4 ml of cytosolic extract (1-3 mg protein) was incubated for 4 hrs at 4°C with [^{3}H]RA (50 nM; 30 Ci/mMol) and layered onto a linear 5-20% sucrose gradient. The gradients were centrifuged at 230,000 x g for 20-22 hrs in a Beckman 50.1 rotor, fractionated into twenty-five 0.2 ml fractions, mixed with 5 ml of aqueous scintillation counting solution (Amersham) and radioactivity determined. To correct for nonspecific binding a 100-fold excess of unlabeled RA was added to duplicate samples before centrifugation, and counts obtained after centrifugation were subtracted from those observed when only [^{3}H]RA was present. After correcting for nonspecific binding in the 2S region of the gradient,

specific binding activity was calculated as fmol of [^{3}H]RA bound per mg of protein in the cytosolic extract. Protein determination was performed using the Bradford assay (Bio-Rad protein assay kit).

Inhibition of Tumorigenesis in Athymic Nude Mice

Outbred athymic nude mice (nu/nu) were purchased from Harlan Sprague Dawley, Inc. (Indianopolis, IN). All mice used in these studies were males at 3-4 weeks of age. These animals were housed in the animal resources facility at the University of Arizona Health Sciences Center in a filtered air environment to reduce exposure to airborne pathogens.

To test the ability of RA to inhibit tumor formation by virally transformed cells we used a technique of intracutaneous injection of tumor cells as previously described by Levine, et al. (1982) with some minor modifications. The nude mice were anesthetized with 0.05 mg Innovar (Pitman-Moore), intramuscularly. A Dermovac suction blister apparatus (Instrumentatium, Helsinki) was then applied to the back of each animal and the pressure was reduced to 75 mm of Hg and maintained for 90 seconds. As soon as the blisters were thus formed, 50,000 Balb/cMSV3T3 cells in 0.05 ml of serum free media were injected into the blister cavity through a 30 gauge needle. Nine animals were used for each of two groups, control and RA treated. Two sites per animal were injected with tumor cells to give a possible total of 18 tumors per group with a 100% incidence of tumor formation. Treatment was begun 24 hrs after injection of the tumor cells. Ten µl of 0.2% RA

(Sigma) in DMSO was applied topically every 24 hours to each tumor site. Control animals received DMSO only. At the end of 28 days the animals were terminated and the tumors were excised and wet weights were recorded. Tissue slices were stained with eosin and hematoxylin for examination of gross histology.

DNA Probes and Anti p21 Antibody

The DNA probes used represent an internal fragment of each viral genome that is specific for the respective v-onc gene described below and shown in Figure 3. In all cases, the v-onc specific probe was isolated free of vector by electrophoresis from agarose gels onto NA45 DEAE membrane (Schleicher and Schuell) (Dretzen et al. 1981; Winberg and Hammarskold 1980). Each fragment thus purified was nick translated (nick translation kit, Bethesda Research Laboratories) using [^{32}P]dCTP, (600 Ci/mmol) to a specific activity of 5 x 10^7 to 2 x 10^8 dpm/μg DNA for use in dot blot hybridization experiments. The EcoRI fragment from the HiHi3 plasmid, containing the v-ras^k sequence was used for KMSV transformed cells (Ellis et al. 1981). For Balb/cMSV transformed cells, a v-bas specific Hind III-Bam HI fragment previously cloned into pBR322 was used (Eva et al. 1982). A Pst I fragment from SSV was previously cloned into pBR322. This plasmid was a generous gift from Dr. K. Robbins at the National Cancer Institute in Bethesda, MD. This fragment has some viral sequences in addition to v-sis which are liberated by cutting the insert with XbaI to yield a PstI-XbaI v-sis specific fragment which was used for hybridization (Devare et al. 1983).

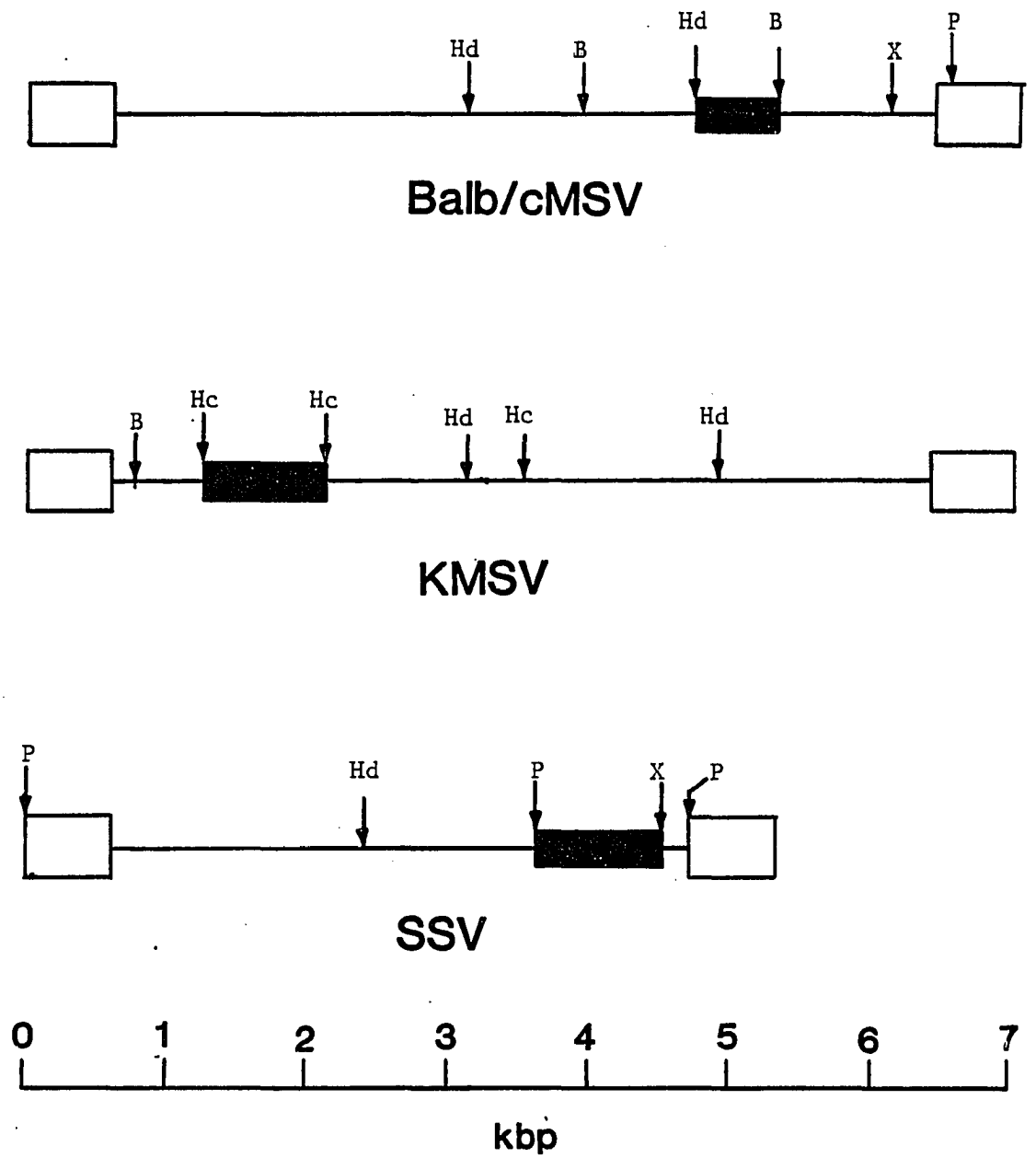


Figure 3. Partial restriction endonuclease maps of Balb/cMSV, KMSV and SSV. The oncogene of each virus are indicated by the solid boxes. The restriction enzyme sites are indicated as: Hd, Hind III; B, Bam HI; X, Xba I; P, Pst I; Hc, Hinc II.

The monoclonal antibody used in these studies is a rat IgG molecule being produced by a rat lymphocyte-myeloma hybrid cell line generated by Furth et al. (1982). This antibody designated, Y13-259, reacts specifically with the 21,000-dalton transforming protein (p21) encoded by the v-ras and v-bas genes of KMSV and Balb/cMSV respectively (Furth et al. 1982). This antibody was a generous gift from Dr. S. Srivatava (NIH, Bethesda, MD). Serum free medium, from hybridoma cells, containing Y13-259 was concentrated by ammonium sulfate precipitation. The dissolved precipitate consists of 30-40% antibody protein (personal communication with Dr. S. Srivastava). This gives about 300 μg of Y13-259 per ml of antibody solution.

Measurement of v-onc Specific mRNA Concentration

Total cytoplasmic RNA was isolated by lysing cells in 10% Nonidet P40, 0.2 M NaCl, 8 mM $MgCl_2$, 0.05 M Tris-HCl, pH 7.4 and 10 mM vanadyl-ribonucleoside complex (Bethesda Research Labs). The nuclei and debris were removed by centrifugation at 16,000 x g for 1 min at room temperature. The cytoplasmic extracts were treated with 1% sodium dodecyl sulfate and extracted with phenol in the presence of chloroform-isoamyl alcohol as previously described (Spector and Baltimore 1975). All RNA samples were adjusted to 0.4 M sodium acetate and precipitated with 2.5 volumes of absolute ethanol and stored as a slurry at -20°C. Poly(A^+)mRNA was isolated by a single pass of total cytoplasmic RNA over oligo(dT)cellulose (Bethesda

Research Laboratories) (Aviv and Leder 1972). Greater than 95% of cytoplasmic RNA is composed of RNA other than poly(A^+)mRNA (Brandhorst and MConkey 1974). We obtained [^{3}H]RNA by labeling cells with [^{3}H]uridine as previously described (Farmer et al. 1978). Poly(A^+)mRNA was removed from the labeled cytoplasmic RNA by two passes over oligo(dT) cellulose. Trace amounts of this non-poly(A^+) [^{3}H]RNA were added to our cytoplasmic RNA preparations before oligo (dT) chromatography to give a measurable specific activity of non-poly(A^+)RNA in the preparation. The specific activity of the poly(A^+)mRNA obtained after a single pass over oligo(dT) was used to determine the degree of contamination by non-poly(A^+)RNA. The mRNA obtained was 40-70% contaminated. All mRNA samples were corrected for contamination before application to nitrocellulose paper (BA85; Schleicher & Schuell). RNA dot blot analysis was done according to the method of Thomas (1980) using [32p] DNA probes described above. The range of heat denatured (100°C; 5 min) mRNA used was 0.16 g to 1.25 μg spotted in 4 μl of sterile H_2O. After hybridization and washing, the nitrocellulose filters were exposed to Kodak X-Omat RP X-ray film at -70°C for 6-16 hours with an intensifying screen.

Metabolic Labeling of Cells, Immunoprecipitation and SDS-PAGE Analysis

The transforming protein of KMSV and Balb/cMSV, p21, was labeled with [^{35}S] methionine by growing KNRK and K3T3 cells were grown in 100 mm plastic Petri dishes in DMEM with 10% heat inactivated

calf serum. Treated cells contained 10 μM RA while control plates received 0.1% DMSO. Balb/cMSVNRK and Balb/cMSV3T3 cells were grown under the same conditions as the KMSV transformants at 1 μM RA. The treatment period for all cells was 72 hours and the initial seeding density was adjusted such that cells were nearing confluency at the time of [^{35}S] Methionine labeling.

At the end of the RA treatment period the cells were washed once with 5 ml of methionine free DMEM (Gibco, Grand Island, NV) and incubated with 5 ml of the same medium at 37°C for 30 minutes. This preincubation in methionine free medium allows for the depletion of intracellular pools of methionine in order that a greater amount of [^{35}S] methionine will be incorporated into newly synthesized proteins during the labeling period. The cells were labeled by removing the medium and adding back 3.5 ml of the same medium containing 150 μCi/ml of [^{35}S] methionine (1167 Ci/mmole; New England Nuclear). The labeling was continued for 3 hours at 37°C. At the end of the incubation period the medium was removed and the cells were washed once with 10 ml of phosphate buffered saline. One ml of lysing buffer containing 20mM phosphate (pH7.5), 1% Triton X-100, 0.1% sodium dodectyl sulfate, 15mM NaN_3, 0.5% sodium deoxycholate, 0.1 M NaCl, 1mM PMSF, and 100 Kallikren units/ml of aprotinin was added per plate. The cells were scraped from the plates with a rubber policeman and homogenized in a tight-fitting Doune (Kontes) homogenizer for 25 strokes. The homogenates were then placed in a TY65 Beckman rotor and centrifuged at 45,000 rpm for 30 minutes. A small aliquot of each

lysate was removed for determination of protein concentration and [^{35}S] methionine incorporated while the remainder was frozen in liquid nitrogen for subsequent analysis. The range of protein concentrations of the lysate was 1.5-2.5 mg/ml and the amount of [^{35}S] methionine incorporated was 1-2 x 10^5 dpm/μg protein. No change in the specific activity of total protein from RA treated cells compared to control was observed (data not shown).

Immunoprecipitation of p21 from cell lysates was accomplished by incubating cell lysates with 5 μl of Y13-259 antibody (1.5 g) in a volume of 0.2 ml for 30-60 minutes at 4°C. This represented 275-475 g of protein per reaction or 1-5 x 10^7 dpm of incorporated [^{35}S] methionine. For each cell line an equivalent number of dpms were included per reaction for control and RA treated lysates. The precipitating reagent used in these experiments was Protein A-Sepharose CL-4B (Pharmacia) which was first incubated in swelling buffer (lysing buffer previously described with 1 mg BSA/ml without PMSF and aprotinin) and then coupled with goat anti-rat IgG (Capell). Excess IgG was removed by washing and centrifugation and then the coupled Protein A-Sepharose was resuspended in swelling buffer to give 85 μg of protein A per ml. Each lysate was then incubated with 200 μl of the precipitating reagent (17 μg of protein A) while agitating at 4°C for 1 hour. The tubes were centrifuged, the supernatant was aspirated and 1 ml lysing buffer minus PMSF and aprotinin was added per tube. The immune complex was resuspended and precipitated by

centrifugation. This was repeated for a total of 3 washes. After the final wash, 25 µl of SDS-PAGE buffer (18% glycerol, 2.6% sodium dodectyl sulfate, 0.02% bromphenol blue, 50mM Tris-HCl, pH 6.8 was added to each protein A sepharose pellet and the immune complex was dissociated by heating at 100°C for 2-5 minutes. The supernate from each sample was subsequently used for SDS-PAGE analysis. The polyacrylamide concentrations used were 3% in the stacking gel and 15% in the resolving gel. Unlabeled molecular weight standards ranging from 14,300-44,600 daltons were included in the sample lanes and identified by staining the gel with 0.025% Coomassie blue in methanol and acetic acid. After destaining the gel was treated with Enhance (New England Nuclear) and dried. The dried gels were exposed to Kodak X-O mat RP x-ray film at -70°C for 2-4 days.

GDP Binding Assay

KNRK and SSVNRK cells were grown in 100 mm petri dishes in DMEM with 10% heat inactivated calf serum. Control plates received 0.1% DMSO while treated plates contained 10 M RA. Cells were treated for 72 hours with a daily medium change. At the end of the treatment period the cells were reaching confluency. GDP binding assay was performed as previously described (Scholnick et al. 1979) with some modifications. At the end of the treatment period each plate was washed with 10 ml of PBS and 0.6 ml of lysing buffer (5 mM $MgCl_2$, 100 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 20 mM Tris-HCl pH 7.5) was added, and the plates were placed at 4°C for 5-10 minutes.

The cells were scraped from the plates and passed through a 22 gauge needle three times. The cell lysate was then centrifuged in a Beckman TY65 rotor at 45,000 rpm for 30 minutes. The upper 50% of the supernate was used at the time of preparation. Aliquots of the lysates were saved for protein determination according to the method of Lowry et al. (1951). Each reaction contained 10-300 µl of cell lysate (0.03-1.05 mg protein). All volumes were adjusted to 300 µl with GDP binding buffer (3mM $MgCl_2$, 100 mM NaCl, 0.1% Triton X-100, 0.02% sodium deoxycholate, 20 mM Tris-HCl pH 7.5). Fifteen µl (1.5 µg IgG) of a 1 to 3 dilution of Y13-259 antibody (described previously) and 7.8 µCi of [^{3}H] GDP (7.9 Ci/mmol; New England Nuclear) were added to each reaction to give a final GDP concentration of 2 µM. After 1-2 hours incubation at 4°C, 200 µl of protein A-sepharose coupled to goat anti rat IgG (this is the same precipitating reagent described in the preceding section but in the presence of GDP binding buffer) was added and the tubes were shaken vigorously for 45 minutes at 4°C. The immune complexes were washed twice with 1 ml of GDP binding buffer by centifuging and resuspending. The immune complex was then placed over a 0.4 µm nitrocellulose filter (Schleicher and Schuell) and washed 3 times with 5-10 ml of GDP binding buffer. The filters were placed in 5 ml of aqueous counting solution (Amersham) and radioactivity determined.

RESULTS

The Effect of Retinoic Acid on Anchorage-dependent Growth of Cell Lines Containing an Activated c-ras Gene

The effect of RA on anchorage-dependent growth falls into two categories: (a) effects on growth rate and (b) effects on density-dependent growth (Lotan 1980). A decrease in growth rate and/or saturation density of transformed cells is evidence for RA induced biological activity (Lotan 1980). The effect of 1 μM RA on the anchorage-dependent growth of the cell lines which contain an activated c-ras gene is shown in Table 4. A stimulation of cell growth was observed in the human carcinoma cell lines (EJ, A2182, and LX1), as well as, in the NIH/3T3 transformants (44-9-1-1 and 118-4-3-1) which have acquired an activated c-ras gene via transfection with carcinoma DNA. Retinoic acid had no effect on the parent NIH/3T3 cells.

The Effect of Retinoic Acid on Anchorage-independent Growth of Cell Lines Containing an Activated c-ras Gene

The ability of cells to form colonies in soft agar, anchorage-independent growth, has consistently been shown to be the best *in vitro* correlate of tumorigenicity (Colburn et al. 1978; Freeman and Shin 1974; Kahn and Shin 1979; Shin et al. 1975). The only cell line with an activated c-ras gene which showed a reduction in colony number was the NIH/3T3 transformant, 44-9-1-1, which was transformed by

Table 4

THE EFFECTS OF 1 μM RETINOIC ON CELL LINES WHICH CONTAIN AN ACTIVATED c-ras GENE

Cell line	Change in anchorage dependent growth as % Control[a]	Change in anchorage-independent growth as % Control[b]	Colony forming efficiency of control cells in soft agar[c]	CRABP fmol/mg protein[d]
NIH/3T3	101± 7.9	BDL[e]	BDL[e]	1100
EJ/T24	122± 5.2	111± 9.8	0.3%	BDL[e]
44-9-1-1	136± 6.0	26± 3.4	4.0%	1800
A2182	133±9.0	126±19	0.2%	BDL[e]
118-4-3-1	156± 14	108± 7.1	16%	2800
LX1	137± 5.9	104± 2.2	21%	700

[a]Cells were seeded at 5 x 10^4 to 2 x 10^5 cells/35 mm dish. Daily cell counts were done and % of Control (0.1% ethanol) for cells treated with 1 μM RA was determined at the time the cells were reaching the end of log phase growth, usually day 6. The number represents the average of 3 dishes ± S.E.

[b]The cells were suspended in 0.33% of agar at 10^3-10^5 cells/35 mm dish. Control received 0.1% ethanol while treated cells received 1 μM RA. Colony number was determined after 7-14 days of incubation at 37°C. (see Materials and Methods). The number represents the average of 5 dishes ± S.E.

[c]Colony forming efficiency = number of colonies formed/number of cells seeded x 100.

[d]Cleared cytosolic extracts (1-3 mg protein) were labeled with [^{3}H]RA (50 nM) and separated by centrifugation on a 5-20% linear sucrose gradient. The amount of CRABP binding activity was determined by estimating the amount of specifically bound [^{3}H]RA under the 2S region of the gradient (see Materials and Methods).

[e]Below detectable limits.

c-ras^{H} (Table 4). The number of colonies which formed at 1 μM RA was 25% of control.

Cytoplasmic Retinoic Acid-binding Protein in the Cell Lines Containing an Activated c-ras gene

A small intracellular 14,600 dalton protein which specifically binds RA has been purified and characterized (Ong and Chytil 1978; Saari et al. 1978; Sani and Banerjee 1978). The protein, termed CRABP, has subsequently been shown to be present in a wide variety of normal and malignant tissues both *in vivo* and *in vitro* (Lotan 1980). The binding of RA by CRABP is of high specificity and affinity giving a Kd value in the nanamolar range indicating that CRABP may act as the physiological receptor in a manner analogous to the steroid hormone model (Chytil and Ong 1978; Lotan 1980; Mehta et al. 1980).

To investigate the potential role of CRABP in RA induced changes in cell growth, binding protein assays were performed. The number of fmol of binding activity per mg of protein for all cell lines is shown in Tables 4 and 5. The human carcinoma cell lines, EJ and A2182, did not contain detectable levels of CRABP. The lung carcinoma line, LX1, had 700 fmol of binding activity per mg of protein which corresponds to approximately 37,000 molecules of binding protein per cell. The parent NIH/3T3 mouse embryo fibroblast contained 1100 fmol binding activity per mg of protein, while the NIH/3T3 transformants, 44-9-1-1 (c-ras^{H}) and 118-4-3-1 (c-ras^{K}), showed comparable levels of binding activity at 1800 and 2800 fmol per

and an effect of RA on the transformed phenotype was observed when the c-ras^{H} gene was involved in the transformation. The presence of CRABP was not sufficient to elicit an effect by RA on anchorage-independent growth of c-ras^{K} transformed NIH/3T3 cells (118-4-3-1).

Retinoic Acid Effects on Anchorage-dependent Growth of Retrovirally Transformed Cells

RA induced changes in anchorage-dependent growth of the virally transformed NRK cells is presented in Table 5. In contrast to the cell lines which contain an activated c-ras^{K} gene, the NRK cells transformed by KMSV (v-ras^{K}) are sensitive to growth inhibition by RA. The cell density achieved was 70% and 38% of control at 1 and 10 μM RA, respectively, compared to non-treated cells (Table 5 and Fig. 4). This inhibition of cell growth was not due to cytotoxicity because the ability of KNRK cells to form colonies on plastic was not effected by 10 μM RA (Fig. 5). The NRK cells transformed by SSV were slightly inhibited, whereas the Balb/cMSV transformants and the parent NRK cells were unaffected.

The effect of 1 and 10 μM RA on anchorage-dependent growth of the NIH/3T3 viral transformants is presented in Table 5. Even at the highest RA concentration, 10 μM, only small changes in cell growth were observed for all NIH/3T3 transformants. The parent NIH/3T3 cells displayed no change at 1 μM RA.

TABLE 5

THE EFFECTS OF RETINOIC ACID ON VIRALLY TRANSFORMED CELLS

	Change in anchorage dependent growth as % Control[a]		ED_{50} for RA inhibition of colony formation in soft agar[b]	CRABP[c] (fmoles/mg protein)
	1 μM RA	10 μM RA		
NRK	96 ± 3.3	NT[d]	-[e]	BDL[g]
NIH/3T3	101 ± 7.9	NT[d]	-[e]	1100
KNRK	70 ± 2.6	38 ± 3.3	5 μM	BDL[g]
K3T3	128 ± 5.3	121 ± 4.9	>10 μM	1660
Balb/cMSVNRK	84 ± 6.9	57 ± 1.6	NI[f]	BDL[g]
Balb/cMSV3T3	87 ± 4.8	77 ± 1.7	1 nM	1940
SSVNRK	101 ± 5.1	69 ± 6.4	5 μM	BDL[g]
SSV3T3	91 ± 5.7	93 ± 7.2	20 nM	930

[a]Cells were seeded in triplicate at 5 x 10^4-2 x 10^5/35 mm dish. Daily cell counts were done and % of control (0.1% DMSO) for the treated group (1 μM RA or 10 μM RA) was determined at the time the cells were reaching the end of log phase growth, usually day 6. The numbers represent the average of 3 dishes ± S.E.

[b]Cells were plated in 0.33% agar at 10^3-10^4 cells/35 mm dish in 0.1% DMSO for control or a range of RA concentrations of 0.1 nM to 10 μM. Five dishes were used per concentration. The value reported above is the concentration of RA which gave a 50% inhibition of colony formation, ED_{50}. Colony number was determined after 7-14 days of incubation (see Materials and Methods).

[c]Cleared cytosolic extracts (1-3 mg protein) were labeled with [^{3}H]RA (50 nM) and separated by centrifugation on a 5-20% linear sucrose gradient. The amount of CRABP binding activity was determined by estimating the amount of specifically bound [^{3}H]RA under the 2S region of the gradient (see Materials and Methods).

[d]Not tested.

[e]No colony formation was observed in the presence or absence of RA at a seeding density of 2 x 10^5 cells/35 mm dish.

[f]Not inhibited; a maximal 3.5 fold increase in colony number was observed at 1 μM RA.

[g]Below detectable limits.

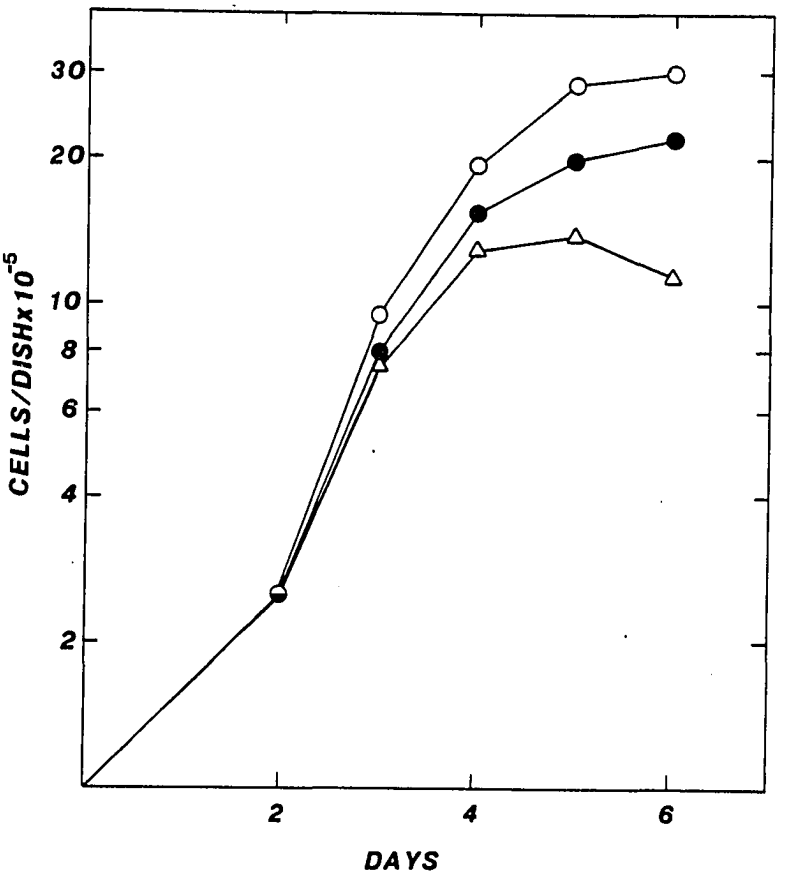


Figure 4. Growth curves of KNRK cells grown in the absence (O) or presence of 1 μM (●) and 10 μM (Δ) retinoic acid. The cells were seeded at 1 x 10^5 cells/35 mm dish in complete medium. Starting at 48 hours cells were counted each day through day 6. The points represent the mean from triplicate dishes.

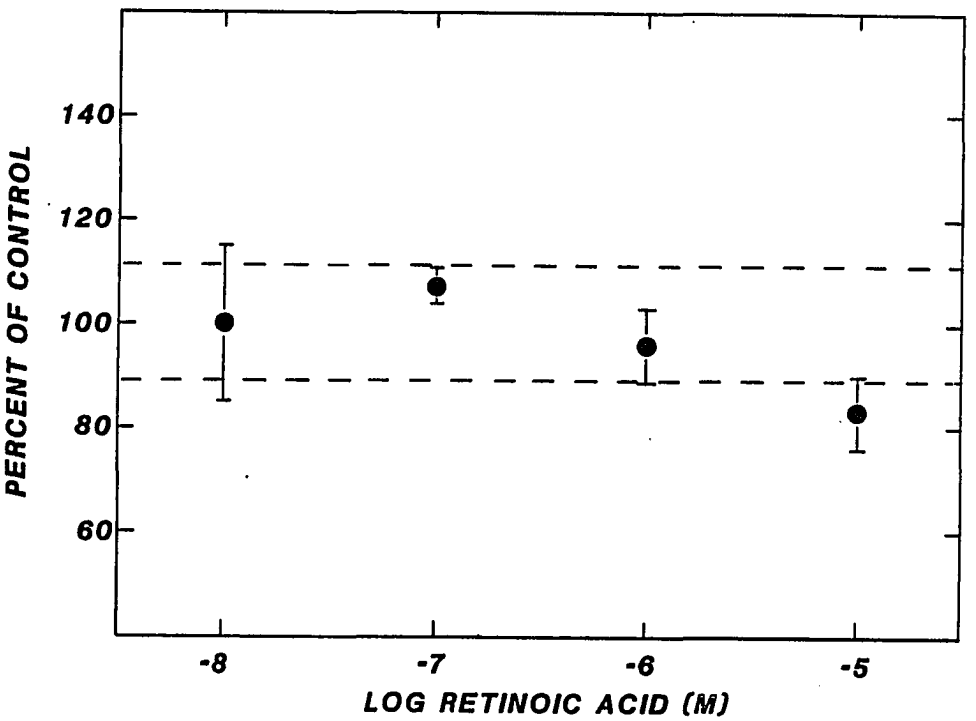


Figure 5. The effects of RA on colony forming effeciency of KNRK cells on plastic Petri dishes. KNRK cells (500) were seeded onto 60 mm dishes in DMEM with 10% FBS. After 7 days of incubation at 37°C the colonies were stained with 0.5% crystal violet in 95% ethanol. Points, 5 dishes were seeded at each RA concentration and expressed as a percentage of the number of colonies observed on the control plates (215 colonies per dish). Bars, S.E. of colony number at each RA concentration. Dotted lines, the area between the lines represents S.E. of the control dishes. Only colonies 50 cells or larger were counted.

The Effect of Retinoic Acid on Anchorage-independent Cell Growth of Retrovirally Transformed Cells

The ability of virally transformed cells to grow in soft agar is highly correlated with their tumorigenicity as stated above. To determine the sensitivity of each of the virally transformed cell lines to inhibition of colony formation in soft agar by RA, cells were grown in the presence of various centrations of RA. The dose-response curves generated were used to estimate the concentration of RA required to give 50% inhibition of colony formation, ED_{50}, which is presented in Table 5.

The NRK transformants demonstrated a relatively low level of sensitivity to inhibition of anchorage-independent growth or were stimulated to give an increase in colony number by RA. Both SSVNRK and KNRK cell lines gave an ED_{50} of 5 μM; whereas, Balb/cMSVNRK showed a 3.5 fold increase in colony number at 1 μM RA.

A much greater degree of sensitivity to RA was observed when the NIH/3T3 transformants were tested. An example of dose response curves for both SSV transformed cell lines is shown in Fig. 6. The SSV3T3 cells were greater than 200 fold more sensitive to inhibition of colony formation by RA than SSVNRK cells. The most striking difference in response to RA was observed with Balb/cMSV transformation. Of the cell lines studied, Balb/cMSV3T3 is the most sensitive to inhibition of anchorage-independent growth with an ED_{50} of 1 nM RA. On the other hand, 1 μM RA gives a maximal stimulation (3.5 fold) of anchorage-independent growth of Balb/cMSVNRK cells. At

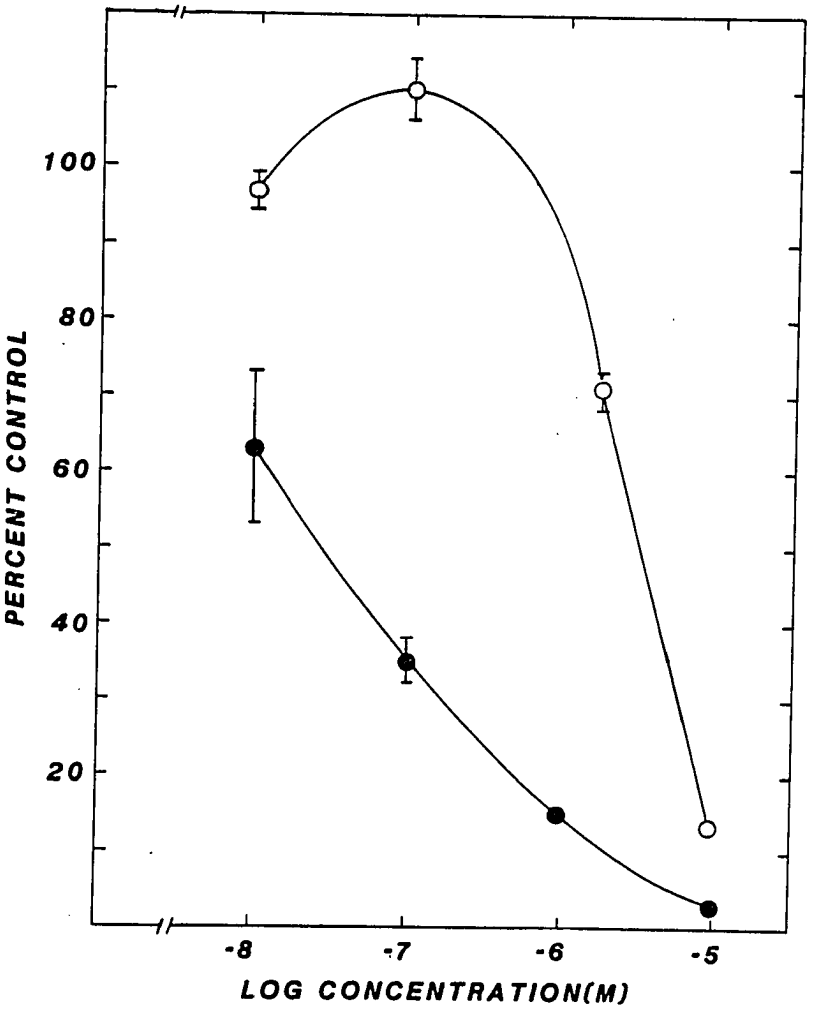


Figure 6. Effect of Retinoic Acid on anchorage-independent growth of SSVNRK (O) and SSV3T3 (●) cells. The cells were suspended in 1 ml of complete medium containing 0.33% agar and 4000 cells/35 mm dish. The concentration of retinoic acid used is indicated on the abscisa. Colonies greater than 60 μM in diameter were counted on day 14 as described in the "Materials and Methods". Points, 5 dishes were seeded at each retinoic acid concentration and expressed as a percentage of the number of colonies observed on control plates (200-300 colonies per dish). Bars, S.E. of colony number at each retinoic acid concentration. Where S.E. bars are absent S.E. fell within the point.

1 μM RA, anchorage-independent growth of Balb/cMSV3T3 is completly inhibited. Neither of the parent cell lines, NRK or NIH/3T3, formed colonies in the presence or absence of RA when seeded at a level 10 fold higher than any of the transformants.

Levels of Cytoplasmic Retinoic Acid-binding Protein in the Retrovirally Transformed Cells

It has been suggested that the biological effects of RA may, at least in part, be mediated via an interaction with CRABP as previously noted. The parent NRK cells and the NRK viral transformants did not have detectable levels of CRABP. In contrast, NIH/3T3 cells had 1100 fmol of binding activity per mg of cellular protein while the transformants have levels that ranged from 930 to 1940 fmol per mg protein (Table 1). This corresponds to 50,000-100,000 CRABP molecules per cell. Figure 7 shows the sucrose gradient analysis of both parent cell lines, NRK and NIH/3T3, and their KMSV transformants.

Inhibition of Intracutanous Sarcoma Formation in Nude Mice by Retinoic Acid

Our initial investigation of a potential role for RA in the inhibition of oncogene induced cellular transformation employed several *in vitro* assays, namely anchorage-dependent and -independent growth. The acquisition of anchorage-independence is generally felt to be the best *in vitro* indicator of oncogenic transformation as stated before. We found that human carcinoma cell lines containing an activated c-ras gene were not responsive to inhibition of anchorage

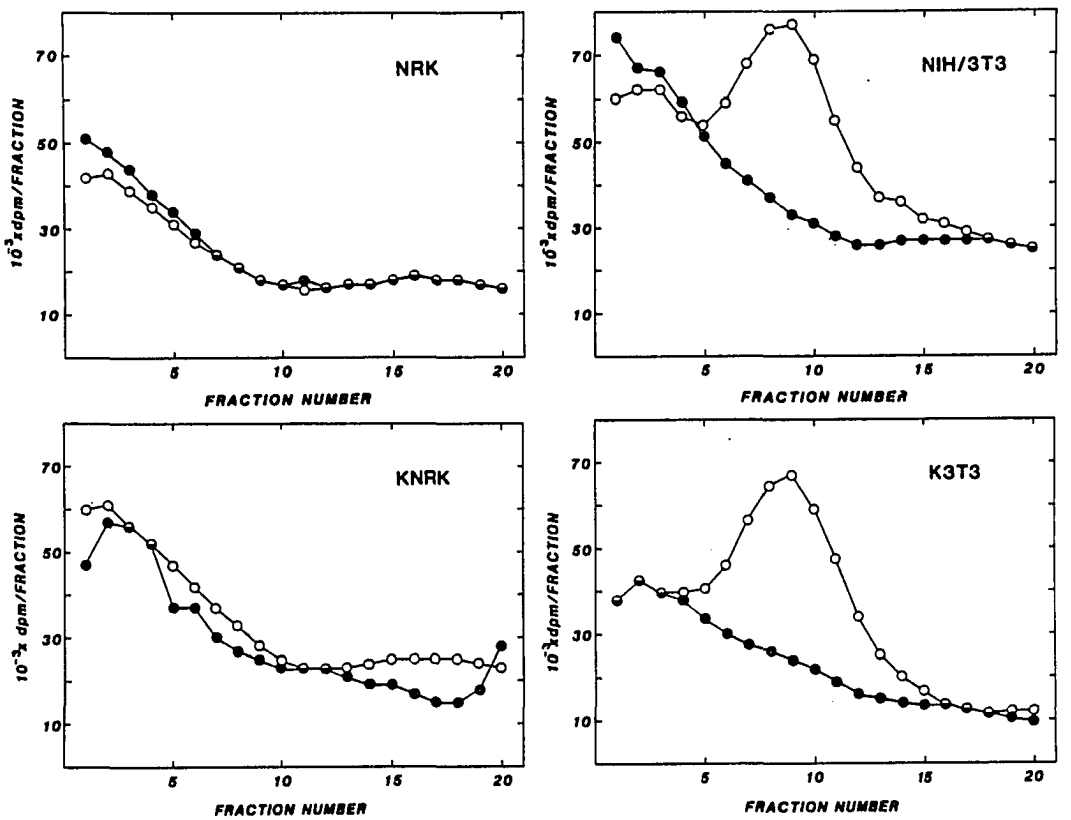


Figure 7. Sucrose gradient analysis of CRABP. Cell extracts (2 mg of protein in 0.4 ml of 10 mM Tris-HCl, pH 7.4, 1 mM EDTA) were incubated for 4 hours with 50 nM [^{3}H]retinoic acid in the dark in the absence (O) or presence (●) of a 100 fold excess of cold retinoic acid. At the end of the incubation period, the extract was layered onto a 5 to 20% sucrose gradient and centrifuged at 230,000 x g for 20-22 hours. Then 0.2 ml fractions were collected and radioactivity was counted. The gradients were fractionated form top to bottom. The lactoglobulin 2.0 S standard peak was at fraction 9.

independent growth by RA. Conversely, when cells transformed by KMSV, Balb/cMSV, or SSV were tested for their ability to form colonies in soft agar, in the presence of RA, we observed a dramatic inhibition of anchorage-independent growth at concentrations as low as 1 nM. Therefore, RA was able to block the expression of the transformed phenotype of retrovirally transformed cells in vitro. To conclusively demonstrate an antineoplastic effect of RA on retrovirally induced tumors, tumorigenesis in vivo in the athymic nude mouse was examimed. NIH/3T3 cells transformed by Balb/cMSV (Balb/cMSV3T3) demonstrated the greatest sensitivity to RA in vitro. Anchorage-independent growth was inhibited by 50% at 1 nM RA. Tumors were initiated in animals by injecting 50,000 cells into a suction blister which had been formed on the backs of the mice (see Materials and Methods). Nine animals were used for each control and RA treated groups. Each animal received an injection of tumor cells at two sites, therefore, a 100% incidence of tumor formation in either group should result in a total of 18 tumors. Twenty-four hours after the injection of Balb/cMSV3T3 cells treatment was initiated by applying topically 10 µl of 0.2% RA in DMSO to each tumor site. The control group received 10 µl of vehicle alone to each site. Treatment was continued on a once a day schedule and animals were sacrificed 28 days after the initiation of the study. The antitumorigenic activity of RA was apparent upon visual inspection of the animals. Figure 8 is a picture taken of 3 representative animals from each group. Even when a tumor had formed at some RA treated sites, they were too small to be observed upon visual inspection.

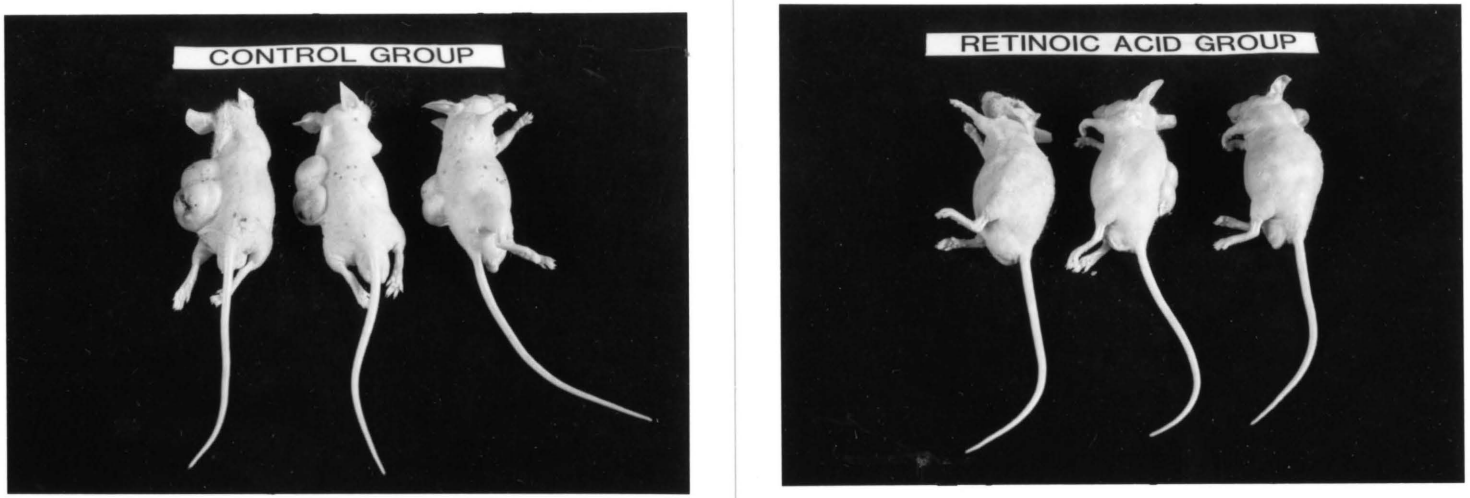


Figure 8. Effect of retinoic acid on tumor formation in nude mice injected with Balb/cMSV3T3 cells. Tumor cells (50,000) were injected intracutanously at two sites per mouse. The treated group recieved 10 µl of 0.2% retinoic acid in DMSO applied topically to each site daily. The controls recieved vehicle alone. At 28 days after treatment began the animals were sacrificed and pictures were taken of three representive animals from each group of 9.

Each tumor site was excised, and the tumors were dissected free of surrounding normal tissue. We observed a 100% induction of tumor formation in the control group while the RA treated group formed tumors at 14 of a possible 18 sites. This was confirmed upon histological examination. The 20% reduction in the incidence of tumor formation was shown by Chi-Square analysis to be significant at $p=0.034$. The wet weight of each tumor was determined so that the effect of RA upon tumor size could be evaluated. The mean tumor weight of the control group was 1.29 gm while that of the RA treated group was 0.67 gm. This represents a significant 53% reduction in mean tumor size ($p < .005$, Students t-test, see Table 6). If the median tumor weights are compard an 83% reduction in weight due to RA treatment is observed (Table 6).

All tumors were fixed in formalin in order that hematoxylin and eosin stained slices could be prepared to determine if gross histological differences existed between control tumors and tumors that formed under the influence of RA. Histological evaluation revealed that all tumors that formed were well circumscribed and displayed a disordered array of cell growth characteristic of neoplastic tissue. There was no evidence of invasion or metastasis. Tumors that formed in the RA treated group did not appear to be a significant effect upon both tumor incidence and weight confirming the anti-neoplastic activity of RA first observed *in vitro*.

TABLE 6

Effects of Retinoic Acid on Tumorigenicity of BALB/cMSV3T3 Cells in Nude Mice[a]

	Control	Retinoic Acid	% of Control
Mean±SE	1.29±0.26[b]	0.61±0.24[b]	47
		($p<0.005$)[c]	
Median	0.80[b]	0.13[b]	16
(range)	(0.2-3.6)	(0-4.2)	
#Tumors/#sites	18/18	14/18	80
		($p=0.034$)[d]	

[a]50,000 tumor cells were injected intradermally at two sites on each animal. Each site was treated topically with 10 l of 0.2% retinoic acid in DMSO daily. Controls received DMSO alone (see Materials and Methods). Tumors were excised and weighed on day 28.

[b]Values represent the wet weight of the tumors in grams.

[c]Student's t-test was used to analyze the means.

[d]Chi-Square analysis was used to determine the significance of the reduction in tumor incidence.

The Effect of Retinoic Acid on v-onc Specific mRNA Levels

Initiation and maintenance of transformation by acute transforming retroviruses is dependent upon expression of their respective v-onc genes (Bishop and Varmus 1982). Inhibition of viral transformation by RA could be the result of decreased v-onc mRNA concentration. To test this possibility, we used dot blot hybridization analysis to estimate changes in mRNA levels. All cell lines were treated for 72 hours with 10 μM RA for the KMSV transformants or 1 μM RA for the SSV and Balb/cMSV transformants. This treatment period was chosen because past reports have shown that morphological and biochemical changes induced by RA are usually maximal by 48-96 hours (Lotan 1980). The RA concentration chosen was that concentration which gave a maximal response in the anchorage-independent growth assay. The only cell line in which a change in v-onc mRNA concentration was observed was Balb/cMSVNRK (Fig. 9). This cell line is stimulated 3.5 fold by RA in the anchorage-independent growth assay and the v-bas mRNA level was increased 2-4 fold over control cells. Surprisingly, the level of v-onc mRNA in the other cell lines is unchanged when treated with RA, even those highly sensitive to inhibition of anchorage-independent growth.

The Effect of Retinoic Acid on the Relative Rate of Synthesis of the Transforming Protein, p21, in Cells Transformed by KMSV and Balb/cMSV

The studies presented on the expression of the v-onc genes revealed that the retrovirally transformed cells which displayed

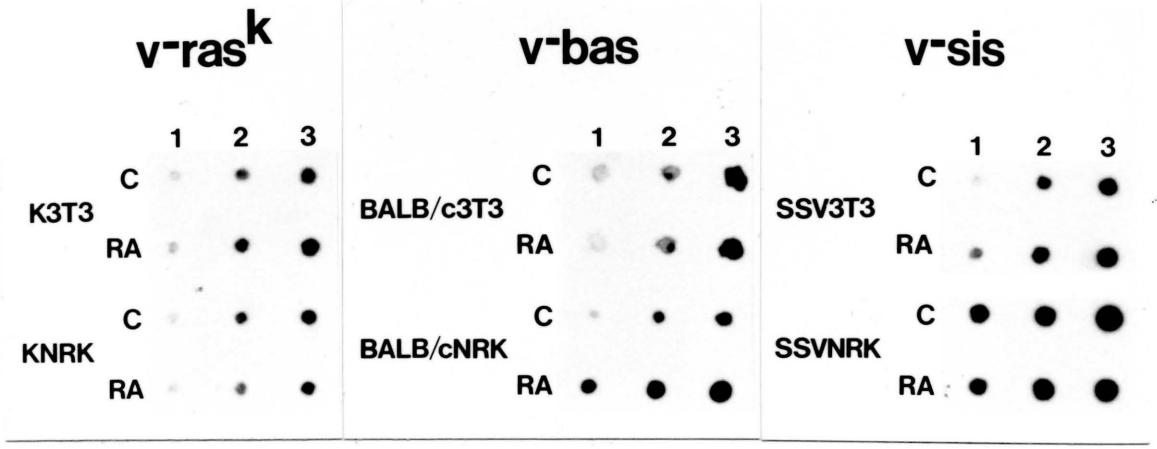


Figure 9. Effects of retinoic acid on transcriptional expression of v-onc genes in virally transformed cells. The cells were grown for 72 hours in the presence of 1 μM (Balb/cMSV and SSV transformants) or 10 μM (KMSV transformants) retinoic acid. The controls received 0.1% vehicle only. Poly A^+ mRNA was isolated as described in the Materials and Methods. RNA from cells transformed by KMSV and SSV was spotted onto nitrocellulose filters: Lane 1, 0.16 μg; Lane 2, 0.31 μg; Lane 3, 0.63 μg. RNA from Balb/cMSV transformed cells was spotted: Lane 1, 0.31 μg; Lane 2, 0.63 μg; Lane 3, 1.25 μg. The ^{32}P nick translated v-ras^K, v-bas, and v-sis probes (0.5-2.0 x 10^8 dpm/ μg) described in the Materials and Methods section were hybridized with the immobilized RNA. After washing the filters, they were exposed to Kodak X-Omat RP X-ray film an -70°C for 6 to 16 hours with an intensifying screen. No signal was observed when RNAs from each cell line were hybridized to v-onc probes representative of the viruses not involved in their transformation (data not shown).

sensitivity to growth inhibitory effects of RA did not have a reduced level of v-onc specific mRNA (Fig. 9). If the relative concentration of a viral transforming protein was being decreased by RA this effect could be changed by RA treatment. If the relative concentration of a viral transforming protein was being decreased by RA this effect could potentially explain the growth inhibitory and antitumorigenic effects of RA that we have observed on retrovirally transformed cells. This has been shown to be true for a temperature sensitive mutant of KMSV (Shih et al. 1979). At the nonpermissive temperature p21 levels drop dramatically and transformation is reversed. Therefore, we measured the relative rate of p21 synthesis in KMSV and Balb/cMSV transformed cells. Both NRK and NIH/3T3 cells transformed by these viruses were grown for 72 hours in the absence or presence of 10 μM (KMSV transformants) or 1 μM (Balb/cMSV transformants) RA. The cells were labeled in the presence of [^{35}S]methionine for 3 hours and cell lysates were prepared as described in Materials and Methods. To examine the relative rate of p21 synthesis an amount of lysate (135-200 μg protein) which gave an equivalent amount of incorporated [^{35}S] methionine was used for each control and treated groups. The amount of lysate used contained subsaturating amounts of p21 in order that the immunoprecipitations were always done at antibody excess. This was determined by titrating each lysate (data not shown). After immunoprecipitating p21 from each lysate the precipitated protein was subjected to SDS-PAGE analysis. Fig. 10 is the resulting

autoradiogram. Lane 9 represents SSV3T3 lysate and the very faint p21 band observed is probably the result of the expression of endogenous c-ras genes as previously described for untransformed NIH/3T3 cells (Furth et al. 1982). This indicates that background interference from the expression of c-ras genes is very low. Lanes 10 and 11 represent Balb/cNRK control and K3T3 RA treated lysates, respectively. Anti p21 antibody was left out of the immunoprecipitation procedure and no p21 band was observed in these lanes. Specificity of the immunoprecipitation was further demonstrated by replacing anti p21 antibody in the reaction with anti vitamin D_3 receptor antibody (a gift from Dr. J. Wesley Pike, University of Arizona). KNRK control lysates treated in this manner gave no p21 band (data not shown). The p21 immunoprecipitated from KNRK cells (Fig. 10, Lane 1 and 2) was different from that observed in K3T3 cells (Fig. 10, Lanes 3 and 4). K3T3 cells give a p21 doublet with a size difference of about 1,500 daltons. This is consistant with the observations of Furth et al. (1982) who demonstrated a slower migrating p21 band in K3T3 cells due to p21 phosphorylation. They also observed only a single, faster migrating p21 band in KNRK cells. This is consistant with our observations. These results indicate that posttranslational modification of KMSV coded p21 can vary depending upon the cell type this virus infects. In the Balb/cMSV transformed cells, p21 migrated as a fast moving singlet independent of cell type. Shorter exposure of Lanes 7 and 8 (Balb/cMSVNRK cells) did not reveal a potential doublet. Balb/cMSV coded p21 is apparently not phosphorylated

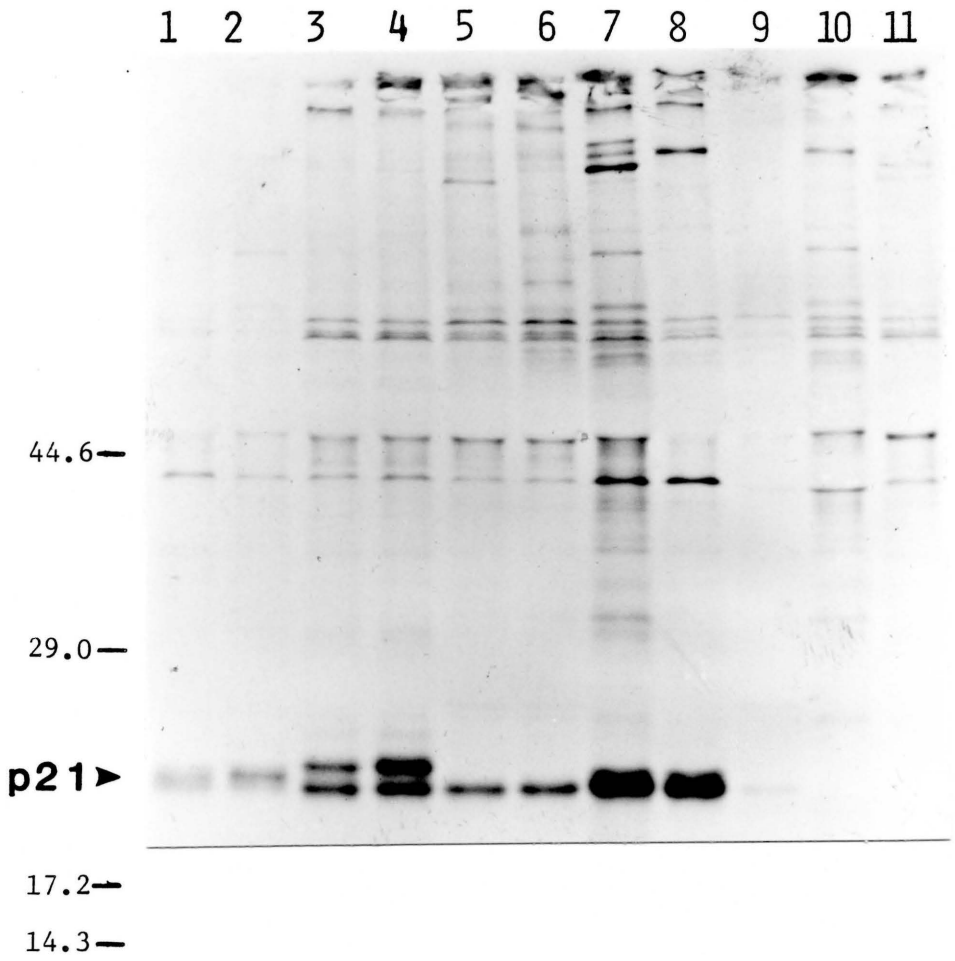


Figure 10. Effect of retinoic acid on p21 sythesis in KMSV and Balb/cMSV transformed cells. Cells were treated for 72 hours with RA prior to pulse labeling with [^{35}S] methionine. Lysates were prepared and p21 was immunoprecipated with Y13-259 monoclonal antibody and subjected to SDS-PAGE analysis as described in Materials and Methods. Lanes 1 and 2, KNRK; lanes 3 and 4, K3T3; lanes 5 and 6, Balb/cMSV3T3; lanes 7 and 8, Balb/cMSVNRK. The odd numbered lanes are control and the even numbered lanes are RA treated. Lane 9, SSV3T3; lane 10, same as lane 7 without antibody; lane 11, same as lane 3 without antibody. Arrowhead indicates the major p21 species. Bars indicate the relative mobilities of marker proteins of known molecular weight (X 10^{-3}).

(personal communication with Dr. S. Srivastava, NCI, Bethesda, MD). The effect of RA on the relative rate of p21 synthesis in each of the cell lines is shown in Lanes 1-8, Fig. 10. Very little if any change in the rate of p21 synthesis was observed. This was concluded due to the lack of an effect of RA on the intensity of the p21 band(s). These results indicate that the inhibition of transformation and tumorigenesis by RA of retrovirally transformed cells is not due to a decrease in the relative rate of p21 synthesis.

GDP Binding in KNRK Cells After Treatment with Retinoic Acid

The final point at which RA could modify the expression of the v-ras gene directly would be a change in the functional activity of p21, the v-ras gene product. Although little is known about the role of p21 in cellular transformation several properties have been attributed to this transforming protein. Scolnick et al. (1979) showed that p21 of both Kirsten and Harvey MSVs was able to bind reversibly the guanine nucleotides GDP, GTP and dGTP. Studies with a partially purified p21 preparation from Harvey MSV transformed NIH/3T3 cells demonstrated a Kd of binding for GDP of 7 nM (Shih et al. 1980). Also, when incubated in the presence of [^{32}p] GTP autophorylation of p21 was observed. At present the significance of the guanine nucleotide binding or kinase activity is not fully understood but may be associated with the transforming activity of p21.

The previous studies had shown that RA inhibits both anchorage-dependent and -independent growth of KNRK cells without effecting

v-ras mRNA levels or the rate of synthesis of p21. This study was designed to determine if RA could influence p21 GDP binding. KNRK cells were grown in presence or absence of 10 μM RA for 72 hours prior to the preparation of cell extracts. Aliquots of cell extract containing 0.03 to 1.05 mg of protein was incubated with 2 μM [^{3}H]GDP and the amount of specifically bound nucleotide was determined by immunoprecipitation of p21 (see Materials and Methods). RA treatment had no effect on the level of GDP binding activity (Fig. 11). Also, no change in binding activity was observed when control cell extract was incubated in the presence of exogenously added RA at 10 μM (data not shown). Samples from which the antibody was left out gave very low levels of nonspecific binding. SSV transformed NRK cells gave a level of GDP binding equivalent to background demonstrating that a retrovirally transformed NRK cell not producing viral p21 does not express detectable levels of endogenous p21 GDP binding activity. These results also indicate that the inhibition of the transformed phenotype observed after treatment of KNRK cells does not involve a direct effect on the functional activity of p21.

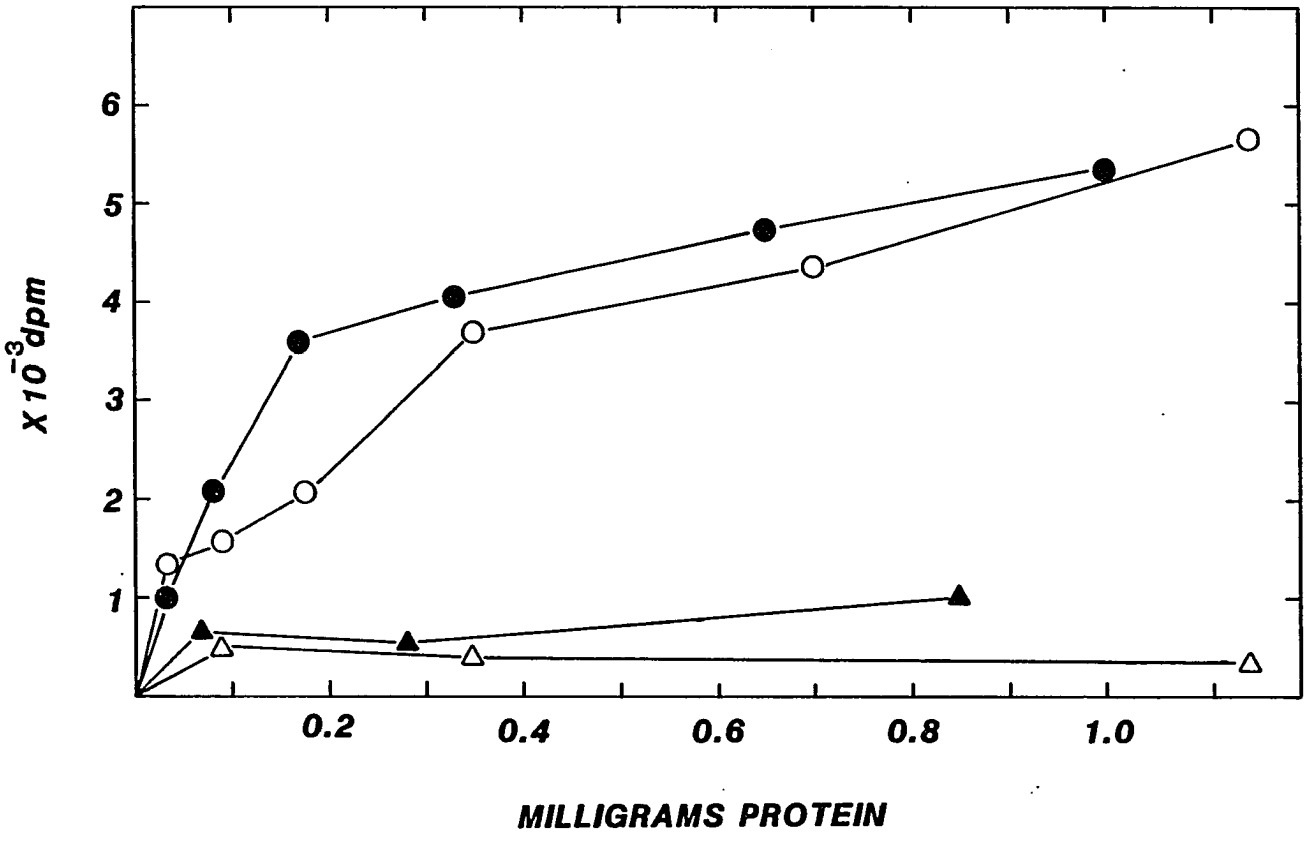


Figure 11. Retinoic acid effects on GDP binding in KNRK cells. The GDP binding assay was carried out as described in the Materials and Methods. Symbols: ○, control KNRK lysate incubated with Y13-259 antibody; ●, retinoic acid treated (10μM) KNRK lysate incubated with Y13-259 antibody; △, control KNRK lysate minus antibody; ▲, SSVNRK lysate incubated wity Y13-259 antibody.

DISCUSSION

The pioneering studies of Mori (1922) and Wolbach and Howe (1925) clearly defined a role for Vitamin A in cellular proliferation and differentiation. It was also noted that gross tumors were evident in a variety of epithelial tissues of vitamin A deficient animals. Because of these early observations and the current view that cancer is a fundamental defect in cellular differentiation which leads to abnormal cell growth (Mintz and Fleischman 1981; Pierce et al. 1978) it is only natural that investigations would be conducted on the potential role of vitamin A in carcinogenesis. The potential antineoplastic effects of vitamin A have been addressed in a wide variety of *in vivo* and *in vitro* systems. These studies have clearly demonstrated that retinoids are capable of suppressing cellular transformation that has been induced by chemical carcinogens, gamma and ultraviolet radiation, growth factors or viruses (Lotan 1980). In all of these cases the specific changes in the expression of genetic information leading to the transformed state remain largely unknown. Therefore, the aim of the research presented in this dissertation was to investigate the potential role of RA in inhibiting cellular transformation in systems in which specific genetic lesions had been previously defined, namely, transformed cells which express an

activated cellular or viral oncogene. If RA proved to be an effective inhibitor of transformation and tumorigenesis in these cells, it would be possible to investigate potential effects on the specific molecular events known to be necessary for the maintenance of transformation.

The first studies initiated were designed to detect any inhibitory effects RA may display when applied to human carcinoma cells which had been shown to contain an activated c-onc gene. The best evidence in the literature that retinoids may have a role in inhibiting in vitro cellular transformation that involves the activation of a c-onc gene was presented by Merriman and Bertram (1979). In this study it was shown that 3-methylcholanthrene-induced neoplastic transformation of 10T1/2 mouse fibroblasts could be completely blocked by treating the cells with 0.3 μM retinyl acetate starting 7 days after carcinogen exposure. The delay between carcinogen exposure and retinoid treatment indicates that the mechanism of inhibition does not involve a block in initiation. This point is further supported by the observation that the inhibition of transformation is fully reversible upon withdrawal of the retinoid. When purified DNA is prepared from cells that have been transformed in this system and used to transfect NIH/3T3 cells, activated genes capable of inducing cellular transformation are detected which are not present in the DNA of the parent cells (Shilo and Weinberg 1981). The identity of the activated gene is unknown but restriction endonuclease enzyme inactivation indicates that the same gene is activated in several clones that were tested. There are three possible

explanations for these results. First, retinyl acetate may be inhibiting the expression of the c-onc gene whose activation restulted from a direct interaction between 3-methylcholanthrene and the gene. Secondly, the activation of the gene may occur later in the transformation process due to an indirect effect of the chemical carcinogen. In this case, retinyl acetate may inhibit the activation and/or expression of the gene due to activation by secondary events. Evidence for the indirect activation of the c-ras by chemical carcinogens has been recently presented (Sukumar et al. 1984). In relation to these studies, it has been shown that RA can inhibit the formation of fibrosarcoma in mice induced with 3-methylcholanthrene (Chauvenet and Pague 1982). In a separate study 3-methylcholanthrene induced fibrosarcomas from mice were shown to contain an activated c-ras^{K} gene (Eva and Aaronson 1983).

The effects of retinoids on mammary tumorigenesis has been the subject of much investigation (reviewed by Moon et al. 1983). One of the most commonly employed model systems of mammary tumorigenesis in which retinoids have proven antineoplastic effects was developed by Gullino et al. (1975). Nitroso-methylurea is administered to 50 day old rats with 90% of the animals forming mammary tumors within 60 days. Using this experimental model Sukumar et al. (1983) demonstrated that 9 out of 9 tumors tested contained an activated ras^{H} gene. The activated c-ras^{H} gene from one tumor was cloned and the activation was shown to be the result of a point mutation in the 12th amino acid codon.

One of the most widely studied systems in cancer biology is the two-stage model of mouse skin carcinogenesis (Boutwell 1983). Tumor formation is first initiated with a single application of a chemical carcinogen and then promoted by repeated applications of a tumor promoter, usually a phorbol ester. Benign papillomas first appear some of which progress to malignant carcinomas. Retinoids have been very effective in blocking the promotion stage in this model system (Verma and Boutwell 1977) and regression of existing tumors has also been reported (Bollag 1972). These is now ample evidence that c-ras^{H} gene activation is involved in mouse skin carcinogenesis. It was first shown that a transplantable squamous cell carcinoma induced in the two-stage model system contained an activated c-ras^{H} gene (Balmain and Pragnell 1983). It has also been shown that the first benign lesions to appear, primary papillomas, also have the same activated gene indicating that c-onc gene activation may represent an early event in the carcinogenic process (Balmain et al. 1984). All of these studies taken together clearly indicate that retinoids have a role both <u>in vitro</u> and <u>in vivo</u> in suppressing cellular transformation involving the activation of c-onc genes. The examples cited above specifically implicate a role for retinoids in the inhibition of carcinogenesis in systems involving the activation of c-ras^{H} and c-ras^{K} genes. Therefore, cell lines containing these activated genes were employed in these studies.

A bladder carcinoma (EJ/T24) and two lung carcinoma (LX1 and A21812) cell lines which have been shown to contain an activated cellular oncogene belonging to the ras gene family were chosen for these studies (Der et al. 1982; Parada et al. 1982a; Pulciani et al. 1982; Reddy et al. 1982; Tabin et al. 1982; Taparowsky et al. 1982). The EJ/T24 cell line has an activated c-ras^{H} gene so designated due to its homogy to v-ras^{H} of Harvey MSV (Parada et al. 1982; Pulciani et al. 1982b; Reddy et al. 1982; Taben et al. 1982; Taparowsky, et al. 1982). Molecular cloning and DNA sequencing have shown that a point mutation in the coding region of the first exon is responsible for its activation (Reddy et al. 1982; Tabin et al. 1982; Taparowsky et al. 1982). It should be pointed out that this mutation in the human c-ras^{H} gene is in the same codon as that previously described for the rat mammary tumor c-ras^{H} gene.

Both LX1 (Der et al. 1982) and A2182 (Pulciani et al. 1982a) contain an activated c-ras^{K} gene which is homologous to v-ras^{K} of KMSV. The specific genetic alteration in the c-ras^{K} gene of these cell lines has not been defined, but a recent study with another lung carcinoma cell line in which c-ras^{K} is activated has shown that the activation is due to a mutation in the coding sequence, as was the case for c-ras^{H} of EJ/T24 (Shimizu et al. 1983). Schwab et al. (1983) have recently shown the c-ras^{K} is greatly amplified in a mouse adrenocortical carcinoma. Amplification of the gene leads to an increased level of expression which the authors suggest may have been involved in the genesis of this tumor. This last example indicates

that gene dosage or transcriptional activation of a cellular oncogene may lead to its transforming potential.

When tested for effects on anchorage-dependent growth, RA is capable of reducing the growth rate and/or saturation density of many cultured transformed cells (Lotan 1980). At 1 μM RA cell density increased slightly (20-40%) when applied to EJ/T24, A2182 and LX1 cells. When the same concentration of RA was tested on the NIH/3T3 transformants, 44-9-1-1 (transformed with c-ras^{H} from EJ/T24) or 118-4-3-1 (transformed with c-ras ^{K} from A2182), an increase in cell density was also observed, while the parent NIH/3T3 cells were uneffected. In the cell lines, which contain an activated c-ras gene RA was not able to restore normal density dependent growth control.

A second <u>in vitro</u> parameter was investigated, anchorage-independent growth. The ability of cells to grow in soft agar is highly correlated with tumorigenicity as stated previously. The three human carcinoma cell lines showed no change in colony forming efficiencey at 1 μ M RA. The NIH/3T3 cells transformed with the c-ras^{K} gene were also insensitive to RA in the soft agar assay. However, 44-9-1-1, which was transformed with the c-ras^{H} gene of EJ/T24, displayed a 74% reduction in colony number in the presence of 1 μM RA. Many cells contain a cytoplasmic binding protein for RA, CRABP (Chytil and Ong 1979; Lotan 1980). Although a role for CRABP has never been defined, it has been proposed that CRABP may be the physiological receptor which may mediate at least some of the biological effects of

RA (Chytil and Ong 1978; Lotan 1980). We measured CRABP levels in the cell lines containing an activated c-ras oncogene to determine if any correlation exists between the presence of CRABP and the ability of RA to alter properties of the transformed phenotype. Of the two lung carcinoma cell lines containing an activated c-ras^{K} gene, LX1 had significant levels of CRABP while A2182 was negative for this protein. None of these cell lines displayed a change in anchorage-dependent or -independent growth. This indicated that the lack of sensitivity to RA was not due to the absence of CRABP. This view is strengthened by the observation that when the c-ras^{K} of A2182 is transfected into NIH/3T3 cells (118-4-3-1) inducing transformation, this CRABP positive cell line remains insensitive to RA. The analogous example with c-ras^{H} did not give the same results. The bladder carcinoma cell line, EJ/T24, which contains c-ras^{H}, is CRABP negative and unresponsive to RA. However, when this gene is transferred into a CRABP positive cell, NIH/3T3, the subsequent transformant (44-9-1-1) is sensitive to RA, showing a 75% reduction in anchorage-independent colony forming ability at 1 μM. Therefore, it is possible that CRABP could be involved in RA effects on transformation involving the c-ras^{H} gene but apparently does not mediate RA effects on cells transformed by c-ras^{K}.

These studies failed to show any inhibitory effects of RA on the anchorage- or density-dependent growth of human carcinoma cell lines which contain an activated c-ras gene. This observation was the same in the presence or absence of CRABP. In the previous studies cited in which retinoids were effective in inhibiting cellular

transformation or tumor formation, the retinoid was administered prior to the phenotypic expression of neoplastic transformation. In the 10T1/2 mouse fibroblast system, Merriman and Bertram (1979) reported that they did not observe a change in cell morphology if retinyl acetate was applied after transformation had occurred. In the in vivo systems cited the carcinogen induced mouse fibrosarcoma and rat mammary tumor induction by nitro-methylurea, stimulation of the immune system may have played a role in retinoid inhibition of tumorigenesis. Evidence supporting immune stimulation was presented in the fibrosarcoma study (Chauvenet and Paque 1982). In the two stage model of mouse skin carcinogenesis retinoids are the most effective at blocking promotion indicating that the effects are most pronounced on the early steps in the progression to a fully transformed malignant carcinoma.

The studies reported here employed well established, fully transformed carcinoma cell lines and were conducted in the absence of any immunological influences. This may explain why RA failed to exhibit any antineoplastic activity in these studies of transformation involving activated c-onc genes.

The second portion of the work presented in this dissertation has dealt with the role of RA in inhibiting retrovirally induced cellular transformation and tumorigenesis. Previous studies have addressed this question in a number of systems. In mice, prior administration of vitamin A has been shown to block tumor formation

due to inoculation with Moloney MSV (Seifter et al. 1973; Seifter et al. 1976). Similar results have been reported with Rous sarcoma virus in both chickens (Frankel et al. 1979) and hamsters (Frankel et al. 1979a; Frankel et al. 1979b). In these *in vivo* studies a reduction in tumor number could be due to a change in the infectivity or physiological distribution of the virus. It has also been suggested that immunological stimulation by the retinoid could account for the antineoplastic response seen (Seifter et al. 1973; Seifter et al. 1976).

A number of studies have been conducted on retrovirally transformed cells *in vitro* where the physiological and immunological responses mentioned above would not play a role. Mukherjee et al. (1983) have looked at the ability of RA to alter cellular transformation of KMSV and Rous sarcoma virus transformed cells. RA was able to block the expression of the transformed phenotype and somewhat restore density dependent growth. The retrovirally transformed cell lines they used were shown to contain CRABP (Mukherjee et al. 1983). On the other hand, Todaro et al. (1978) were unable to show a decrease in soft agar colony formation by retinal acetate or RA on Moloney MSV transformed mouse and rat cells.

In the present study, the effects of RA on retrovirally transformed NRK and NIH/3T3 cells has been investigated. Transformation by the same virus of each cell line enabled us to examine RA effects in both CRABP positive and negative cells. Three

RNA tumor viruses were employed; the murine sarcoma viruses, KMSV and Balb/cMSV, as well as the simian virus, SSV.

KMSV is a replication defective retrovirus which was isolated after two passages of the erythroblastic Kirsten strain of murine leukemia virus in Wister/Furth rats (Kirsten and Meyer 1967). In rats, this virus is capable of causing sarcomas, osteolytic lesions and petechial hemorrhages. The transforming potential of KMSV arose through recombination with rat genomic sequences in which the rat c-ras^{K} gene was acquired (Scolnick et al. 1973). The v-ras^{K} gene codes for a 21,000 dalton guanine nucleotide binding protein known as p21 whose continued expression is required for the maintenance of transformation (Bishop and Varmus 1982).

Balb/cMSV was originally isolated from a hemangiosarcoma in a Balb/c mouse (Peters et al. 1974). This virus is derived from Balb:virus-1, a known endogenous virus of Balb/c mice (Aaronson and Barbacid 1978). The transforming gene present in this virus, v-bas, has been shown to be closely related to v-ras^{H} of Harvey MSV which was derived from the mouse c-ras^{H} gene (Anderson et al. 1981). The v-bas gene is also related to the v-ras^{K} gene of KMSV showing about 75% homology in nucleotide sequence. The protein coded for by v-bas is also known as p21, but it apparently lacks quanine nucleotide binding activity (personal communication with Dr. S.A. Aaronson, NCI, Bethesda, MD).

The third virus employed in these studies, SSV, was first isolated from a spontaneous fibrosarcoma of a pet woolly monkey (Theilen et al. 1971). SSV causes well differentiated fibrosarcomas and fibromas in a variety of primates (Wolf et al. 1971). The transforming gene of SSV, v-sis, codes for a 28,000 dalton protein known as p28 (Robbins et al. 1982). Recent studies have made the very interesting observation that there is close structural and immunological similarity between p28 and human platelet-derived growth factor (Doolittle et al. 1983; Robbins et al. 1983). The implication of these findings in relation to retinoids will be discussed later in this section.

The first parameter we examined was anchorage-dependent growth. RA has been shown to inhibit the rate of proliferation and saturation density of many transformed cells (Lotan, 1980). These effects represent a marker for biological activity of RA. We found that the only cell line which showed a significant level of anchorage-dependent growth inhibition was KNRK. This cell line displayed a 60% reduction in saturation density at 10 μM RA.

A very reliable index of virally induced cellular transformation is anchorage-independent growth which is highly correlated with the ability of virally transformed cells to form tumors in a susceptible host (Shih et al. 1975; Weiss 1982). We tested the sensitivity of each of the virally transformed cell lines to inhibition of colony formation in soft agar. Two of the NRK transformants (KNRK and SSVNRK), which are CRABP negative,

demonstrated a low sensitivity to RA with an ED_{50} of 5 μM. The other NRK transformant, Balb/cMSVNRK, showed a 3.5 fold stimulation in colony formation. This indicates that all retrovirally transformed cells do not display an inhibition of anchorage-independent growth in response to RA. The stimulation of anchorage-independent growth by RA observed with Balb/cMSVNRK cells may indicate enhanced tumor forming potential. Several reports of enhanced tumor formation by RA have appeared in the literature (Levy and Polliack 1968; Welsch et al. 1981; Wilson and Reich 1978). The NIH/3T3 transformants (Balb/cMSV3T3 and SSV3T3) which were responsive to inhibition of anchorage-independent growth required a concentration of RA that was 200-5000 times lower than that required for the responsive NRK lines. These NIH/3T3 transformants are CRABP positive, and the concentration of RA required to suppress anchorage-independent growth (1-20 nM) correlates with the K_D (4 nM) for CRABP (Ong and Chytil 1978). This indicates that the binding protein may have a potential role in mediating RA activity. Also, this degree of sensitivity was not observed in any of the CRABP negative lines. The role of CRABP is discussed in more detail later.

The <u>in vitro</u> studies with the retrovirally transformed cells clearly demonstrated that RA was active in blocking the expression of the transformed phenotype. Balb/cMSV3T3 and SSV3T3 cells were responsive to nmolar concentrations of RA which is in the physiological range (DeRuyter 1979) and 100-1000 times lower than the concentrations achievable pharmacologically (Frolic et al. 1975).

To confirm the *in vitro* assays the ability of RA to block tumor formation in athymic nude mice which had been injected with Balb/cMSV3T3 cells was examined. This was the most sensitive cell line in the anchorage-independent growth assay. It was clearly demonstrated in these studies that RA caused a significant inhibition of both tumor incidence and growth. Therefore, it was shown the RA is not only able to block the expression of the transformed phenotype *in vitro* but is also effective at inhibiting tumor formation *in vivo* confirming the antineoplastic effects of RA against Balb/cMSV transformed 3T3 cells.

There are some potential problems in the tumorigenicty assay system which may lead to an underestimation of the efficacy of RA. The tumor cells are administered intracutaneously by injecting the cells into the cavity that was introduced through the formation of a suction blister (see Materials and Methods). RA is administered topically in a 0.2% DMSO solution every 24 hours. The amount of RA which is actually absorbed through the skin or the concentration of retinoid reaching the tumor cells is not known. Therefore, it is possible that a therapeutic concentration of RA is not achieved or maintained leading to a less than optimal inhibition of tumorigenesis. In addition, histological examination of all tumors revealed that two tumors that formed in the RA treated group were inadvertantly placed subcutaneously and it is therefore unlikely that adequate RA concentrations were achieved at the tumor site. Again, this indicates that a less than optimal response may have been obtained.

The remainder of the work presented here was aimed at uncovering the mechanism(s) by which RA was blocking retrovirally induced cellular transformation and tumorigenesis. The initiation and maintenance of transformation is dependent upon the expression of the v-onc genes present in each of the viral genomes (Bishop and Varmus 1982). If RA was having a direct effect upon the expression of the v-onc genes this could be occurring at a number of different levels including: transcription, primary transcript processing and/or transport to the cytoplasm. The expression of the gene product could also be modified directly through an inhibition of translation or an alteration in the functional activity of the protein.

Retinoids have been shown to modify the cellular concentration of a number of proteins including: keratins (Fuchs and Green 1981), collagen (Beach and Kenney 1983), collagenase (Brinckerhoff and Harris 1980), plasminogen activator (Strickland and Mahdavi 1978), alkaline phosphatase (Reese et al. 1981), the receptor for epidermal growth factor (Jetten 1980) and transglutaminase (Yuspa et al. 1982). In the case of the keratins, it was shown that retinol caused specific changes in mRNA levels (Fuchs and Green 1981). In the absence of retinol keratinocytes produced a 67kd keratin mRNA but upon addition of retinol the 67kd mRNA levels decrease and mRNA for smaller molecular weight keratins increases. The expression of the c-myc gene has also been shown to be turned off in response to RA (Westin et al. 1982). When HL60 cells are exposed to RA the c-myc mRNA level drops

dramatically. These two examples indicate that retinoids can effect the expression of specific genes by modifying mRNA concentrations. Whether these changes in gene expression are the result of direct effects upon transcription or are secondary to some other retinoid effect is not known. Also, both of the systems mentioned above involve cellular differentiation. If RA is replaced by DMSO in the HL60 system the same result is obtained (Westin et al. 1982). Whenever differentiation is involved it is possible that the observed change in the concentration of a protein is due to the stage of differentiation irrespective of the inducing agent and therefore represents a secondary effect. Differentiation does not appear to be involved with a number of examples cited above including; alkaline phosphotase, the epidermal growth factor receptor and collagenase.

A complete inhibition of v-onc gene expression may be expected to result in a reversal of the anchorage-dependent growth changes brought about by v-onc expression. This has been shown to occur in an NRK cell line transformed by a temperature sensitive KMSV mutant at the nonpermissive temperature (Shih et al. 1979). In this case, the transforming protein (p21) concentration decreases to very low level compared to cells grown at the permissive temperature. Since we observed little change in anchorage-dependent growth of these cell lines, we did not expect to observe a complete suppression of v-onc mRNA levels. To test the possibility that RA may be altering v-onc gene expression the level of v-onc mRNA all of the virally transformed cells was examined. In all cases cells were treated for 72 hours at

the RA concentration that gave a maximal reduction in anchorage-independent growth. For all three viruses, KMSV, Balb/cMSV and SSV, only one genomic size transcript has been observed (Scheinberg and Stand 1980; Eva et al. 1982; personal communication Dr. S.A. Aaronson of the NCI, Bethesda, MD). Dot blot hybridization analysis revealed that the only changes in v-onc mRNA levels that occurred resulted in a 2-4 fold increase in v-bas mRNA concentration in Balb/cMSVNRK cells. This is consistant with the stimulation of anchorage-independent growth of these cells in the presence of RA. No alteration in v-onc mRNA concentration was observed in any of the other cell lines, indicating that transcriptional effects are not responsible for the antineoplastic activity of RA exhibited by the sensitive cell lines.

Vitamin A has been shown to be associated with ribosomes suggesting a potential direct effect upon translation (Leutskaya and Fais 1973). Lotan, et al. (1982) have shown RA causes a significant reduction in protein synthesis in mouse S91 melanoma cells which are sensitive to growth inhibitory effects of RA. Two-dimensional gel electrophoresis analysis of proteins synthesized by control and treated S91 cells revealed few qualitative differences in the more than 100 proteins detected. In contrast, a number of quantitative differences were easily detected indicating that the relative concentration of some protein were being changed by RA treatment.

To test the possibility that RA may be altering the translation of p21, the relative rate of p21 synthesis in KMSV and Balb/cMSV

transformants was studied. The amount of p21 being synthesized was not influenced by RA. This indicated that the effects of RA are not on the expression of p21 unless the functional activity of the protein was being modified.

The role of p21 in transformation is not understood but several biochemical features of p21 have been characterized. Scolnick et al. (1979) showed that p21 of both Kirsten and Harvey MSV was able to bind guanine nucleotides reversibly. When using partially purified preparations of p21 it was observed that GTP could serve as a substrate for autophosphorylation (Shih et al. 1980). No effect of RA on GDP binding in KNRK cells which are sensitive in the in vitro assays was observed. This observation was important for two reasons. First, this result indicated that the effects of RA were not through an alteration in p21 function. Secondly, the steady state level of p21 appeared unchanged which implies that since p21 synthesis was not altered, p21 turnover in KNRK cells is also uneffected.

The focus of the work presented here was on defining a potential role for RA in inhibiting cellular transformation that involved the expression of v-onc and c-onc genes. Except for the v-sis gene of SSV, all of the transformed cell lines contained either a v-ras or c-ras oncogene and both members of the ras gene family, ras^H and ras^K in the form of c-ras and v-ras, were represented. The v-bas and $v\text{-}ras^H$ are homologous genes and there is no reason to believe that one is more closely related to $c\text{-}ras^H$ than the other. Therefore, to avoid confusion during the remainder of this discussion, v-bas will be

called v-ras^{H}. In addition to the human carcinoma cell lines with activated c-ras genes, NIH/3T3 cells transformed with the c-ras genes via transfection were also examined. This allows for a direct comparison of the effects of RA on NIH/3T3 cells transformed with either v-ras or c-ras.

EJ/T24 (c-ras^{H}), the CRABP negative human carcinoma cell line, was not inhibited by RA in soft agar but the NIH/3T3 transformant 44-9-1-1 (c-ras^{H}) was sensitive as was the viral NIH/3T3 transformant, Balb/cMSV 3T3 (v-ras^{H}). When a CRABP positive fibroblast, NIH/3T3 is transformed by either c-ras^{H} or v-ras^{H} the transformant is sensitive to inhibition of anchorage independent growth by RA. Although it is not certain that CRABP plays a role here, it is worth noting that the CRABP negative Balb/cMSVNRK (v-ras^{H}) cells are stimulated to form colonies in soft agar by RA.

K3T3 and 118-4-3-1 represent the same cell line, NIH/3T3, transformed by v-ras^{K} and c-ras^{K}, respectively. Both of these transformants were unresponsive to RA, although they both had significant levels of CRABP. This indicates that the presence of CRABP is not sufficient to confer RA sensitivity to cells whose transformation involves the expression of the Kirsten member of the ras gene family. These results show that when transformation involving c-ras vs v-ras, in the same cellular background, are compared for their sensitivity to inhibition of transformation by RA the viral or cellular source of the homologous transforming gene is withour major consequence.

As was just pointed out, the presence of CRABP does not appear sufficient to confer sensitivity to RA. It may not be necessary either. KNRK cells which show anchorage-independent growth inhibition in response to RA treatment do not have detectable levels of CRABP. It has been proposed that the action of retinol and RA is mediated through direct interactions with the nucleus and the retinol-CRBP or RA-CRABP complex analogous to the steroid hormone model for gene regulation (Chytil and Ong 1978; Mehta et al. 1982; Liau et al. 1981). The evidence in support of a role for CRBP and CRABP in retinoid action follows several lines of investigation. Liau et al. (1981), using purified CRBP, demonstrated specific and saturable binding sites in rat liver nuclei and chromatin. These studies provided evidence that retinol was delivered to the nucleus, to specific binding sites, in complex with CRBP. In contrast to the steroid hormone model, CRBP does not bind but instead delivers retinol to sites where it specifically binds. For these sites to be recognized by retinol, it must be in complex with CRBP because free retinol does not compete for binding. The RA-CRABP complex has also been shown to interact with tumor cell nuclei (Mehta et al. 1982). The nuclear binding displayed the same characteristics as that described for retinol-CRBP. Several studies have shown that high affinity of retinoid derivatives for CRABP is correlated with their ability to induce differentiation, inhibit papilloma formation, and reverse metaplasia in organ culture (Sherman et al. 1983; Sani et al. 1984; Trown et al. 1980). Also, mouse embryonal carcinoma cell mutants selected for their inability to

differentiate in response to RA lack CRABP (McCue et al. 1983; Schindler et al. 1981). Evidence has also accumulated which brings the biological role of CRBP and CRABP into doubt. Lotan et al. (1980) demonstrated that the binding proteins were neither necessary or sufficient for the inhibition of transformed cell growth in vitro. Retinoids are able to effectively block chemical carcinogen induced cellular transformation in vitro in the apparent absence of binding protein (Libby and Bertram, 1982). Mutant S91 melanoma cells have been selected for their insensitivity to RA (Lotan et al. 1983). Several of the resistant clones contain as much or more CRABP as the highly sensitive parent cells bringing into question the significance of CRABP. It was pointed out that a receptor or acceptor mutation could have occurred that was independent of binding activity. A role for CRABP in differentiation has also been questioned. HL60 cells (promyelocytic leukemia) are very sensitive to induction of differentiation by RA but apparently do not contain detectable levels of CRABP (Douer and Koeffler 1982; Breitman et al. 1980), although there is one report of CRABP in HL60 cells (Takenaga et al. 1981). It has also been demonstrated that human teratocarcinoma cell lines which fail to differentiate in the presence of RA have high levels of CRABP (Matthaei et al. 1983). These studies indicate that with RA induced differentiation, CRABP is neither sufficient or necessary. In defense of CRABP, the present methodology does not allow for the detection of less than 2,000 CRABP molecules per cell (personal communication with Dr. Mark Haussler). This level of binding protein may be adequate for

mediating RA. In cell lines with binding activity that do not respond to retinoids, other factors could be important. Receptor-acceptor interactions could be modified without an effect on binding activity as has already been pointed out, cellular retinoid uptake could be defective or retinoid metabolism by the effector cell may be important. The studies presented here also indicated that CRABP was neither sufficient or necessary for RA inhibition of onc gene induced transformation. But it should be pointed out that in the cases where a high level of sensitivity was observed, indicating specific effects, CRABP was present. In instances where responsiveness to RA was observed in the absence of CRABP, high concentrations (10 μ M) were required indicating that the effects observed may be nonspecific.

Studies presented here have shown that RA can inhibit cellular transformation and tumorigenesis induced by retroviruses but that no alteration was observed in v-onc mRNA levels. The synthesis of the v-ras gene product, p21, was not decreased and that in one case, KNRK, the functional properties of p21 were not modified. How then may RA be inhibiting the transformed state?

A number of recent studies have shown that oncogene induced cellular transformation requires other cooperating events in the cell before tumorigenicity is expressed. When primary cultures of rat embryo fibroblasts are transfected with the EJ/T24 c-ras^{H} gene, they are not converted into tumor cells (Ruley 1983; Land et al. 1983). When the EJ/T24 gene is cotransfected with the viral or cellular myc gene, polyoma large T antigen, or adenovirus early region 1A gene, the

fibroblasts did become tumorigenic. The EJ/T24 gene is capable of inducing tumorigenic transformation of chemically immortalized hamster embryo fibroblasts but does not transform normal fibroblasts (Newbold and Overell 1983). These studies demonstrate that other processes, expression of a second oncogene or immortalization, must work in concert with the ras gene before complete neoplastic transformation occurs. NIH/3T3 and NRK cells are long term, established cell lines in which the process of immortalization has already occurred. What makes NIH/3T3 and NRK cells susceptible to oncogene induced transformation is not known but these studies show that other cellular processes are required to work in concert with the oncogene. The studies reported here indicate that RA does not directly effect v-ras gene expression and, therefore, must modify these undetermined cellular functions that are necessary for tumorigenic transformation.

Retinoids have been shown to effect other areas of cellular physiology that may have a role in cooperating with retroviral oncogenes. It has been demonstrated that RA regulates the expression of the c-myc gene (Westin et al. 1982). This is especially important in view of the fact that c-myc has been shown to cooperate with the c-ras^H gene to bring about tumorigenic transformation. This could be an important area for further investigation especially since the specific probes needed are available.

The effects of retinoids on the cell surface has received much attention. Events occurring at the cell surface (e.g., ion transport and receptor interactions) are known to be important in regulating

cellular proliferation and differentiation and, therefore, could provide the cooperating signals. Retinol is known to be involved in glycoprotein synthesis. Sugar transfer is mediated via mannosyl retinyl phosphate (DeLuca et al. 1979; Wolf et al. 1979). Although this may be important for retinol, it is much less likely to play a role in activity of RA or the highly active RA derivatives (Sporn and Roberts 1983). Membrane microviscosity has also been shown to be altered by retinoids (Jetten et al. 1983). Again, this could lead to changes in membrane structure and function.

Very recent studies have opened up a new area of retinoid research which could be important. It has been suggested that RA induction of F9 embryonic carcinoma differentiation may involve calcium and phospholipid-dependent cyclic AMP-dependent protein kinase activity (Anderson et al. 1983). This is supported by the in vitro study with partially purified kinase (Ohkubo et al. 1984). RA stimulated kinase activity while retinol and retinal were much less effective which correlates with their potency to induce differentiation. Because of the known role of protein kinase activity in the regulation of cellular proliferation, this may represent a very important site of action for retinoids in the inhibition of cellular transformation.

The role of peptide growth factor in cellular transformation is rapidly gaining a greater appreciation (Bradshaw and Sporn, 1983). Retinoids have been shown to alter epidermal growth factor receptor number and decrease the mitogenic response (Jetten 1982). The

phenotypic and morphological transformation of cells to sarcoma growth factor has been shown to be blocked by retinoids (Todaro et al. 1978). Welsch et al. (1984) have demonstrated that retinoids can suppress peptide hormone induced stimulation of mammary carcinoma cells in vivo and in vitro. These observations are especially important in view of the fact that the SSV v-sis gene product, p28, is structurally and immunologically related to the mitogenic peptide platelet derived growth factor (Doolittle et al. 1983; Robbins et al. 1983). Therefore, the inhibition of transformation by RA observed with the SSV transformed cells, SSVNRK and SSV3T3, could have resulted from retinoid induced alterations in the PDGF receptor number or p28 PDGF receptor interactions. Alternatively, secondary events involved with p28 induced mitogenesis such as receptor internalization could have been effected.

In conclusion, when one reviews the bewildering number of biological activities that have been ascribed to the retinoids as a potential mechanism to explain their antineoplastic effects, it should become obvious that a clear understanding is still not at hand. This also indicates that retinoids are involved either directly or indirectly in a wide variety of cellular functions. Therefore, it is entirely possible that for retinoids to express their antineoplastic effects, a number of cellular events must occur in response to retinoid exposure and that these events may vary from one transformed cell to another. This concept is supported by the fact that transformation is such an ill defined state and the endpoints used to

measure it are also ill defined (e.g., growth in soft agar). There are no specific molecular or biochemical markers for transformation that apply to all systems. The lack of specific biochemical markers for retinoid activity has also greatly hindered progress in this area, but due to the reasons stated above, it may not be reasonable to expect to find one marker that will apply to all systems. Retinoid research is very diverse and extremely complex especially in view of the fact that systems of cellular differentiation and transformation are usually employed in retinoid studies. These processes have been a major enigma in biology for a long time and until recent advancements in cellular transformation occurred, our understanding was only descriptive. Therefore, retinoids may represent important biochemical probes which will be used to gain a better understanding of these complex processes.

LIST OF ABBREVIATIONS

Balb/cMSV	Balb/c murine sarcoma virus
Balb/cMSV3T3	Balb/c murine sarcoma virus infected NIH/3T3 cells
Balb/cMSVNRK	Balb/c murine sarcoma virus infected normal rat kidney cells
c-onc	Cellular oncogene
CRABP	Cytoplasmic retinoic acid-binding protein
CRBP	Cytoplasmic retinol-binding protein
DMSO	Dimethyl sulfoxide
DMEM	Dulbecco's minimum essential medium
DNA	Deoxyribonucleic acid
GDP	Guanosine diphosphate
GTP	Guanosine triphosphate
K3T3	Kirsten murine sarcoma virus infected NIH/3T3 cells
KMSV	Kirsten murine sarcoma virus
KNRK	Kirsten murine sarcoma virus infected normal rat kidney cells
mRNA	Messenger ribonucleic acid
MSV	Murine sarcoma virus
NRK	Normal rat kidney cells
PBS	Phosphate buffered saline
PMSF	Phenylmethyl-sulfonylfluoride
RA	β-all-trans-retinoic acid

RBP	Retinol binding protein
RNA	Ribonucleic acid
SDS-PAGE	Sodium dodectyl sulfate polyacrylamide gel electrophoresis
SSV	Simian sarcoma virus
SSV3T3	Simian sarcoma virus infected NIH/3T3 cells
SSVNRK	Simian sarcoma virus infected normal rat kidney cells
v-onc	Viral oncogene

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