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REGULATION OF MOUSE MAMMARY TUMOR VIRUS GENE EXPRESSION

by

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REGULATION OF GENE EXPRESSION OF THE MOUSE MAMMARY TUMOR VIRUS

by

Gordon Ringold

ABSTRACT

REGULATION OF MOUSE MAMMARY TUMOR VIRUS GENE EXPRESSION

by Gordon Ringold

I have studied the regulation of mouse mammary tumor virus (MMTV) gene expression by measuring the production of virus and the transcription of viral genes in cultured cells. Measurements of viral RNA in the growth medium (i.e., virus production) or in the cell were made by hybridizing radioactive MMTV complementary DNA (cDNA) with cell RNA or RNA extracted from the tissue culture fluids. In cell lines and primary explants derived from mammary tumors of mice the amount of virus produced correlates with the level of intracellular virus-specific RNA. This suggests that in these cells, transcriptional controls are of primary importance in regulating the production of MMTV. In contrast, a lymphoma cell line (S49) from a BALB/c mouse contains large quantities of MMTV-specific RNA yet produces extremely low levels of virus. In these cells, mechanisms other than transcriptional controls may be important in regulating virus production.

The production of MMTV by mammary tumor cells has been shown to be under the control of corticosteroid hormones. Using a cell line derived from a spontaneous mammary tumor in a mouse of the GR strain, I have investigated the mechanisms by which dexamethasone, a synthetic glucocorticoid, stimulates virus production. Initial experiments demonstrated that the increase in virus production was a consequence of increased concentrations of intracellular viral RNA. The accumulation of viral RNA occurs rapidly ($t_{\frac{1}{2}} \sim 2\frac{1}{2}$ hours) and is dependent on

RNA synthesis but not protein or DNA synthesis. The effect of dexamethasone also appears to be mediated by a specific and saturable glucocorticoid receptor similar to those described in other steroid-sensitive mouse cells. These experiments suggest that the accumulation of MMTV RNA is a primary response to dexamethasone.

The accumulation of viral RNA consequent to treatment with dexamethasone could be due to an increase in its rate of synthesis, a decrease in its rate of degradation, or a combination of the two. Using a new assay to measure newly synthesized viral RNA I found that the rate of synthesis of MMTV RNA was increased 3-4 fold by treatment with dexamethasone; however, the concentration of viral RNA was increased 15-20 fold. Furthermore, experiments designed to test the turnover of MMTV RNA suggest that the viral RNA present in dexamethasone treated cells is significantly more stable than the viral RNA in uninduced cells. Thus it appears that the dexamethasone mediated accumulation of MMTV RNA in GR cells is due to both an increase in its rate of synthesis and a decrease in its rate of degradation.

Lastly, I have found that MMTV RNA in dexamethasone treated cells is preferentially associated with polyribosomes. The viral RNA in treated and untreated cells appear to be similar with respect to their polyadenylation and their distribution between cytoplasm and nuclei. These results suggest that the stabilization of MMTV RNA in dexamethasone treated cells may be due to its preferential incorporation into polyribosomes.

ACKNOWLEDGMENTS

I am grateful to Mike Bishop and Harold Varmus for having directed me through the intricacies and subtleties of experimental design. Without their help I suspect I would still be running sucrose gradients to assay virus production. For having given me their advice and friendship I thank them.

I thank all of the people in the lab for their help, their tolerance, and their companionship (especially late at night). In particular I appreciate Ed Stavnezer's assistance in the use of his assay and apologize for always being one step behind him. I also thank Ed Medeiros for encouraging me to try things when I first began this project. Without his moral support I would never have begun this work.

Several other people have played key roles in my progress. I thank Brian McCarthy for giving me a hard time when it was warranted and for serving as a teacher in and out of the classroom. I thank Keith Yamamoto for his help in performing many experiments and for his desire and enthusiasm in discussing new ideas. I also acknowledge many fruitful conversations with Leon Levintow and Warren Levinson.

Most importantly I thank Carol, my resident zoologist, for her patience, understanding, and encouragement. She knew when to keep me home and didn't complain when I stayed in the lab throughout the early hours of the morning.

Lastly, I dedicate this work to the memory of Gordon Tomkins. His overwhelming enthusiasm and support for my work provided the single greatest impetus to its success. When others were skeptical Gordon was excited, and when others were reluctant Gordon always said "try it". His creativeness, his wit, and his charm continue to live through his work and hopefully, my work will reflect that special quality which we all knew was Gordon's.

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INTRODUCTION

Steroid hormones, which include the estrogens, progestins, androgens, corticoids, and ecdysones, play an important role in the regulation of developmental and physiological processes in metazoan organisms (King and Mainwaring, 1974). Shortly after Jensen and his colleagues (Jensen and Jacobsen, 1962) succeeded in synthesizing radioactively labeled steroids of high specific activity a unifying theory evolved which has attempted to explain the diverse actions of this group of hormones. A large body of evidence has accumulated which suggests that steroids diffuse through cell membranes and subsequently bind tightly and specifically to receptor proteins present in the cell cytoplasm (Gorski et. al., 1968; Jensen et. al., 1968; Gorski and Gannon, 1976; Yamamoto and Alberts, 1976). Upon binding they act as allosteric effectors to alter the structure or conformation of the steroid receptor thereby increasing its affinity for DNA containing sites in the cell nucleus. By some unknown mechanism(s) the interaction of the steroid-receptor complex with the genetic material of the cell triggers the biological response characteristic of the particular hormone and target tissue.

That the receptor is required for steroid hormone action is evidenced by several types of experiments. In early studies (Jensen and Jacobsen, 1962; Jensen and DeSombre, 1972) it was found that receptor proteins with high affinity for estradiol were present only in target tissues (e.g. uterus contains receptors for estradiol but not for testosterone). Similar observations were subsequently made for the other steroid hormones (see King and Mainwaring, 1974). Secondly, a strong correlation exists between the biological effectiveness of the

hormone and its affinity for the receptor. In a series of elegant experiments (Baxter and Tomkins, 1971; Samuels and Tomkins, 1972), Tomkins and his colleagues were able to demonstrate that the effectiveness of a particular glucocorticoid in stimulating the accumulation of tyrosine amino transferase (TAT) in a rat hepatoma cell line (HTC) could be correlated with its affinity for the glucocorticoid receptor. Moreover, they were able to demonstrate that other steroids (e.g. progesterone) which could bind to the receptor without eliciting the biological response would act as competitive inhibitors of the active hormones. Thirdly, in all systems which have been carefully studied, the magnitude of the biological response is dependent on the amount of hormone which is bound to receptors. Each of these approaches has provided convincing evidence that the receptor is a biologically significant molecule and that its presence is required for hormone activity.

However, it has proven considerably more difficult to evaluate the biological significance of the second step in the scheme outlined above. That is, how important is the nuclear localization of the steroid-receptor complex? Using ^{3}H-estradiol in uterine cells, Gorski and Williams (1972) demonstrated that 85% of the label (virtually all of which was bound to receptor) was localized in the nucleus, irrespective of the degree to which the receptors were saturated. In addition, both Tomkins' and Gorski's groups have shown that the magnitude of the hormone response increased as a function of the number of receptors bound in the nucleus. It is also suspected that receptors must be complexed with hormone in order to bind to nuclei since anti-inducing hormones which bind to receptors but are inactive biologically

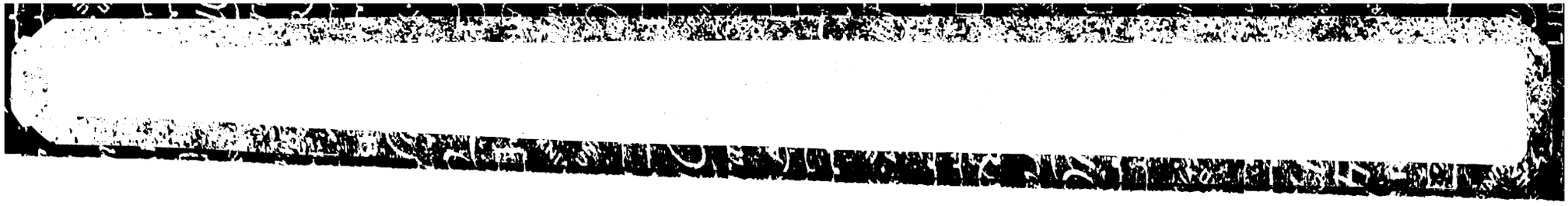
(e.g. progesterone in HTC cells) do not promote nuclear localization of the steroid-receptor complex (Rousseau, 1975). A more appealing approach which combines the tools of genetics and biochemistry has recently been used. Since receptor-binding to nuclei can be prevented by treatment with DNAase (Harris, 1971; Musliner and Chader, 1972; Baxter et. al., 1972) (but not RNAase) it is clear that at least part of the nuclear binding site contains DNA. Several groups have demonstrated in vitro that steroid receptors are DNA binding proteins (King and Gordon, 1972; Toft, 1972; Rousseau et. al., 1975). Using a mouse lymphoma line, S49, Sibley and Tomkins (1974 a, b) were able to select variant cells which were resistant to the killing action of these hormones. Two interesting phenotypes were found. In one, nt^-, the hormone was able to bind to the receptor but nuclear binding was extremely poor. In another, nt^i, the steroid bound to the receptor normally, but a greater than normal amount of the steroid-receptor complex was localized in the nucleus. A subsequent series of experiments demonstrated that these variant cell types contained receptors with abnormal DNA binding properties (Yamamoto et. al., 1974). The nt^- had a lower affinity whereas the nt^i had a higher affinity for DNA than the wild-type receptor. Thus for the first time a biochemical property (DNA binding) could be correlated with an observed biological property of the receptor. However, since the processes responsible for cell killing are obscure, this system does not yet provide unequivocal evidence for the direct action of the receptor in the biological manifestations of the response.

In all systems studied to date, the number of nuclear binding sites for the steroid-receptor complex is much greater than the total

number (approx. 10^4) of receptor molecules per target cell (Chamness et. al., 1974; Rousseau et. al., 1975). Therefore a major conceptual problem has been to ascertain how the receptor detects specific nuclear sites (assuming that in fact such specificity exists). Yamamoto and Alberts (1975) have provided an explanation based on similar observations made on the nature of the binding of the lac repressor to E. coli DNA. They suggest that there are a few high-affinity sites in the DNA (which may or may not be) accessible to the steroid-receptor complex but in addition, since the receptor is a general DNA binding protein there are a huge number of low affinity sites. Therefore, a large number of receptor molecules are required thermodynamically in order that they be able to find the high affinity sites within a reasonable period of time. An alternative hypothesis has been presented by Spelsberg et. al. (1972, 1973) who argue that specificity arises from protein-protein interactions which position a DNA binding subunit at or near the proper site on the DNA. They found that the progesterone receptor from chick oviduct is composed of two subunits. One of the subunits has the ability to bind to DNA whereas the other will bind to chromatin (but not free DNA). Furthermore, they claim that the chromatin-binding subunit binds specifically to an acidic protein (AP-3) extracted from chick oviduct nuclei and is incapable of binding to non-target tissue proteins. Although this model is appealing, there has been little experimental data to support it. Thus, at the present time, the means by which the receptor might bind specifically in the nucleus remain to be elucidated.

Based on the evidence for transcriptional modification by

regulatory proteins in prokaryotes it has been suggested that the steroid-receptor complex acts to alter transcription (O'Malley and Means, 1974; Yamamoto and Alberts, 1976). The major problems confronted in proving such a hypothesis are the following; 1) as described above it has been an extremely difficult proposition to establish that the receptor's interaction with the nucleus is biologically significant; 2) the initial events subsequent to interaction of the receptor with its nuclear binding sites have been observed in only a few instances and in most cases it is clear that the observed biological or biochemical response is not a "primary" effect (see below) of the hormone; 3) the process of transcription in eukaryotes is poorly understood and the development of *in vitro* transcription systems has progressed very slowly; 4) the steroid receptors (except recently the progesterone receptor) have proven refractory to purification thus making it impossible to study their properties *in vitro*. In spite of these major impediments there is evidence that one of the earliest effects of steroid hormones is to mediate a selective increase in the rate of transcription from a limited number of genes. The only well-documented case is provided by the puffing behavior of the polytene salivary chromosomes of *Drosophila* after the administration of the moulting hormone ecdysone. Ashburner (1973) has demonstrated that within 10 minutes after the addition of hormone to third instar larvae, one can detect puffing, indicative of active RNA synthesis (Judd and Young, 1973), of six bands (chromomeres). This early puffing is sensitive to inhibitors of RNA synthesis and five of the six puffs are insensitive to inhibitors of protein synthesis. Thus it is characteristic of what I shall call a *primary*



response (i.e. it occurs rapidly and is insensitive to inhibitors of protein synthesis). A secondary response to the hormone begins approximately three hours after addition of the hormone. In a clearly defined temporal order, the first of more than 100 new bands undergoes puffing behavior presumably mediated by one or more of the "early puff" gene products. In addition, one (or more) of these proteins is responsible for shutting off early puff RNA synthesis. Thus, we see that the initial events subsequent to interaction of the steroid-receptor complex with the chromosome is the induction of transient RNA synthesis at a small number of loci. This in turn triggers a defined pattern of puffing at a much larger number of genes (the secondary response).

In contrast to the clearly defined sequence of events in Drosophila, the situation in mammalian cells is much less clear. Although many effects of steroids (e.g. induction of hepatic enzymes by glucocorticoids (Thompson et. al, 1966) and induction of ovalbumin by estrogen (Palmiter, 1975) are dependent on RNA synthesis it is not known whether the steroid-receptor complex is directly affecting transcription. In the best studied case, Schimke's and O'Malley's, groups have demonstrated that the rate limiting factor in the production of ovalbumin in chicken oviducts is the concentration of ovalbumin mRNA (mRNAov) (see reviews by Palmiter, 1975 and Means and O'Malley, 1974). Re-exposure of the estrogen-primed chicken to estrogen (or progesterone) induces ovalbumin synthesis in preexisting tubular gland cells after a lag of approximately 3 hours (Palmiter, 1975); this precedes a second stage of cellular proliferation. The first exposure to estrogen is complicated by the massive proliferation

of tubular gland cells which results in a several hundred-fold increase in the weight of the oviduct. By nucleic acid hybridization and translation assays the concentration of mRNAov was found to be increased several-thousand fold in the cells of estrogen treated oviducts (Rhoads et. al., 1975; Cox et. al, 1974; Harris et. al., 1975). In O'Malley's lab the induction of mRNAov begins with little lag (30') subsequent to treatment with estrogen (Harris et. al., 1975) and is insensitive to inhibitors of protein synthesis (personal communication). In marked contrast, both Palmiter's (Palmiter, 1975) and Schimke's (McKnight et. al., 1975) groups find that the increase in mRNAov occurs with a lag of approximately 3 hours and can be blocked by inhibitors of protein synthesis. Clearly, these contradictory data must be reconciled before one can decide whether the induction of mRNAov is a primary estrogen effect and whether this system will be useful as a model for studying steroid receptor action. In light of this conflict it is interesting that the induction can not be reproduced in isolated oviducts in organ culture (Palmiter et. al., 1971). Thus it is conceivable that estrogen-sensitive processes outside the oviduct could mediate ovalbumin induction.

In addition to the problems described in the preceding paragraph no one has yet been able to demonstrate that the induction of mRNAov is a consequence of an increase in its rate of synthesis. It is conceivable that the hormone acts at some processing step or in altering the turnover rate of the mRNA. In fact, Palmiter has demonstrated that the half-life of mRNAov is considerably longer in the presence of estrogen than after withdrawal of the hormone (Palmiter and Carey, 1974). However, since the processes of mRNA biosynthesis in eukaryotic

cells are poorly understood and the technology for studying their rates of synthesis and degradation are very primitive, the significance of this observation with respect to the induction of mRNAov is unclear. Thus, we see that even in the best studied case, the mechanisms by which steroid hormones induce the accumulation of specific mRNAs are far from clear.

The work presented in this dissertation introduces and characterizes a system which appears to be extremely well suited for analyzing glucocorticoid action. In 1961 Smoller et. al. noticed that the number of mouse mammary tumor virus particles (MMTV) in mammary tumors of mice treated with cortisol was greater than in untreated animals. More recently, McGrath (1971) found that the production of MMTV in primary cultures of mammary tumors increased when cortisol was included in the culture medium.

MMTV is an enveloped virus whose genome consists of 2 or 3 identical single-stranded RNA molecules of approximately 3 x 10^6 daltons (Duesberg and Cardiff, 1968; Morris et. al., in preparation). The virus was first described by Bittner in 1936 as the milk-borne agent responsible for causing mammary tumors in several strains of mice. Genetic (Bentvelzen et. al., 1970) and biochemical (Varmus et. al., 1972) evidence has indicated that all strains of mice, regardless of their incidence of mammary tumors, harbour an endogenous virus in the form of a DNA provirus (Temin and Baltimore, 1972) covalently linked to the host genome. Although MMTV DNA is present in all mouse tissues, production of infectious virus is <u>generally</u> limited to mammary tissues (for a few exceptions see review by Nandi and McGrath, 1973). In accord with this, Varmus et. al (1971) found that high concentrations

of MMTV-specific RNA were present only in lactating mammary glands, mammary tumors and Leydig cell tumors. Thus it appears that the production of MMTV in vivo is under transcriptional control.

Study of MMTV in vitro has been hampered by the inability to reproducibly infect tissue culture cells with the virus. Recently however, primary cultures and continuous cell lines derived from mammary carcinomas have been used to propagate MMTV (Sykes et. al., 1968; Lasfargues et. al., 1972; Owens and Haskett, 1972). These cell lines spontaneously and continuously produce MMTV (under glucocorticoid control) thus permitting one to study the factors involved in the regulation of virus production. This dissertation attempts to answer some questions about the role of transcription in the production of MMTV in vitro and in particular examines the effects of glucocorticoid hormones on the transcription of MMTV genes in a continuous cell line derived from a spontaneous tumor in the GR strain of mouse (Muhlbock, 1965). The major conclusions that can be drawn from my experiments are as follows:

1) The intracellular concentration of MMTV RNA is a limiting factor in virus production from mammary tumor cells in culture.
2) Dexamethasone (a synthetic glucocorticoid) stimulates virus production by increasing the intracellular concentration of MMTV RNA.
3) The effect of dexamethasone on MMTV RNA is a primary hormone effect and appears to be mediated by a specific and saturable glucocorticoid receptor.
4) The induction of MMTV RNA is due to an increase in its rate of synthesis as well as a decrease in its rate of degradation.
5) The effect of desamethasone on the stability of MMTV RNA may be due

to a preferential synthesis of RNA destined to become polysome associated.

MATERIALS AND METHODS

Buffers and Solutions

Tris : tris (hydroxymethyl) - aminomethane - hydrochloride

TE : 10mM Tris (pH 7.4), 10mM EDTA

STE : 0.1 M NaCl, 20mM Tris (pH 7.4), 1mM EDTA

RSB : 10mM NaCl, 1.5mM $MgCl_2$, 10mM Tris (7.4)

M buffer: 10mM Tris (7.4), 5mM $MgCl_2$, 0.25M Kcl

P buffer: 25mM NaCl, 25mM Tris (7.4), 5mM $MgCl_2$

DNA buffer: 0.1 M NaCl, 50mM Tris (8.2), 10mM EDTA

10X annealing buffer: 3M NaCl, 0.25 M Tris (7.4), 10mM EDTA

Tris-glucose: 140 mM NaCl, 5mM Kcl, 5.5mM glucose,
5mM Na_2PO_4 - 20 mM Tris (pH 7.4)

Puck's: EDTA: 0.8% NaCl, 0.04% KCl, 0.035% $NaHCO_3$,
0.1% Glucose, 0.02% EDTA

MUP buffer: 8M Urea, 0.2M sodium phosphate buffer pH 7,
1% SDS

S1 buffer: 3M NaCl, 30 mM Na Acetate (pH 4.5), 3mM $ZnCl_2$,
10 μg/ml denatured calf thymus DNA.

Cells and viruses. Various lines of cultured cells producing MMTV were obtained as follows. The GR cell line was derived from a spontaneous mammary tumor in the GR strain of mouse (Muhlbock, 1965) as previously described (Lasfargues et. al., 1972). The TA3 cell line, a mammary tumor line derived from the A/HeHa strain of mouse (Keydar et. al., 1973) was received from Drs. P. Kimball and J. Schlom, Meloy Labs, Rockville, Maryland. Medium from the "296" cell line derived from a C3H spontaneous mammary tumor by Dr. A. Hackett (Owens and Hackett, 1972) was received from Drs. W. Parks and E. Scolnick, Meloy Labs. The primary explants of BALB/cf C3H mammary tumors, also referred to as "dome" cultures (McGrath, 1971), were kindly supplied by Dr. R. Cardiff, University of California, Davis, California. GR and TA3 cells were grown in medium 199 and primary tumor cultures in Dulbecco's modified MEM. All media (Grand Island Biological Co.) were supplemented with 10% fetal calf serum and, where noted, with 10 μg/ml dexamethasone (Sigma) and/or 10 μg/ml bovine insulin (Schwarz-Mann).

Mouse cells derived from tissues other than mammary gland were obtained as follows: 3T3 cells (Todaro and Green, 1963) were supplied by Dr. P. Vogt and were grown as previously described (Varmus et. al., 1973b). JLS-V11 cells (Wright et. al., 1967) were a gift from Dr. A Hackett, Oakland, California and were maintained in Dulbecco's modified MEM supplemented with 10% calf serum. S49 lymphoma cells (Horibata and Harris, 1970) (grown in suspension in Dulbecoo's modified MEM supplemented with 10% horse serum) were kindly provided by Drs. K. Yamamoto and G. Tomkins, University of California, San Francisco. Nucleic acids from L cells (Earle et. al., 1943) were generously provided by Drs. J.

Siegel and B. McCarthy, University of California, San Francisco.

MMTV purified from the milk of RIII mice was kindly supplied by Dr. D. Moore, Institute for Medical Research, Camden, New Jersey, and by Meloy Labs, Rockville, Maryland (provided through the Office of Program Resources and Logistics, National Cancer Institute). The B77 strain of avian sarcoma virus (B77-ASV) was propagated in chick embryo fibroblasts and purified as previously described (Bishop et. al., 1970). The Moloney strain of murine sarcoma virus (Mo-MSV) was purchased from Electro-Nucleonics Laboratories, Inc., Bethesda, Maryland.

Preparation of virus specific DNA. DNA complementary (cDNA) to the RNA genomes of B77-ASV, Mo-MSV, or RIII-MMTV was synthesized using the endogenous DNA polymerase activity of detergent activated virus. The cations used were as follows: 1mM Mg^{2+} for MMTV, 8mM Mg^{2+} for ASV, and 2mM Mn^{2+} for MSV. The optimal concentration of NP-40 (gift of Shell Oil Co.) was determined individually for each virus. Specific activities of 2-3 X 10^7 cpm/μg of cDNA were achieved using 10^{-5} M ^3H dTTP (59 Ci/mM, Schwarz-Mann) and 10^{-4} M unlabeled dGTP, dCTP, and dATP. Polymerase reactions were allowed to proceed for 18 hr at 37°C in the presence of 100μg/ml Actinomycin D (Calbiochem) to reduce the synthesis of double-stranded DNA (Garapin et. al., 1970; McDonnel et. al., 1970). Despite this treatment, I found that some double-stranded product was synthesized; consequently, single-stranded DNA was purified by batch elution from hydroxyapatite (BioRad) (Fanshier et. al., 1971). The isolated single-stranded DNAs were treated with NaOH (0.3 N for 18 hr at 37°C), neutralized with HCl, precipitated with ethanol and dissolved in a small volume of 3 mM EDTA. All such

preparations were at least 95% sensitive to the single strand-specific nuclease, S1, from Aspergillus oryzae (Leong et. al., 1972; Ando, 1966).

Determination of viral RNA concentrations in culture fluids by hybridization with complementary DNA (cDNA). Medium was harvested from virus-producing cultures and clarified at 8500 rpm for 10 min at 4°C in a Sorvall HB-4 rotor to remove cells and debris. This prevents possible contamination with RNA from broken cells if the medium is to be frozen. Virus was subsequently pelleted at 4°C in a Spinco SW 27 rotor by centrifugation at 25,000 rpm for 60 min (or smaller volumes in an SW 39 rotor at 35,000 rpm for 30 min). To extract RNA the pellet was resuspended in STE (0.1 M NaCl, 0.02 M Tris:HCl pH 7.4, 0.001 M EDTA) and then treated with 500 μg/ml Pronase (self-digested by incubating 10 mg/ml at 37°C for 2 hr in 0.02 M Tris:HCl pH 7.4) and 1% (w/v) sodium dodecyl sulfate (SDS) at 37°C for 45 min. Proteins were removed by two phenol extractions (phenol equilibrated with .02 M Tris, pH 7.4) at 25°C. Approximately 150 μg of carrier RNA (from yeast or HeLa cells) were added prior to the extraction procedure in order to monitor RNA recoveries as well as to protect the viral RNA from trace amounts of RNase. After precipitation with ethanol, the RNA was resuspended in small (35-100 μl) volumes of 0.02 M Tris, pH 7.4, for hybridization with virus specific DNA (cDNA). Recoveries of RNA were 75-100% as measured by the optical density of the carrier RNA at 260 nm.

Dilutions of RNA prepared from virus-containing culture fluids were made in 0.02 M Tris:HCl (pH 7.4) containing yeast RNA (8 mg/ml). Each dilution was then annealed with 500-600 cpm of cDNA at 68°C in 0.6 M NaCl, 50 mM EDTA. Reactions were performed in a final volume

of 10 μl in sealed capillary pipettes and hybridization was assayed by conversion of labeled cDNA into S1 nuclease-resistant material. Control reactions (cDNA hybridized with yeast or HeLa RNA) gave values of less than 5% self-annealing.

Percent hybridization at each dilution was plotted as a function of the volume of culture medium (V_o, in ml) from which the RNA was derived, multiplied by the time (t, in hr) of hybridization. We have designated this parameter at the "V_ot" and have called the point at which the hybridization is half complete the $V_ot_{\frac{1}{2}}$ in analogy with the previously described functions $C_ot_{\frac{1}{2}}$ (Britten and Kohne, 1968) and $C_rt_{\frac{1}{2}}$ (Birnstiel, 1972). None of the V_ot values has been corrected for relative RNA recovery since recoveries were always greater than 75%.

Preparation of cell RNA and determination of intracellular viral RNA concentration. Confluent cultures of cells were treated in STE with 500 μg/ml of Pronase and 0.5% (w/v) SDS for 1 hr at 37°C. The cell lysate was extracted twice at 60°C with phenol (equilibrated with 0.02 M Tris, pH 7.4). Nucleic acids were precipitated with ethanol, resuspended in 0.02 M Tris, pH 7.4 plus 0.01 M $MgCl_2$, and then treated with 20 μg/ml of RNase-free DNase (Worthington) for 2 hr at room temperature. The RNA was reextracted with phenol, precipitated with ethanol, and resuspended in a small volume of 0.02 M Tris:HCl, pH 7.4. Yields were approximately 150 μg RNA/10^7 cells.

The method for establishing viral RNA concentration within cells as a function of C_rt (concentration of RNA X time of hybridization with cDNA) has been previously described (Leong et. al., 1972). In brief, increasing amounts of RNA were annealed with 600 cpm of cDNA at 68°C in

0.6 M NaCl/0.05 M Tris:HCl, pH 7.4-0.002 M EDTA. The extent of hybridization was assayed by resistance of labeled cDNA to the single strand-specific nuclease, S1 (Leong et. al., 1972). Analysis of purified B77-ASV 70S RNA (prepared as described in Quintrell et. al., 1974) was also performed in this manner to provide a standard curve with which to compare unknown samples. Although these reactions can be performed by varying the time of hybridization and maintaining a constant RNA concentration, I have chosen not to do so in order to avoid being misled by artificially low plateaus (i.e., premature termination of the hybridization) due to insufficient RNA excess (Hayward and Hanafusa, 1973).

Assay for RNA-directed DNA polymerase (reverse transcriptase). DNA polymerase activity of ASV and MMTV was measured by the incorporation of ^{3}H TMP into acid-insoluble material using poly-(rA): oligo(dT) as template-primer (Baltimore and Smoler, 1971). Medium was harvested from confluent cultures and clarified at 8500 rpm for 10 min. Virus was then pelleted at 25,000 rpm for 60 min at 4°C in an SW 27 Spinco rotor or at 35,000 rpm for 30 min in an SW 39 Spinco rotor. Virus pellets were resuspended in 0.1 ml of reaction mixture and incubated at 37°C. For B77-ASV the reaction mixture contained 90 mM Tris, 0.1% (v/v) NP-40, 8 mM $MgCl_2$, 2% (v/v) 2-mercaptoethanol, 1 μg/ml poly(rA), 1 μg/ml oligo$(dT)_{12-18}$, and 1.5 M ^{3}H TTP (16 Ci/mM, Schwarz-Mann). The reaction mixture for MMTV was identical except for the $MgCl_2$ concentration (3mM) and the NP40 concentration (0.25%). Samples were taken at 0, 30, and 60 min and incorporation of ^{3}H TMP into acid precipitable material was measured as previously described (Garapin et. al., 1970).

Preparation of Cell-Free Extracts and Labeling with ^{3}H dex. Frozen cell pellets were thawed and homogenized with a motor-driven Teflon pestle in one volume of TEGNO5 buffer (10mM Tris pH 8.1, 1mM Na_3 EDTA, 10% (v/v) glycerol, 50mM NaCl, 1mM (mercaptoethanol, and 100 μg/ml crystalline bovine serum albumin). Homogenates were centrifuged first at 33,000 x g (10 min), then at 160,000 x g (75 min). The clear final supernatant was transfered to tubes containing various concentrations of ^{3}H dex (27-35Ci/mmol; New England Nuclear Corp.), and steroid bound by macromolecules was detected by sucrose gradient sedimentation or by exclusion from Sephadex G25 columns.

DNA-Cellulose Chromatography. DNA-cellulose columns were prepared and run according to Alberts and Herrick (1971), with Whatman CF-11 cellulose and native calf thymus DNA (Worthington; 0.5 mg DNA per packed ml). Little or no free ^{3}H dex or ^{3}H dex-receptor complex binds to blank cellulose controls under these conditions.

Measurements of DNA, RNA, and Protein Synthesis After Treatment with Metabolic Inhibitors.

Cytosine arabinoside, puromycin, and cycloheximide were purchased from Sigma. Actinomycin D was purchased from Calbiochem. Protein synthesis was measured one hour after addition of cycloheximide (2 μg/ml) or puromycin (5 μg/ml) by labeling GR cells for 30 minutes with 10 Ci/ml of ^{3}H-amino acids (Schwartz-Mann). The effect of actinomycin D (10 μg/ml) on RNA synthesis was determined by labeling cells for 30 minutes with 2 Ci/ml of ^{3}H-uridine (Amersham-Searle), 1 hour after addition of the drug. DNA synthesis was measured 1 hour after addition of 10-^{3}M cytosine arabinoside by labeling cells with 0.5 μCi/ml of ^{3}H-methyl thymidine (Schwartz-Mann). At the end of the labeling period,

cells were washed with an isotonic Tris-glucose solution then incubated with pronase (500μg/ml) and SDS (0.5%) for 1 hour at 37°C. An equal volume of 10% TCA was subsequently added, acid-precipitable material was collected on glass fiber filters and counted in a toluene based fluor (Ommifluor, New England Nuclear).

Preparation of "tailed duplex" DNA. MMTV was pelleted from 7.5 liters of GR culture medium by centrifugation in the Spinco type 19 angle-head rotor at 19K rpm for 90 min at 4°C. The virus (approximately $3x10^{12}$ particles) was resuspended in 7.5 ml of 0.1M Tris (pH 8.2) and then added to 110 ml of a DNA polymerase reaction mixture containing 10^{-4}M deoxynucleoside triphosphates (dCTP, dATP, dGTP, TTP), 0.3% NP-40, 3mM $MgCl_2$ and 1.5% B-mercaptoethanol. To label a sample of the DNA, ^{3}H-TTP ($2x10^{-6}$M) was added to an aliquot of the enzyme reaction. DNA synthesis was allowed to proceed for 11 hours at 37°C. The reaction was terminated by addition of SDS (0.5%) and the DNA was deproteinized by incubation with Pronase (37°; 60'; 500μg/ml) followed by extraction with phenol. Approximately 300μg of salmon sperm DNA were added as carrier during the EtOH precipitation. The DNA was then resuspended in 5 ml TE buffer and incubated at 37°C for 1 hr with 100μg/ml of pancreatic ribonuclease. RNAase was removed by phenol extraction in the presence of 1% SDS and the DNA was EtOH precipitated. The DNA was resuspended in 100μl of TE buffer, diluted with 1 ml of MUP buffer (8M urea, 1% SDS, 0.2M phosphate), and passed over a column of hydroxylapatite at 40°C. The column was washed extensively with MUP buffer (4-5ml), then the DNA, containing duplex or partial duplex structures, was eluted with 3ml of 0.4 M phosphate buffer. Approximately 200μg of salmon sperm DNA were added and the eluant was dialyzed

for a few hours against 2 liters of 1% SDS and 0.5M NaCl at room temperature. After EtOH precipitation the DNA was resuspended in 100µl of TE buffer. The amount of DNA recovered at each step was monitored by counting aliquots of the ^{3}H labeled material prepared in parallel. The final yield of DNA which bound to hydroxylapatite in MUP buffer was approximately 800 ng, 80-85% of which was resistant to the single-strand specific nuclease, S1. The size of the strands of duplex DNA was determined by sedimentation of the ^{3}H -DNA in an alkaline sucrose gradient (Figure 1). The DNA was composed of a heterogeneous population of molecules, ranging from about 3-19S, with approximately one-third of the molecules being greater than 10S. Attempts were not made to determine whether the longer molecules exclusively represented DNA complementary to the viral RNA.

Measurement of Newly-Synthesized Viral RNA. GR cells (± dexamethasone) were pulse labeled for 10-30 minutes with 750µCi/ml of ^{3}H -uridine (Amersham-Searle, 28 Ci/mM) in nucleoside-free medium 199 containing 10% dialyzed fetal calf serum. Cell RNA was extracted as described above. The specific activity of the RNAs in a 30' pulse ranged from 50-100 x 10^{4} cpm/µg. RNAs were then hybridized at 68°C in 0.6M NaCl for 20-60hr with excess amounts of MMTV "tailed duplex" DNA (described above). Approximately 1000cpm (0.1-0.2 ng) of ^{32}P MMTV 70S RNA were also included in the reaction in order to measure the efficiency of hybridization of RNA to "tailed duplex" DNA. At the end of the incubation, the reaction was diluted into 1 ml. of MUP buffer. The sample was then passed over a column of hydroxylapatite at either 40° or 60° C (see results). The column was washed three times with 1 ml. of MUP buffer. The load and first wash were each diluted with 2 ml. of water

and then counted in 12 ml. of PCS counting solution (Amersham-Searle). The bound material was eluted with 2 ml. of 0.4M phosphate buffer, diluted with an equal volume of 9M urea+ 2% SDS and again applied to the column. The column was washed 2-3 times with MUP buffer, and the bound material was collected by dissolving the hydroxylapatite in 20% TCA. Approximately 80μg of calf thymus DNA were added; acid precipitable material was collected on glass fiber filters and counted in Omnifluor (New England Nuclear). The counting efficiency in PCS was only 10-15% higher than on filters in Omnifluor therefore no corrections were made for the percentage of bound material. RNAs were tested for the presence of free isotope prior to the hybridizations (by comparing acid precipitable counts to total counts) and were found to be free of such in all cases.

Cytoplasmic-Nuclear Fractionation of GR cell RNA. Confluent cultures of GR cells ($\pm$ dexamethasone) were washed with Tris-glucose (Leong et. al., 1972) and then removed from the plates with Puck's: EDTA solution. The cells were washed twice with Tris-glucose and swollen in ice-cold 0.01X RSB (approximately 10^6 cells/ml.) for 5 minutes. Cells were disrupted with 10 strokes in a Dounce homogenizer; inspection by phase contrast microscopy showed that greater than 95% of the cells but less than 5% of the nuclei were broken. Nuclei were pelleted by centrifugation at 2000 rpm for 5 minutes in the Sorvall HB-4 rotor at 4^o C and washed once with 0.1X RSB and once with 0.1X RSB containing 0.5% DOC (deoxycholate) and 1% NP-40. The supernatants from each wash were added to the cytoplasmic fractions. RNA from the nuclear and cytoplasmic fractions was prepared as described above for whole cell RNA. The concentration of RNA in the nuclear preparation

was corrected for the presence of DNA oligonucleotides remaining after the DNAase treatment (as determined by a diphenylamine colorimetric assay (Dische, 1955). After correction, nuclear RNA constituted between 5-7% of whole cell RNA in both dexamethasone treated and untreated cells.

Preparation of Polysomes from GR Cells Confluent cultures of cells (approximately 10^8 cells) were incubated for 1-2 minutes with 200 μg/ml cycloheximide (Sigma) in Tris-glucose at 37° C. Cells were removed from the dish in the same solution and pelleted by centrifugation at 2000 rpm for 5 minutes in the HB-4 Sorvall rotor at 4°C. The cells were resuspended in 1 ml. of "M" buffer containing 200μg/ml heparin (Sigma) and Triton X-100 was added to a final concentration of 1%. Nuclei were removed (after 10 strokes with a Dounce homogenizer) by centrifugation at 2000 rpm as described above. 25mM EDTA was added to one-half of the cytoplasmic preparation (supernatant). Both EDTA and non-EDTA treated samples were layered directly on a 10-40% sucrose gradient (in "P" buffer containing 100μg/ml heparin) and spun for 2¼ hours at 27K rpm in the Spinco SW27.1 rotor at 4°C. Gradients were collected through a Gilford recording spectrophotometer and pools were made as shown in figure 11 of section 3. RNA was extracted from each pool by treatment with SDS(1%) and pronase (500μg/ml) for 1 hour at 37°C followed by a single phenol extraction at room temperature. After EtOH precipitation each RNA sample was resuspended in 75μl of TE buffer. This method is a modification of the one described by Vecchio et. al. (1973).

Oligo-dT Fractionation of GR cell RNA into Poly(A)+ and Poly(A)- RNA. GR cells were treated with 1% SDS and 500μg/ml Pronase in STE

for 60' at 37°C. The cell lysate was extracted twice with phenol (equilibrated at pH 7 with Tris) at room temperature. Nucleic acids were EtOH precipitated, treated with 1μg/ml of DNase in 20mM Tris (7.4) and 10mM $MgCl_2$, reextracted once with phenol and reprecipitated with EtOH. The RNA was resuspended in STE and then 100-300 μg were diluted in 1 ml of binding buffer (0.5M NaCl, 10mM Tris pH 7.4, 0.5% SDS). The solution was passed over a column of oligo-dT cellulose T_3 (Collaborating Research). RNA lacking sufficient poly(A) (less than 50 residues) to bind was collected in the loading buffer and in a 5 ml wash with binding buffer. Bound RNA was eluted with 3 ml of eluting buffer (10mM Tris pH 7.4, 0.5% SDS). The eluant was brought up to 0.5M NaCl and again applied to the oligo-dT column. Approximately 40% of the RNA which bound the first time, rebound. The final recovery of poly(A) containing RNA was between 2.2-2.6% of the cellular RNA. The poly(A)+ and poly(A)- RNAs were concentrated by EtOH precipitation and resuspended in small volumes of TE buffer.

SECTION I

INTRODUCTION

The limited production of mouse mammary tumor virus (MMTV) in tissue culture and the lack of a rapid biological assay have hindered the study of this virus (Nandi and McGrath, 1973). Recently, cell lines derived from mouse mammary tumors have been developed (Lasfargues et. al., 1972; Owens and Hackett, 1972; Parks and Scolnick, 1973), and techniques for obtaining MMTV from primary explants of virus-producing mammary tumors have been refined (Hilgers et. al., 1971; McGrath, 1971; Cardiff et. al., 1968). These MMTV-producing lines and cultures provide potentially useful systems in which to study the genetic and hormonal factors that influence expression of viral genes.

Here, I describe a new biochemical assay for MMTV which is more sensitive than any of the presently available techniques. I have used this assay to characterize MMTV production by cultured mouse cells and to investigate the roles of insulin and glucocorticoid hormones (Parks et. al., 1974; McGrath, 1971) in the regulation of MMTV gene expression. The assay is based on the hybridization of radioactively labeled cDNA (DNA complementary to the viral genome) with RNA extracted from virus released into tissue culture fluids. This approach has proven to be useful in the study of avian and other murine RNA tumor viruses as well and may be applicable to other viruses for which a convenient biological assay is not available.

RESULTS

Concentration of viral RNA in culture fluids of virus-producing

cells: Measurement by molecular hybridization. The concentration of virus-specific nucleotide sequences in cell RNA can be determined by examining the kinetics of hybridization between vast excesses of cell RNA and labeled complementary viral DNA (cDNA) (Leong et. al., 1972). When the hybridization data is plotted as a function of C_rt (concentration of RNA x time) (Birnstiel et. al., 1972), the difference between the rate of annealing of cDNA with cell RNA and the rate of annealing to purified viral 70S RNA is a direct measure of the viral RNA concentration. In analogy with this procedure, I have used the technique of DNA:RNA hybridization to estimate the amount of viral RNA present in the tissue culture fluids of virus-producing cells. RNA was extracted from high speed pellets of tissue culture fluids and hybridized with cDNA as described in Materials and Methods. Since the concentration of RNA is not known, the data (% hybridization) are analyzed as a function of the volume of tissue culture medium from which the RNA was derived (V_o) multiplied by the time of hybridization (t). This convention (V_ot) is analogous to the C_rt convention described above. Since the kinetics of hybridization of cDNA to viral RNA are known ($C_rt = 2 \times 10^{-2}$ mole-sec/liter, Leong et. al., 1972; Varmus et. al., 1973a), the concentration of viral RNA in samples of culture fluids can be computed from the volume of medium and time required to achieve annealing of cDNA. An initial comparison (Fig. 1) shows that medium from B77-ASV infected chick cells contains approximately 200 times as much viral RNA as medium from GR mouse cells producing MMTV (grown in the presence of 10μg/ml insulin). By calculating the concentration of viral RNA necessary to achieve 50% hybridization ($V_ot_{\frac{1}{2}}$) within a specified reaction time, the concentration

of viral RNA in the original sample can be deduced from the amount of medium required to achieve 50% hybridization.[1] By so doing I find that the B77-ASV infected chick cultures are producing 10^{10} virus particles/ml/24 hr and the GR cultures are producing 5×10^7 particles/ml/24 hr, assuming that each particle contains 10^7 daltons of RNA (Robinson et. al., 1967).

It is known that the kinetics of hybridization of DNA with RNA is dependent on the ratio of RNA to DNA (Kennell, 1971). The effect is unpredictable in our case since the cDNA is a heterogeneous representation of the viral genome (Gelb et. al., 1971; Garapin et. al., 1973; Hayward and Hanafusa, 1973). The $C_rt_{\frac{1}{2}}$ for viral 70S RNA has been determined under conditions of vast RNA excess (Leong et. al., 1972; Varmus et. al., 1973a). Calculating the amount of RNA present in the samples described above (Fig. 1) reveals that the hybridization reactions (performed for 20 hr with increasing amounts of RNA) were not performed in conditions of vast RNA excess. To see what effect this had on the observed $C_rt_{\frac{1}{2}}$ (or $V_ot_{\frac{1}{2}}$)hybridizations were performed under two sets of conditions: (1) Reactions in which the RNA/DNA mass ratio was high (ratios of 20:1 at the lowest value of C_rt and 40,000:1 at the highest value of C_rt). These reactions are necessarily completed within short (30-90 min) incubation times. (2) Reactions in which the RNA/DNA ratios range from 0.014 at the lowest C_rt to 140 at the highest C_rt (i.e., 250-400 times lower RNA/DNA ratio than the curves performed in vast RNA excess).

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[1] See Appendix A

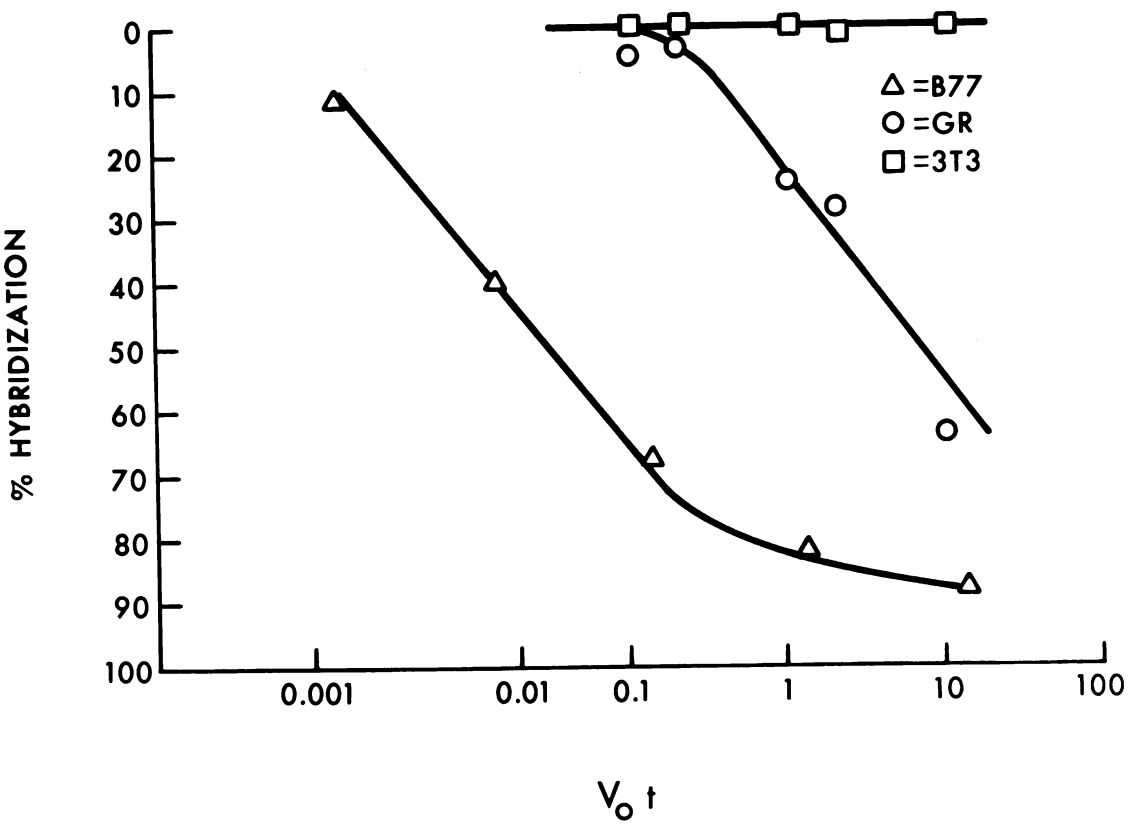


FIGURE I

Comparison of virus production by B77-ASV infected chick cells and MMTV-producing GR mouse cells. Approximately 500 cpm of B77 cDNA were incubated with decreasing amounts of viral RNA extracted from virus derived from 35 ml of infected chick cell culture fluids. MMTV cDNA (500 cpm) were incubated with decreasing amounts of viral RNA extracted from virus derived from 35 ml of GR medium or 3T3 (nonproducing) medium. Hybridizations were carried out in 10 μl reactions for 20 hr in 0.6 M NaCl 0.05 M Tris, pH 7.4, and 0.002 M EDTA at 68°C and assayed by resistance to S1 nuclease.

When B77-ASV cDNA is annealed with B77 70S RNA in conditions of limiting RNA the observed $C_rt_{\frac{1}{2}}$ is 6 x 10^{-2} molesec/liter (Fig. 2a). This is a threefold higher value than the $C_rt_{\frac{1}{2}}$ determined in vast RNA excess. The value of 10^{10} particles B77-ASV/ml/24 hr calculated from The reaction in Fig. 1 should therefore be corrected to 3 x 10^{10} particles/ml/24 hr. Unlike the results for B77-ASV, the kinetics of hybridization of MMTV cDNA to MMTV RNA (Fig. 2b) are affected only slightly by the RNA/DNA ratio. In a series of such reactions, I never observed an appreciable shift in the kinetics of hybridization. The MMTV particle counts determined from the curve in Fig. 1 and in subsequent experiments have therefore not been corrected. I cannot satisfactorily explain the differing effects of RNA/DNA ratio on the kinetics of hybridization of MMTV and B77 cDNA.

Specificity of the assay for viral RNA. A major problem associated with some of the procedures available for studying MMTV production is their lack of specificity. Mycoplasma, cell debris, and the simultaneous presence of two or more viruses are possible sources of artefact in assays for reverse transcriptase, density gradient purification of radioactively labeled virus, and electron microsopic analysis of MMTV-producing cells. The development of the radioimmune assay for viral protein depends on the availability of large amounts of purified virus and its specificity for MMTV is subject to various artefacts arising from impure or nonspecific antiserum.

The specificity of single-stranded DNA complementary to the genome of RNA tumor viruses has been previously documented (Bishop et. al., 1973; Varmus et. al., 1973a). To further substantiate this, I annealed B77-ASV, MMTV, and MSV cDNAs to heterologous and homologous RNAs. I

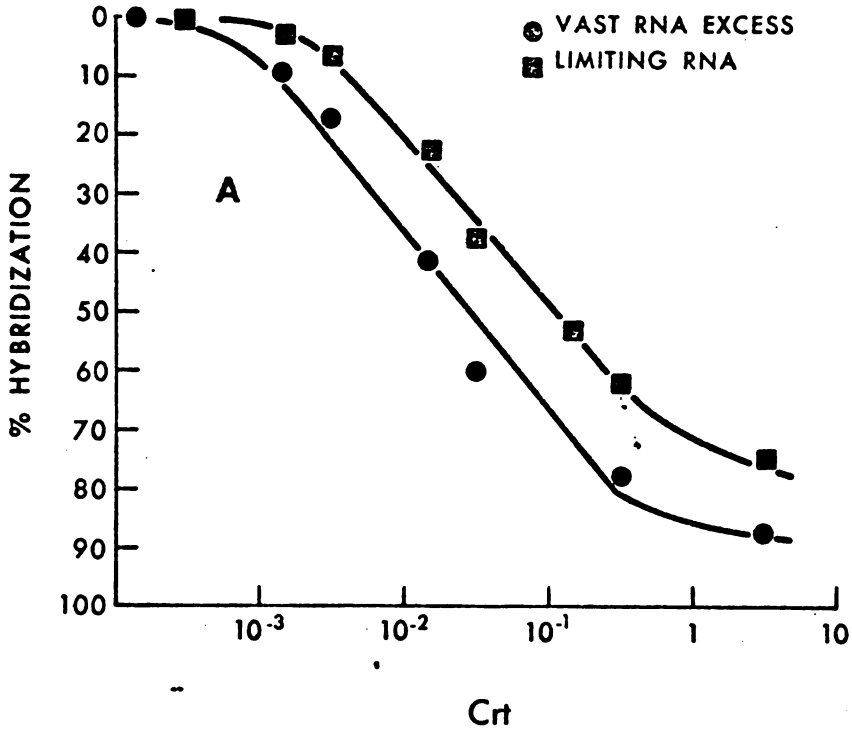
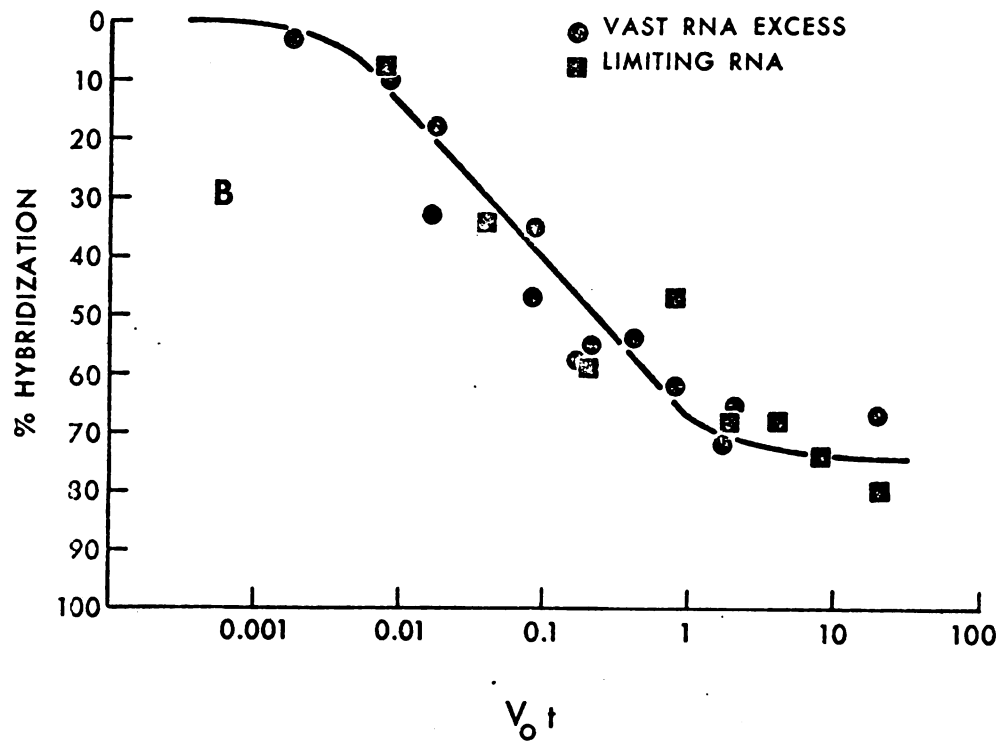


FIGURE 2

Effects of RNA/DNA ratio on the kinetics of hybridization of cDNA with viral RNA. (a) Hybridization of 1000 cpm of B77-ASV cDNA to purified B77 70S RNA under conditions of vast RNA excess (ratio at the lowest C_rt is 40,000:1) and under conditions of limiting RNA (ratios from 0.014 at the lowest C_rt to 140 at the highest C_rt). C_rt refers to the product of the viral RNA concentration and the time of hybridization. (b) Hybridization of 1000 cpm of MMTV cDNA with RNA extracted from 60 ml of 296 cell medium. Ractions were carried out in conditions of limiting and excess RNA as described above. Reactions performed in excess RNA were incubated for 30-70 min and reactions in limiting RNA were incubated for 60-72 hr. All hybridizations were performed at 68°C in annealing buffer and assayed by resistance to S1 nuclease.


VAST RNA EXCESS
LIMITING RNA
B
% HYBRIDIZATION
0
10
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0.001
0.01
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1
10
100
$V_o t$

found (Table 1) that there was no cross reaction among any of the cDNAs. In particular I noted no reaction of the MMTV cDNA with RNA

TABLE I

SPECIFICITY OF REVERSE TRANSCRIPTASE SINGLE STRANDED PRODUCT (cDNA)

RNA	cDNA: Maximum hybridization		
	GR-MMTV	Mo-MSV[d]	B77-ASV
GR-MMTV[a]	80%	0	0
JLS-V11[b]	0	50%	0
B77-ASV[c]	0	0	90%

[a]GR-MMTV = RNA from mouse mammary tumor virus produced by GR cells.

[b]JLS-V11 = RNA from mouse cells infected with the Moloney strain of murine leukemia virus.

[c]B77-ASV = RNA from B77 strain of avian sarcoma virus.

[d]Mo-MSV = Moloney strain of murine sarcoma virus.

from Moloney MuLV-infected JLSV cells, whereas 80% of the cDNA annealed with MMTV RNA. Likewise the Moloney MSV cDNA did not react with MMTV RNA but was capable of reacting with RNA from MuLV infected cells.

Only 50% of the MSV cDNA was capable of reacting with the JLS-V11 RNA, probably because the MuLV produced by the JLS-V11 cells has only partial homology with the MSV cDNA. Similar results have been reported by other investigators (Fan and Baltimore, 1973).

Sensitivity of the hybridization assay for viral RNA. I compared the sensitivity of the hybridization assay for viral RNA with assays for viral reverse transcriptase (Dickson, 1973; Fine et. al., 1974) and viral protein (Cardiff, 1973) the two most common biochemical methods to quantitate MMTV. Typical results for reverse transcriptase activity of B77 avian sarcoma virus and GR MMTV in a standard assay are shown in Table 2. Poly(rA):oligo(dT) was chosen

TABLE 2

REVERSE TRANSCRIPTASE ACTIVITY[a] OF MMTV FROM CULTURED GR MAMMARY TUMOR CELLS AND B77-ASV FROM CHICK EMBRYO CULTURES

Virus	Time of incubation (min)	Acid-insoluble counts of ^{3}H TTP
B77-ASV	0	300
(per 5 ml of medium)	30	98,500
	60	137,150
GR-MMTV	0	200
(per 35 ml of medium)	30	5800
	60	6200

[a]The reaction conditions are described in the Materials and Methods.

as the template:primer for the reaction since it confers greater sensitivity to the assay than does the more specific poly(rC):oligo(dG)

(Baltimore and Smoler, 1971; Fine et. al., 1974).[3] The relative senstivities of the assays for reverse transcriptase, viral RNA, and MMTV protein (Cardiff, 1973) are summarized in Table 3.

TABLE 3

SENSITIVITIES OF BIOCHEMICAL TECHNIQUES FOR DETECTION OF RNA TUMOR VIRUSES: MINIMUM NUMBER OF PARTICLES THAT CAN BE DETECTED

Virus assayed	Minimum number of particles detected		
	Biochemical test		RIP[b]
	V_0t[a]	Reverse transcriptase	
B77-ASV[c]	6×10^5	1.6×10^8	NC[c]
GR-MMTV	6×10^5	1.4×10^8	5×10^6

[a] V_0t = hybridization assay for viral RNA described in text.

b RIP = radioimmune precipitation assay for MMTV protein (from Cardiff, 1973).

[c]NC = not compared.

The number of particles (6×10^5) required to give a positive result in the hybridization assay is calculated from the point on the curve

[3]The conditions for the reverse transcriptase assay are comparable to those commonly reported by other investigators (Fine et. al., 1974). However, I point out that the sensitivity of the assay can be augmented approximately fivefold by raising the labeled precursor concentrations to very high levels (Kelloff et. al., 1972).

at which I observe 10% hybridization above background. A positive result in the assay for reverse transcriptase requires 1-2 x 10^8 particles and is defined as that point at which TCA insoluble counts are two times the background. The radioimmune precipitation assay can detect protein equivalent to 5 x 10^6 particles of MMTV (calculated from the published data of Cardiff, 1973), although the number of intact virus particles present may be lower if the samples contain incomplete virus particles or cross-reacting membrane fragments. From these calculations it is apparent that the hybridization assay for viral RNA is 100- to 1000-fold more sensitive than the assay for reverse transcriptase and approximately tenfold more sensitive than the radioimmune assay for viral protein. However, since I have arbitrarily set the level which defines a positive result for each of these assays, my computations could be altered by a factor of 2 or 3. For example, the sensitivity of the hybridization assay would increase approximately twofold by defining a positive result as 5% annealing (rather than 10%) above background.

Production and regulation of MMTV in cultured mouse cells. (a) Mouse cells of mammary origin. Using the hybridization assay for viral RNA I have studied cell cultures derived from spontaneous tumors of mice to determine which if any are suitable for future use. Since glucocorticoids and insulin have been shown to enhance MMTV production from cultured mammary tumor cells (McGrath, 1971; Parks et. al., 1974; Fine et. al., 1974; and following section) all cultures (except the 296 cells) were grown in 10 μg/ml dexamethasone plus 10 μg/ml insulin. The medium from the 296 cells kindly supplied by Dr. W. Parks was collected from cells grown in 1 μg/ml dexamethasone with no insulin.

Under these conditions, the 296 cell line derived from a spontaneous C3H mammary tumor (Owens and Hackett, 1972) is the best producer of MMTV (Fig. 3), releasing approximately 1-2 x 10^9 particles/ml/24 hr. The GR cell line derived from a spontaneous GR mammary tumor produces five- to tenfold less virus while short-term, high density explants of BALB/cf C3H mammary tumors (McGrath, 1971) and the TA3 mammary tumor ascites line (Keydar et. al., 1973) are producing even lower quantities of MMTV. The BALB/cf C3H primary explants are maintained at much higher cell density (about 10^6 cells/cm^2) than any of the continuous lines; thus, they produce the least MMTV per cell while the 296 cells remain the best producers (Table 4). However, these

TABLE 4

MMTV PRODUCTION BY MOUSE MAMMARY TUMOR CELLS AND ASV PRODUCTION BY B77 INFECTED CHICK CELLS

Cells	Particles/ ml/24 hr	Particles/ cell/24 hr[a]
296	1.5 x 10^9	500
GR	2 x 10^8	100
BALB/cf C3H explants	4 x 10^7	5
TA3	1 x 10^7	10
B77-infected chick cells	3 x 10^{10}	7500

[a]This is the average number of particles released per cell; virus production may vary from cell to cell (Parks and Scolnick, 1973).

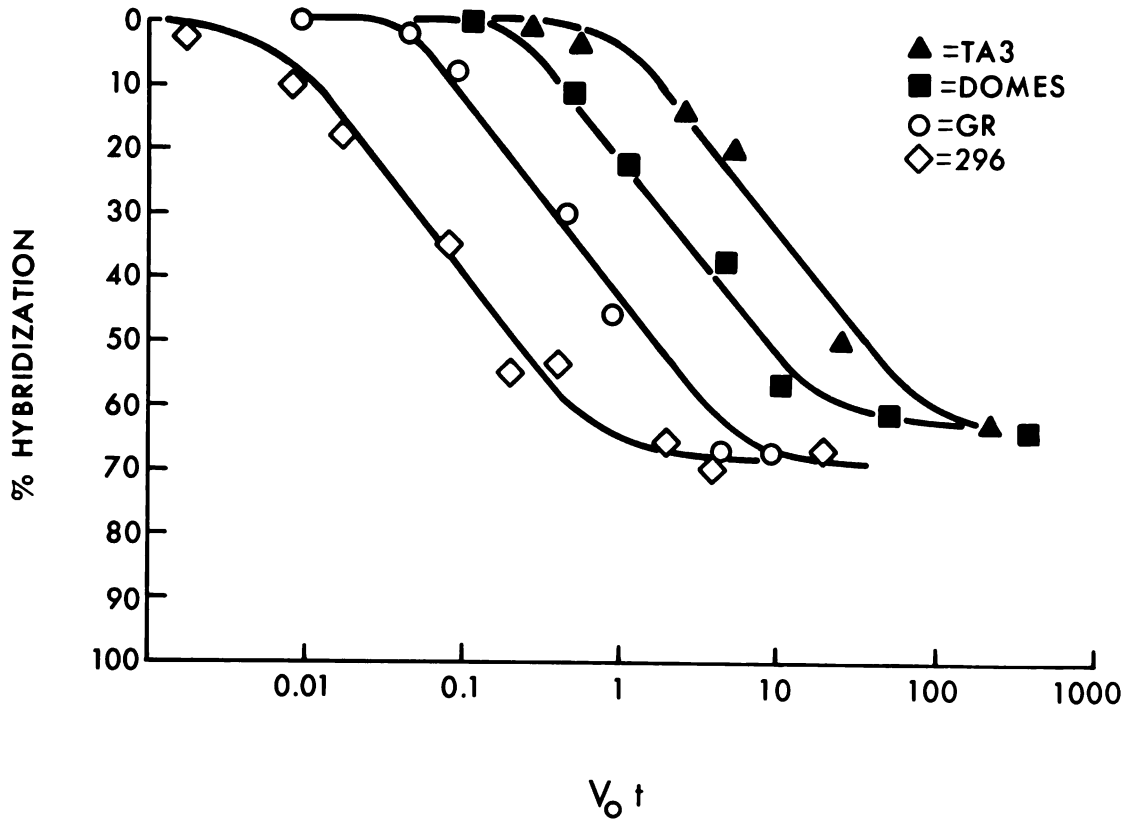


FIGURE 3

MMTV production by cultured mouse cells derived from mammary tumors. Dilutions of RNA extracted from virus pelleted out of tissue culture fluids of cells were hybridized with 1000 cpm of MMTV cDNA. Reactions were performed as described in the legend to Figure 1. "Domes" refers to the primary explants of BALB/cf C3H tumors.

differences may only reflect varying degrees of heterogeneity within each of the cell populations, since cloning continuous lines of MMTV producing cultures reveals large differences in virus production among the cells (Parks and Scolnick, 1973).

I further characterized these four sources of MMTV for production of C-type particles (MuLV/MSV) and found (Fig. 4) that the 296 and GR lines are not producing any detectable MuLV, while explants from BALB/cf C3H tumors produce very little C-type virus (at least 1000-fold less MuLV than MMTV). The TA3 cell line on the other hand is producing at least as much C-type virus as MMTV. However, it is difficult to quantitate the amount of MuLV produced since only 20% of the cDNA was capable of reacting with RNA from the medium of TA3 cell cultures. I ascribe this to the limited reaction of the cDNA with its complementary RNA (described in a previous section) and to the fact that the C-type virus being produced by the TA3 cells most likely has only partial homology with the Moloney virus from which the cDNA was derived.

Transcriptional controls have been implicated in the enhancement of MMTV production by glucocorticoids in tissue culture (Parks et. al., 1974). I asked whether the amounts of MMTV produced by the GR and TA3 cells and by BALB/cf C3H tumor explants reflect levels of virus-specific RNA in these cells. The results shown in Table 5 indicate that of these three, the cells with the highest concentration of viral RNA (GR cells) are the best producers of MMTV. Likewise, the lowest MMTV producing cells (the BALB/cf C3H cultures) contain the lowest concentration of virus specific RNA. The differences observed between the levels of virus production and levels of virus specific RNA are not

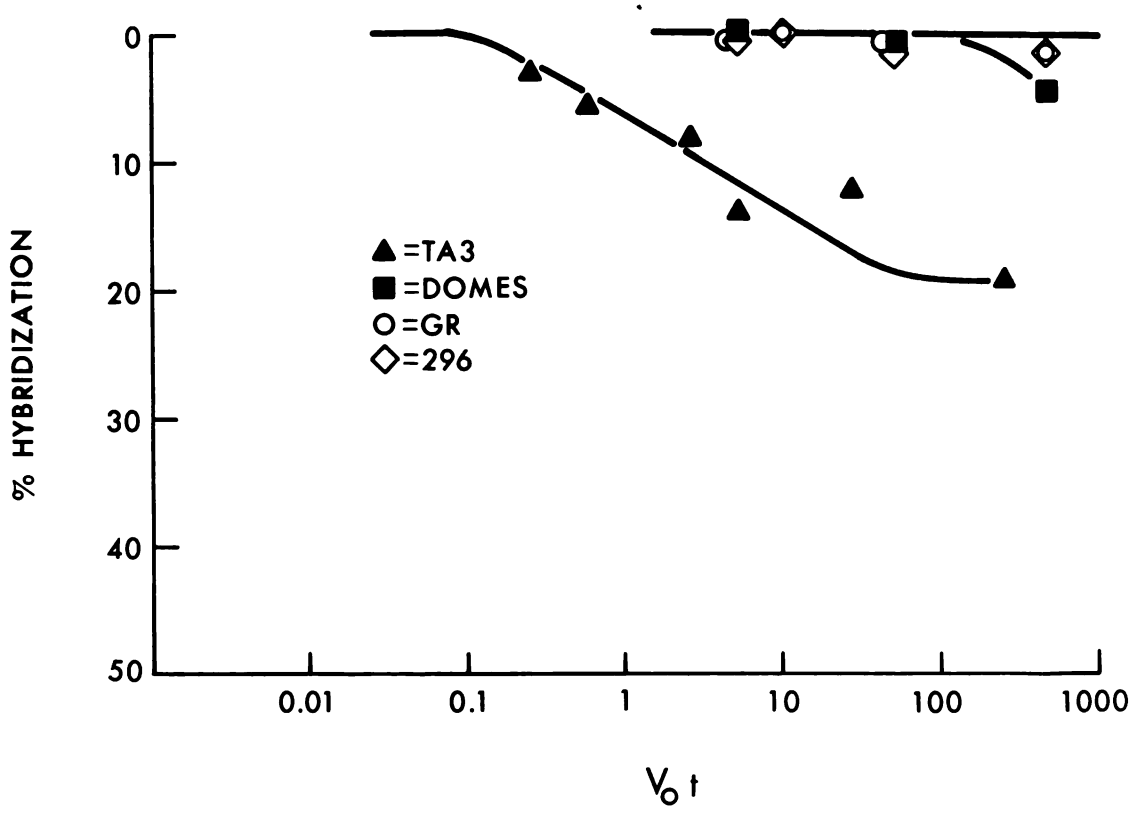


FIGURE 4

Production of C-type virus (MuLV/MSV) by mammary tumor cells of mice grown in tissue culture was determined by hybridizing 1000 cpm of Moloney MSV cDNA with RNA extracted from virus pelleted out of tissue culture fluids. Reactions were performed as described in the legend to Figure 1. "Domes" are described in the legend to Figure 3.

TABLE 5

SYNTHESIS OF MMTV RNA AND PRODUCTION OF MMTV BY CULTURED MOUSE CELLS

Cells	Particles/ ml/24 hr	$C_rt_{\frac{1}{2}}$[a]	MMTV RNA (% of cell RNA)
(A) Cells derived from mammary tumors			
GR	100	3	0.67
TA3	10	10	0.20
BALB/cf C3H explants	5	50	0.04
(B) Cells of non-mammary origin			
JLS-V11	0	-	0
LI	0	-	0
3T3	0	-	0
S49	0.5	10	0.20

[a] C_rt is the product of the concentration of cell RNA and the time of hybridization, expressed as mole-sec/liter.

always equal, however. The TA3 cells for example are producing ten times less virus than the GR cells yet they contain only three to four times less virus specific RNA. The mechanisms responsible for this discrepancy are not known.

(b) Mouse cells of nonmammary origin. Previous work has shown that mouse tissues other than mammary tissues contain MMTV RNA,

generally at very low levels (Varmus et. al., 1973a). I asked whether mouse cells in tissue culture derived from nonmammary tissue also transcribe MMTV genes. Three fibroblast cell lines, BALB 3T3 (Todaro and Green, 1963), "L" (Earle et. al., 1942), and JLS-V11 (Wright et. al., 1967) all of which contain large amounts of MMTV DNA (unpublished observations), have no detectable MMTV-specific RNA (Table 5). In contrast, the S49 cell line derived from a mineral oil-induced lymphoma (Horibata and Harris, 1970) in a BALB/c mouse contains large amounts of MMTV specific RNA (Fig. 5a). Since the level of virus-specific RNA in these cells is similar to the levels in the mammary tumor cultures I asked if virus was also produced in comparable amounts. I found (Fig. 5b) that the level of virus production in these cells is extremely low. The S49 cells produce at least 200-fold less MMTV than is produced by the GR cell line, yet they contain only three to four times less virus-specific RNA. Thus, the correlation between the amount of virus-specific RNA and level of virus production observed in mammary tumor cells appears not to be valid for all mouse cells.

Hormonal regulation of MMTV gene expression. Insulin and glucocorticoids have been implicated as modulators of MMTV production (McGrath, 1971; Parks et. al., 1974). The absence of C-type virus production and the advantages afforded by a continuous line make the GR cells a desirable system in which to study the effects of these hormones. Using the assay described above for viral RNA, I have found (Fig. 6) that removal of insulin from the growth medium (normally present at 10 μg/ml) resulted in a seven- to eightfold decrease in virus production without a concomitant decrease in cell number. In

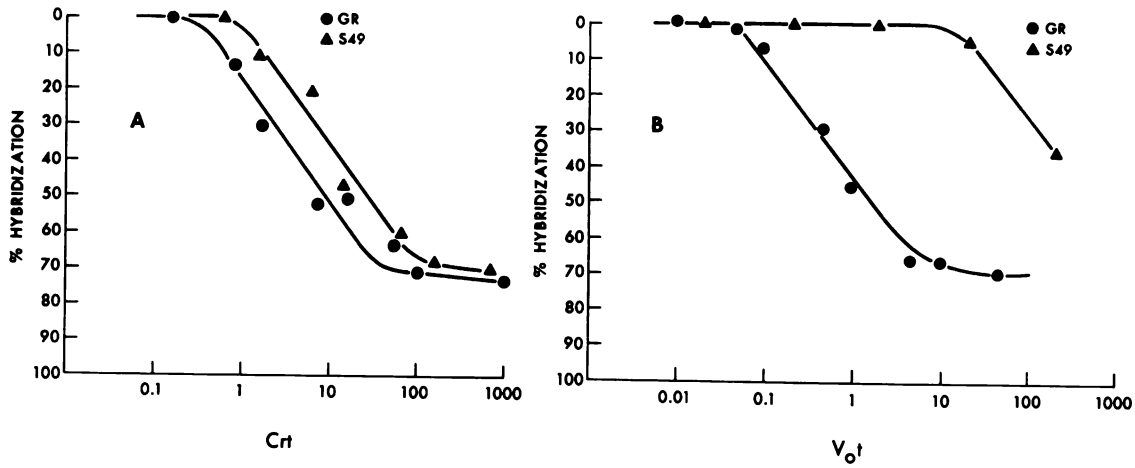


FIGURE 5

(a) Comparison of the intracellular concentration of MMTV specific RNA in GR mammary tumor cells with that of S49 lymphoma cells. Total cell RNA was extracted and dilutions were annealed with MMTV cDNA. C_rt refers to the product of the total RNA concentration and the time of hybridization. (b) Comparison of MMTV production by GR and S49 cells. RNA was extracted from virus pelleted out of the tissue culture fluids of these cells and dilutions were annealed with 1000 cpm of MMTV cDNA. Reactions were performed as described in Materials and Methods and in the legend to Fig. 1.

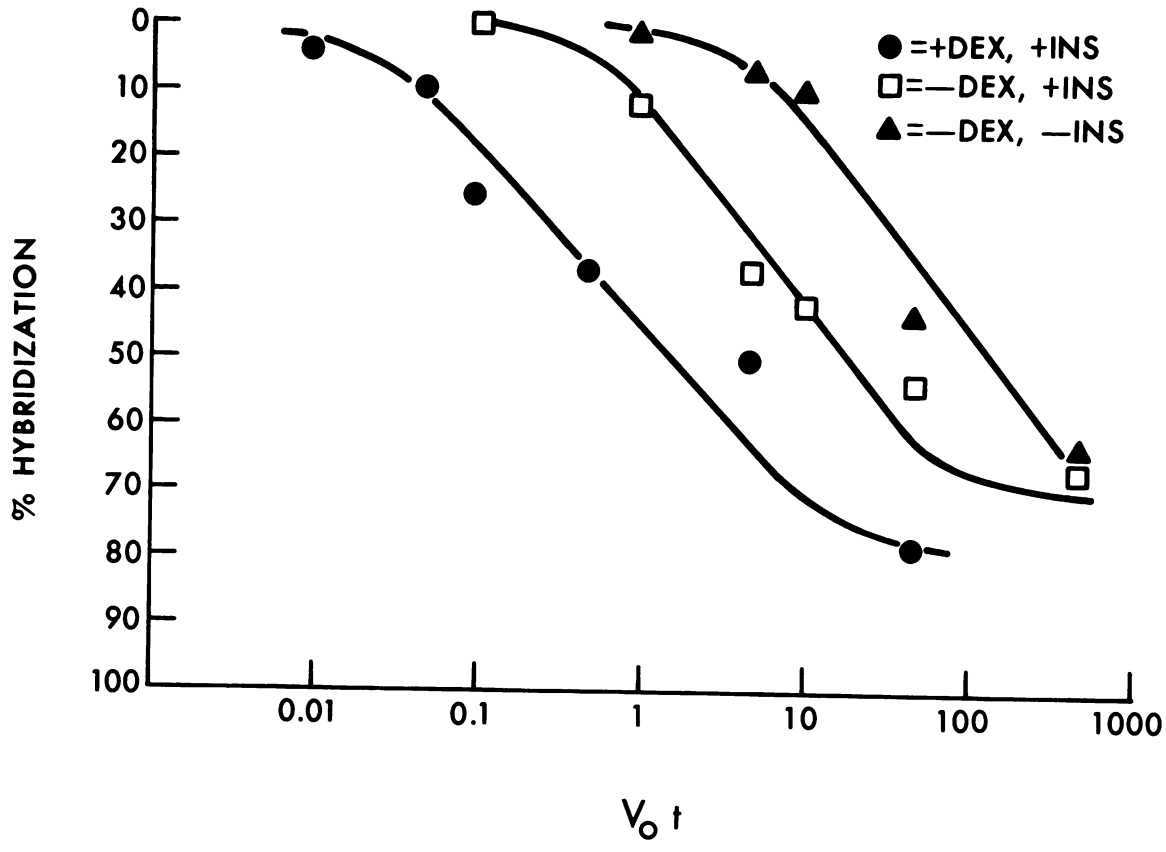


FIGURE 6

Effects of insulin and dexamethasone on MMTV production by GR cells. To determine the effect of insulin, cells were plated in the presence of the hormone. Two days after plating, half of the cells were maintained with insulin (10 μg/ml) and the other half grown in the absence of insulin. Virus production was measured in both groups 5 days later. The effect of dexamethasone was determined by adding the steroid (10 μg/ml) to cells grown with insulin (10 μg/ml) and assaying 48 hr later for the amount of MMTV produced. Assays were performed as described in the legend to Fig. 1.

the presence of insulin, the synthetic glucocorticoid, dexamethasone (10 μg/ml), produced a 5- to 20-fold increase in virus production which could be detected within 24 hr after addition of the hormone. Estradiol at similar doses (10 μg/ml) had no effect on MMTV production.

To determine whether the effect of dexamethasone was due to an increase in intra-cellular virus-specific RNA, I determined the concentration of MMTV RNA in control and dexamethasone-stimulated GR cells (Table 6). In this experiment, I observed a five- to sixfold

TABLE 6

STIMULATION OF MMTV PRODUCTION AND INTRACELLULAR VIRAL RNA CONCENTRATION BY DEXAMETHASONE IN THE GR CELL LINE

Treatment	$V_o t_{\frac{1}{2}}$	Particles/ cell/24 hr[c]	$C_r t_{\frac{1}{2}}$[b]
+Dex[a]	0.35	200	5
-Dex	1.80	35	30

[a]Dexamethasone was added to a concentration of 10 μg/ml.

[b]Virus production and virus specific RNA concentrations within the cells were assayed 40 hr after addition of the hormone.

[c]See footnote to Table 4.

stimulation of MMTV production accompanied by an identical increase in the concentration of virus-specific RNA within the dexamethasone

treated cells. Thus, the increase in virus production is probably consequent to increased synthesis of virus-specific RNA; this conclusion conforms to the general view that steroid hormones act to increase transcription from specific genes (O'Malley, 1974; Liarakos et. al., 1973).

DISCUSSION

Study of the regulation of production of mouse mammary tumor virus (MMTV) depends upon the availability of a reliable assay for virus production. Radioimmune assays for viral proteins (Cardiff, 1973; Verstraeten et. al., 1973) and assays for viral reverse transcriptase (Dickson, 1973) have recently been used for this purpose (Parks et. al., 1974; Fine et al., 1974). In this section I have described an alternative assay for MMTV based on the hybridization of radiolabeled cDNA with RNA extracted from MMTV present in the tissue culture fluids of producing cells. This assay has proven to be highly specific (Table 1) and more sensitive (Table 3) than the other available techniques for measuring MMTV. In addition, I have demonstrated that the hybridization assay can be applied to the study of other RNA tumor viruses (e.g., avian sarcoma viruses, murine leukemia-sarcoma viruses). Although it is not as sensitive as focus or plaque assays it does provide a rapid and reproducible method for viral identification and quantitation. For example, this assay has been of particular use to us in titering avian leukosis viruses and transformation-defective avian sarcoma viruses (unpublished observations of D. Stehelin), a task normally accomplished by technically demanding viral interference tests (Rubin, 1960). Similarly,

reasonable estimates of specific infectivity (particles/infectious unit) can be computed by comparing the number of particles determined by the hybridization assay with titers obtained by focus or plaque assays.

The major problem with the hybridization assay is that each cDNA probe must be standardized. Since many cDNAs generated by the endogenous polymerase reaction of RNA tumor viruses are heterogeneous representations of the genome (Varmus et. al., 1971, 1973a; Hayward and Hanafusa, 1973; Gelb et. al., 1971; Garapin et. al., 1973), the effects of the RNA to DNA ratio on the kinetics of annealing of cDNA with homologous RNA cannot be entirely predicted. Particle counts determined by molecular hybridization are dependent on the $C_rt_{\frac{1}{2}}$ for viral RNA; thus, it is essential that the $C_rt_{\frac{1}{2}}$ be determined under conditions of both limiting and excess RNA (see Fig. 2). If these controls are done, the assay, provides reasonable estimates of the amount of virus in any given sample.

I have used MMTV cDNA to investigate the mechanisms by which the expression of MMTV genes is regulated. By studying the concentrations of virus-specific RNA within mammary tumor cells from different strains of mice, I have found that the level of virus production generally reflects the level of virus-specific RNA within the cells. Cells with low amounts of virus-specific RNA (for example, BALB/cf C3H tumor explants) have correspondingly low levels of virus production. These results confirm observations made by Parks and Scolnick (1973) in their study of two clones of mammary tumor cells derived from the same parental population and suggest that the primary regulation of MMTV gene expression occurs at the level of transcription.

Susceptibility to mammary tumorigenesis in mice appears to be controlled by hormones and the genetic constitution of the mouse (Bentvelzen, 1970; Nandi and McGrath, 1973). Certain hormones, in particular insulin and glucocorticoids, have been implicated as possible regulators of MMTV gene expression (McGrath 1971; Parks et. al., 1974; Fine et. al., 1974). In the case of dexamethasone, a synthetic glucocorticoid, I provide further evidence that transcriptional controls might play a major role in regulating the expression of MMTV since increased virus production in dexamethasone treated cells is accompanied by a similar increase in the intracellular concentration of virus-specific RNA. These findings are consistent with those of Parks et. al. (1974) and suggest that glucocorticoids may act by altering rates of transcription from specific genes. An alternative explanation however is that glucocorticoids may act by inhibiting the breakdown of MMTV-specific RNA. Experiments described in the following sections will hopefully indicate which of these models is correct.

In contrast to mouse cells derived from mammary tumors, most cultured mouse cells do not produce MMTV. In three fibroblast lines we have studied, the inability to produce virus is very likely a result of transcriptional controls since the cells contain no MMTV-specific RNA. However, a lymphoma line (S49) (Horibata and Harris, 1970) produces very little MMTV, yet contains large amounts of MMTV RNA. This observations suggests that in these cells, factors other than the synthesis of viral RNA may play a significant role in regulating the production of MMTV.

SECTION 2

INTRODUCTION

Steroid hormones play an important role in regulating specific gene expression in metazoan organisms. It is commonly believed that the various steroids function via a unified mechanism (for review, see King and Mainwaring, 1974) in which the hormone diffuses into the target cell and becomes tightly associated with a soluble, steroid-specific receptor protein; the complex then binds to DNA-containing nuclear sites (Harris, 1971; Yamamoto and Alberts, 1972) to initiate the biological response. Thus, steroids may act as allosteric effectors, altering receptor properties to increase the affinity of the steroid-receptor complex for the nuclear binding sites (Jensen et. al., 1969; Samuels and Tomkins, 1970).

Although evidence consistent with this model has been reported, the initial molecular events subsequent to interaction of the steroid-receptor complex with the nuclear sites remain obscure. In no case has it been unambiguously shown that the first event detected biochemically actually represents the primary biological effect of the hormone. In fact, in several systems, there is evidence that the primary event is not being directly monitored. For example, the induction of specific proteins by glucocorticoids and estrogen is blocked by inhibitors of RNA synthesis (Peterkofsky and Tomkins, 1968; Gorski and Notides, 1969), and the estrogen-mediated induction of ovalbumin mRNA in the chick oviduct is interrupted, throughout the response, by inhibitors of protein synthesis (R. Schimke, personal communication; R. Palmiter, personal communication).

Cultured cells derived from mammary carcinomas of mice spontaneously

release mouse mammary tumor virus (MMTV), and RNA tumor virus which probably replicates via a DNA intermediate integrated into the chromosomal DNA of the host (see reviews by Nandi and McGrath, 1973 and Bentvelzen, 1974). Treatment of these cells with dexamethasone, a synthetic glucocorticoid, results in a 10-20 fold increase in the intracellular concentration of MMTV RNA (Parks et. al., 1974, 1975; Ringold et. al., 1975) and a coordinate rise in the production of mature virus particles (McGrath, 1971; Parks et. al., 1974, 1975; Ringold et al., 1975). In this section, I present evidence that the glucocorticoid induction of MMTV RNA is mediated by a specific receptor mechanism and that the accumulation of virus-specific RNA represents a primary hormone response. Thus, this system may be well-suited for detailed investigation of the mechanisms by which steroid hormones regulate the expression of specific genes.

RESULTS

Evidence for Receptor Involvement in MMTV Induction. Steroid-specific receptor proteins have been extensively characterized in many systems, and have been implicated in the mechanism of action of all steroid hormones (King and Mainwaring, 1974). As a preliminary test for the involvement of receptor proteins in MMTV RNA induction, several types of experiments were carried out.

Figure 7 shows sucrose gradient sedimentation of a ^{3}H dex-labeled extract of GR mammary tumor cells. A portion of the radioactivity is associated with a macromolecule which sediments at 4S. Only a limited amount of radioactivity can be specifically bound, and Figure 8 reveals that about 5×10^{-8}M ^{3}H dex is required for apparent half-saturation.

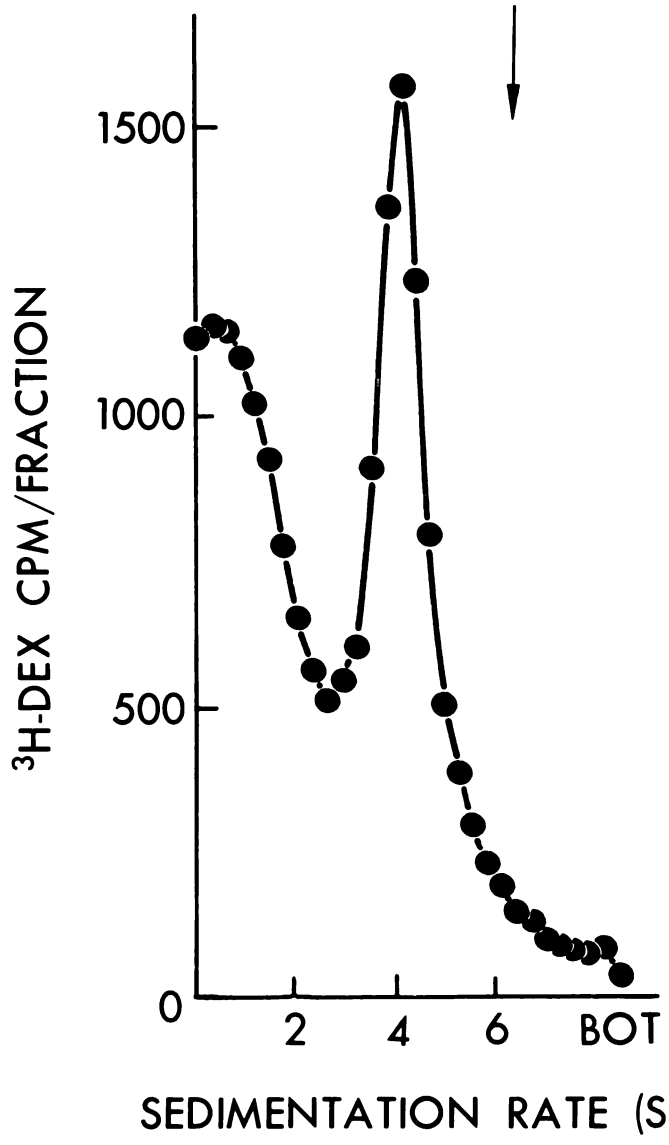


FIGURE 7

Rate zonal centrifugation of ^{3}H dexamethasone labeled GR cell extracts. Cell extracts prepared in TEGNO5 were incubated with 10^{-7}M ^{3}H dex. 90 min at 0°C; most of the unbound ^{3}H dex was then removed by filtration through Sephadex G25. The extracts were made 0.25M in NaCl (TEGN25 buffer) and a 100 µl aliquot was sedimented at 234,000 x g for 20 hr in a 5-20% sucrose gradient containing TEGN25. The arrow denotes the enzyme activity peak of a 6.2S internal standard (E. coli alkaline phosphatase). Radioactivity at the top of the gradient represents residual, unbound dex.

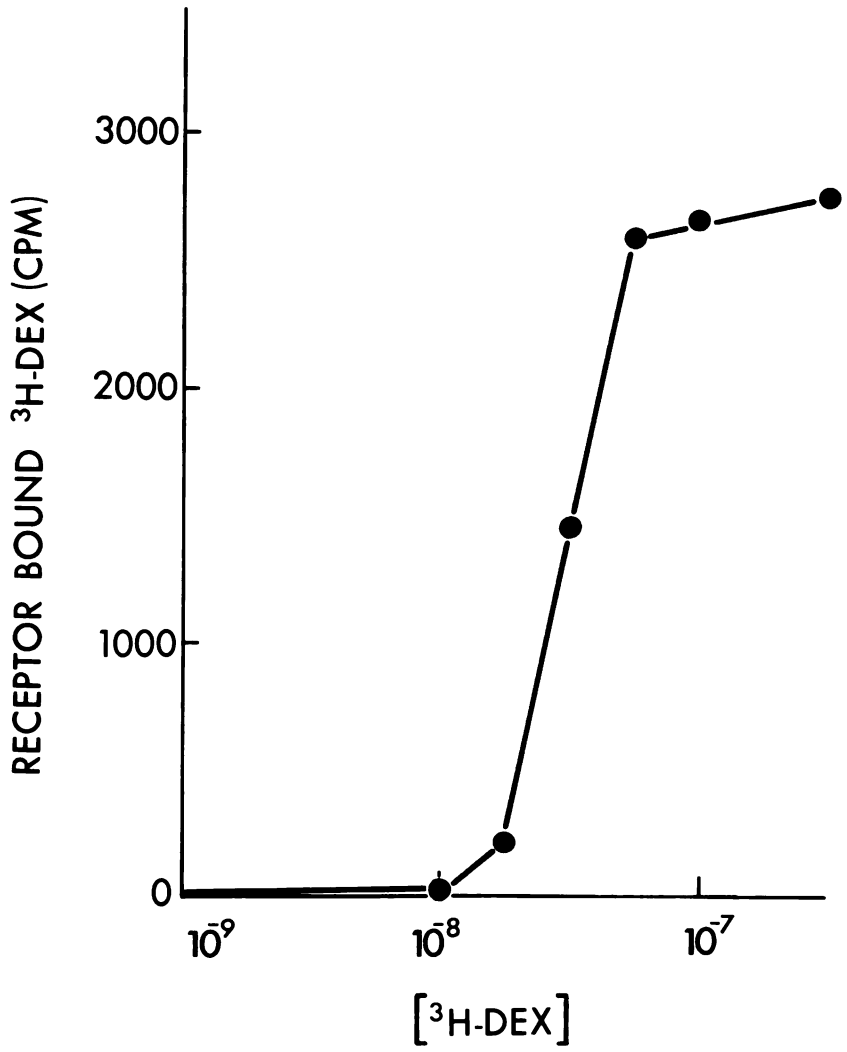


FIGURE 8

Binding of ^{3}H dex to macromolecules in GR cell extracts. Aliquots of an extract prepared in TEGNO5 were incubated with ^{3}H dex at the concentrations shown at 0°C for 90 min. In each case, a parallel aliquot was also incubated with ^{3}H dex, but in the presence of a 500-fold excess of unlabeled dex. Macromolecular-bound steroid was determined by collecting the flowthrough from Sephadex G-25 columns. Specifically-bound hormone is defined as the difference in ^{3}H dex cpm bound in the presence and absence of unlabeled dex, and represented 80-90% of the total bound radioactivity.

If the labeled extract is incubated at 20° for 35 min (conditions which make glucocorticoid receptors competent for nuclear and DNA-binding (Higgins et. al., 1973; Yamamoto et. al., 1974), then chromatographed on DNA-cellulose, 85% of the bound radioactivity absorbs to the column, and can be eluted by buffer containing 160 mM NaCl (Fig. 9). When the extract is kept at 0°, the amount of DNA binding is reduced by approximately ten-fold (data not shown). Each of these properties (sedimentation rate, apparent affinity for DNA, activation-dependent DNA-binding and relative DNA-binding affinity) is similar to the characteristics of a glucocorticoid receptor molecule from another line of cultured mouse cells, S49 (Horibata and Harris, 1970), in which the receptors have been genetically defined (Sibley and Tomkins, 1974; Yamamoto et. al., 1974).

I have measured the concentrations of dex required to augment production of MMTV and to effect an increase in the intracellular concentrations of viral RNA. Figure 10 shows that half-maximal induction is observed at 5-6 x 10^{-8}M dex, the same concentration at which half of the receptors bind the hormone (Fig. 8). Further evidence that the induction is mediated via a specific receptor is shown in Figure 11; a 500-fold excess of progesterone, a competitive inhibitor of glucocorticoid receptor-mediated responses (Rousseau et. al., 1972), completely abolishes the viral RNA accumulation induced by dex in GR cells. Moreover, the characteristics of the observed hormone response correlate well with those expected for a receptor-mediated function.

Induction of MMTV RNA Does Not Require DNA Synthesis. Owens et., al., (1973) have demonstrated that glucocorticoids play a complex role in the differentiation of secretory precursor cells in normal mammary glands.

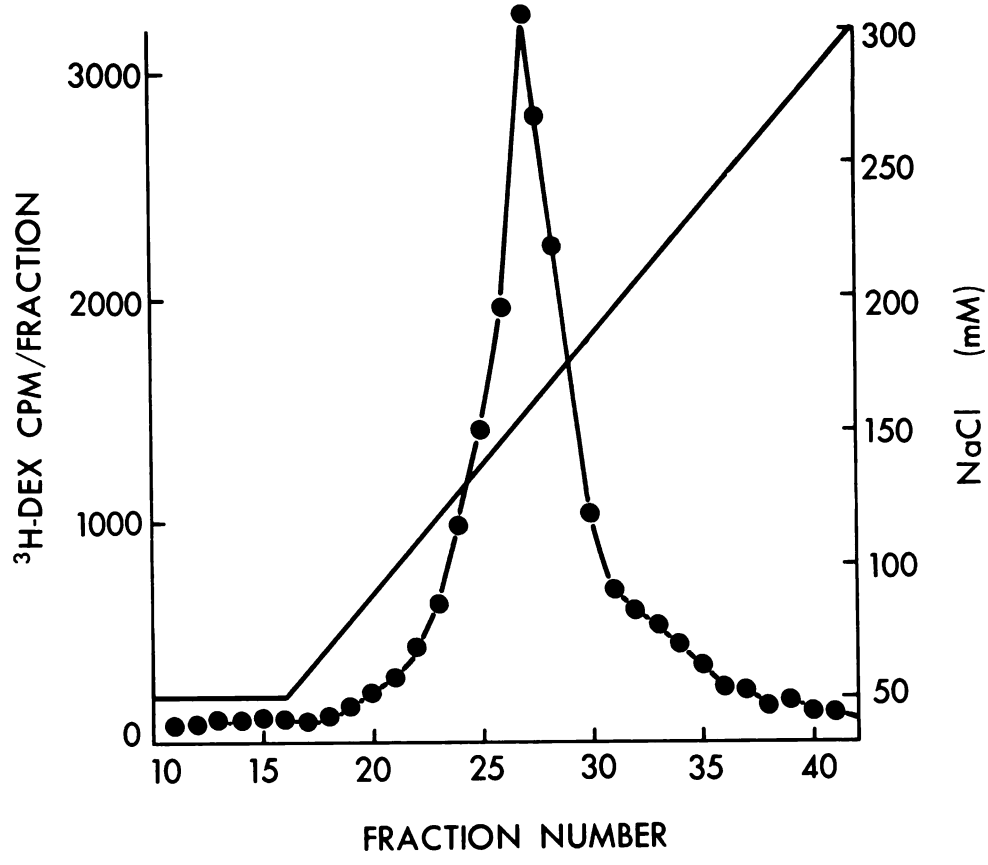


FIGURE 9

Elution of ^{3}H dex-labeled receptors from DNA-cellulose with a 50-250mM NaCl gradient. A labeled extract (0.5 ml), prepared in TEGN05, was incubated at 20° for 35 min, then returned to 0-4° and loaded onto a 1.0 ml DNA-cellulose column. After rinsing the column free of unbound material, adsorbed receptors were eluted with a linear NaCl gradient. Fractions were collected directly into counting vials; the NaCl gradient was monitored by measuring conductivity on a 5 µl aliquot of each fraction.

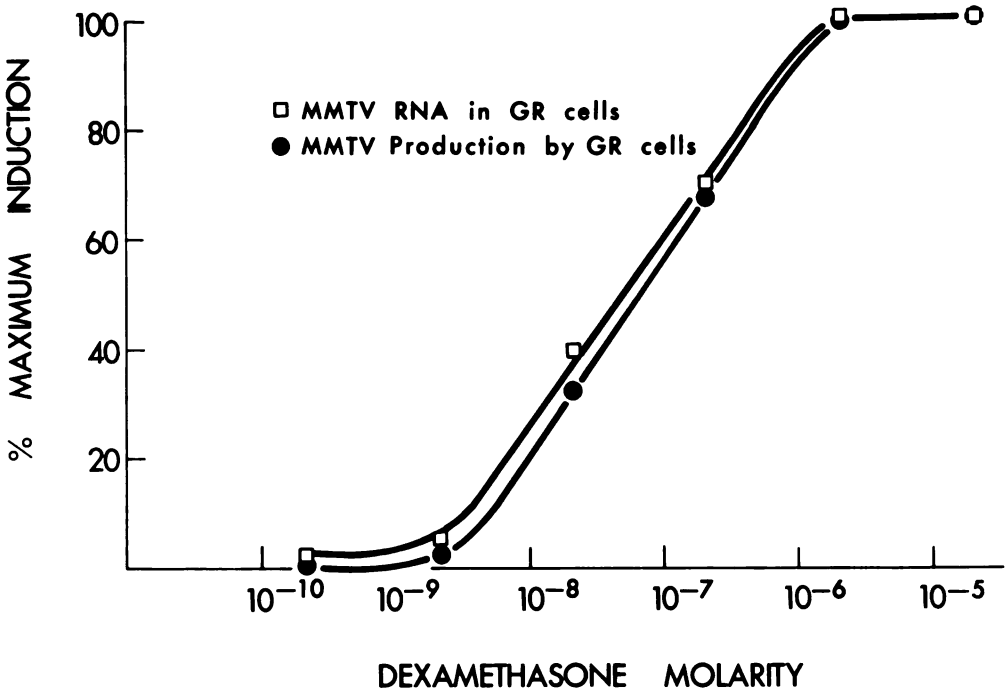


FIGURE 10

Effect of dexamethasone concentration on MMTV production and intracellular MMTV RNA in GR cells. The concentrations of viral RNA in culture fluid and intracellular viral RNA concentrations were determined by molecular hybridization as described in the Materials and Methods section. Annealings were performed in 0.6M NaCl at 68° for 15-20 hr. Cells were treated with dex for 48 hr.

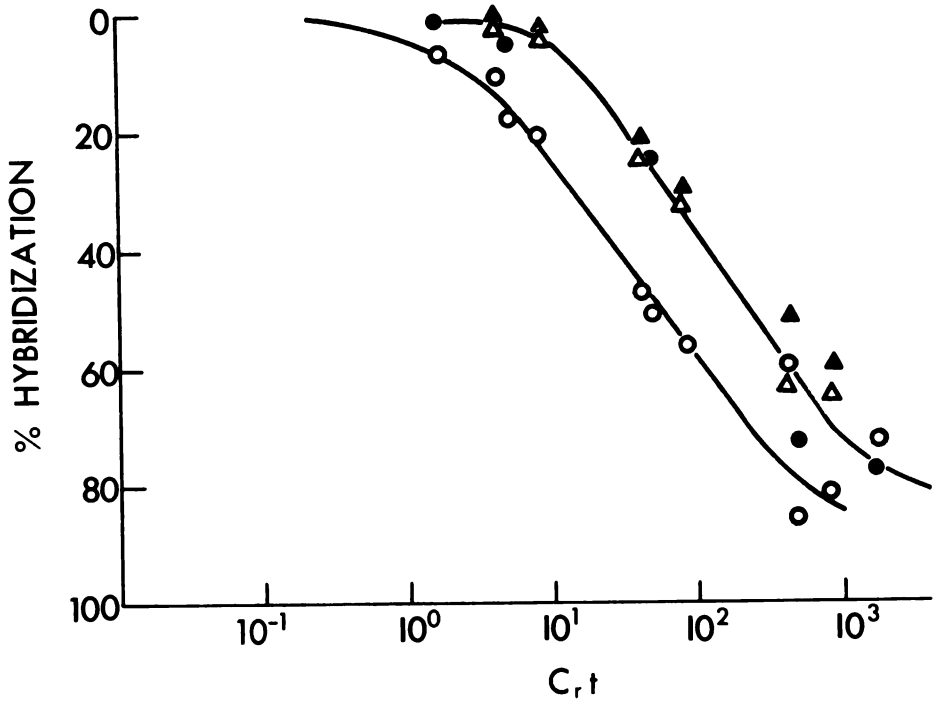


FIGURE 11

Progesterone inhibition of dexamethasone mediated induction of MMTV RNA. Approximately 1000 cpm of MMTV cDNA were annealed with increasing amounts of GR cell RNA for 16 hours at 68° in 0.6M NaCl. The experimental details are described in the Materials and Methods section.

(△) RNA from untreated cells.

(○) RNA from cells treated for 5 hours with 10^{-7}M dexamethasone.

(▲) RNA from cells treated with 5 x 10^{-5}M progesterone for 5 hours.

(●) RNA from cells treated with 10^{-7}M dexamethasone and 5 x 10^{-5}M progesterone.

Thus, it seemed conceivable that the molecular aspects of this mechanism might also be involved in the dex-mediated induction of viral RNA in GR cells. Since the differentiation event occurs only after a round of DNA replication, I examined the effects of cytosine arabinoside, a potent inhibitor of DNA synthesis, on MMTV RNA accumulation. When dex is added to cells that have been pretreated for one hour with 10^{-3}M cytosine arabinoside (sufficient to inhibit 93% of ^{3}H-thymidine incorporation into acid insoluble material), MMTV RNA accumulates to the same extent as in the control cultures (Table 7).

TABLE 7

Induction of MMTV RNA in the Absence of DNA Synthesis[a)]

Inhibitor	$C_r t$ (+Dex)[b)]	$C_r t$ (-Dex)	Relative increase in MMTV RNA concentration
None	30	200	6.7
araC (10^{-3}M)[a)]	30	200	6.7

a) Cells were treated with cytosine arabinoside (araC) for 1 hour prior to addition of dexamethasone.

b) Dexamethasone was added at a concentration of 10^{-7}M in control and araC treated cultures. MMTV RNA concentrations were measured, as described in the Materials and Methods, 4 hours after addition of dexamethasone.

Inhibitors of Protein Synthesis Do Not Affect MMTV Induction. The possibility that dex might act indirectly by inducing the synthesis of a protein, which in turn is responsible for mediating the accumulation

of MMTV RNA, was tested. Figure 12 shows that when GR cells are pre-treated for 1 hour with cycloheximide (2μg/ml; sufficient to inhibit ^{3}H amino acid incorporation by 90%), and are subsequently exposed to dex for 5 hours in the continued presence of the inhibitor, MMTV RNA accumulates as in the untreated cultures. Puromycin, another inhibitor of protein synthesis, is also unable to prevent the induction of MMTV RNA (Table 8). It has been reported that inhibitors of protein synthesis can by themselves induce the accumulation of certain C-type viral RNAs (Aaronson et. al., 1974); however, I found (experiment #1 of Table 8) that cycloheximide alone does not alter the basal levels of MMTV RNA in GR cells. Thus, these results suggest that the dex-induced accumulation of MMTV RNA is not dependent on protein synthesis.

Kinetics of MMTV RNA Induction. If dex directly alters the level of intracellular viral RNA, then the concentration of MMTV RNA should increase with little or no delay upon addition of the hormone. A study of the kinetics of RNA accumulation subsequent to treatment with saturating concentration ($10^{-5}M$) of dex, revealed that maximal induction is attained within 7 hr ($t_{\frac{1}{2}} \sim 2.5$ hr) (Fig. 13). In these experiments, the concentration of MMTV RNA in the cells doubled within 30 min after hormone treatment. Moreover, the apparent lag was quite short (15 min), and might be attributed to the time required for nuclear-localization of the hormone receptor complex, and to an inability to detect small increments in MMTV RNA concentrations at very early times. Thus, the rapid appearance of increased MMTV RNA concentrations provides further support for the idea that I am observing a primary glucocorticoid effect.

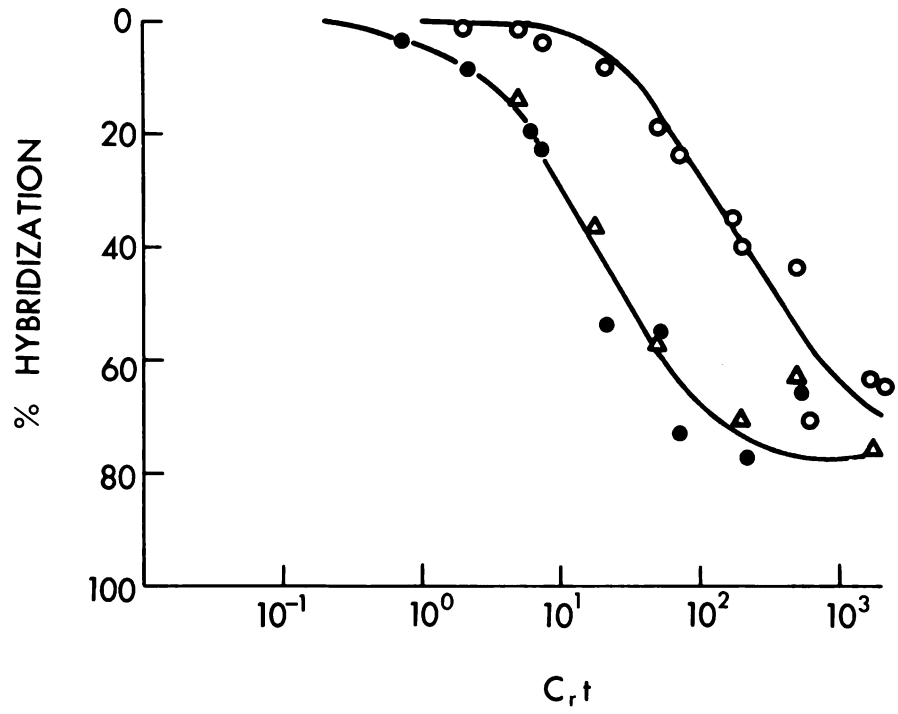


FIGURE 12

Induction of MMTV RNA in the presence of cycloheximide. Hybridizations were performed as described under Materials and Methods.

(O) RNA from untreated cells.

(●) RNA from cells treated for 5 hours with 10^{-5}M dexamethasone.

(Δ) RNA from cehls pretreated for 1 hour with 2 μg/ml cycloheximide, then exposed to 10^{-5}M dexamethasone (in the continued presence of cycloheximide) for 5 hours.

TABLE 8

Effect of Inhibitors of Protein Synthesis

on Induction of MMTV RNA

		C_rt (+Dex)	C_rt (-Dex)	Relative increase in MMTV RNA concentration	Inhibition of ^3H amino acid incorporation[c]
Expt.#1	None	10	120	12	0%
	Cycloheximide[a] (2 μg/ml)	10	120	12	90%
Expt.#2	None	20	200	10	0%
	Cycloheximide[b] (2 μg/ml)	20	N.T.[d]	10	88%
	Puromycin[b]	20	N.T.	10	75%

a) In experiment #1 cells were treated for 1 hour with cycloheximide prior to addition of dexamethasone and then incubated for 13 additional hours with cycloheximide.

b) In experiment #2 cells were pretreated for one hour with puromycin or cycloheximide then incubated for 5 hours after addition of dexamethasone.

c) Protein synthesis was measured 1 hour after addition of drugs by labeling cells for 30 minutes with 10 μCi/ml of ^3H amino acids and then counting acid precipitable material.

d) N.T. = not tested.

Actinomycin D Blocks the Effect of Dexamethasone. The accumulation of MMTV RNA in dexamethasone treated GR cells may be due to an increase

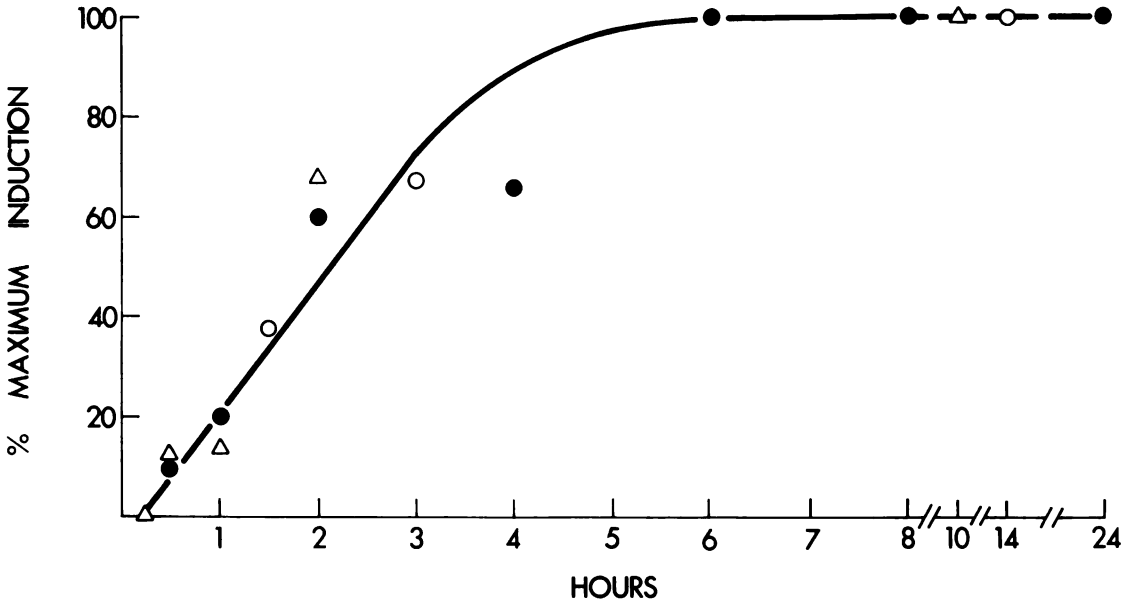


FIGURE 13

Kinetics of accumulation of MMTV RNA in GR cells after treatment with 10^{-5}M dexamethasone. Different symbols refer to separate experiments. Viral RNA concentrations were determined by annealing MMTV cDNA for 15-20 hours with increasing amounts of cell RNA, as described in Materials and Methods. In all of these experiments, MMTV RNA concentrations were increased 15-20 fold at maximal induction.

in its rate of synthesis, a decrease in its rate of degradation, or a combination of the two. As a preliminary test, the effects of actinomycin D (10μg/ml; sufficient to inhibit ^{3}H uridine incorporation by 98%) on the induction were tested. Figure 14 shows that a one hour pre-incubation of GR cells in actinomycin D, followed by a 4 hr exposure to dex in the continued presence of the inhibitor completely prevents the accumulation of MMTV RNA. In addition, the 5 hour exposure to actinomycin D effects at most a two-fold decrease in MMTV RNA concentrations in both dex-treated and untreated cells. I ascribe this to turnover of the viral RNA. Since the time required to see a two-fold increase in MMTV RNA concentrations after treatment with dex is only 30 minutes (Fig. 13), and the induction is dependent on RNA synthesis, it seems likely that the rate of synthesis of MMTV RNA is increased in dex treated GR cells. These experiments do not rule out the possibility that dex might also be altering the stability of MMTV RNA.

DISCUSSION

Steroid hormones have large and specific effects on the metabolism and differentiation of their target tissues; numerous biochemical observations (King and Mainwaring, 1974) as well as some recent genetic studies (Sibley and Tomkins, 1974; Yamamoto et. al., 1974) have implicated hormone-binding receptor proteins in steroid action. Because the ligand-receptor interaction promotes binding of the complex to nuclear sites, it is tempting to speculate that receptors may regulate gene expression at the level of transcription. However, due to the general complexity of eukaryotic cells, and their still poorly defined pathways

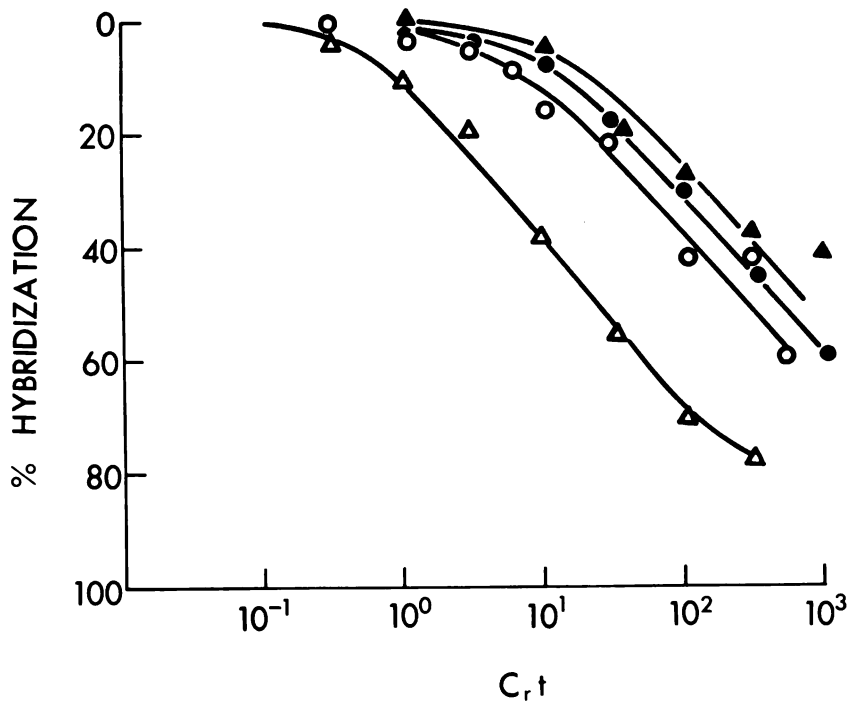


FIGURE 14

Inhibition of dexamethasone mediated accumulation of MMTV RNA by actinomycin D. Cells were pretreated for 1 hour with 10μg/ml of actinomycin D and then exposed for 4 hours to 10^{-5}M dexamethasone. Whole cell RNA was extracted and hybridized as described in Materials and Methods.

(O) RNA from untreated cells.

(Δ) RNA from cells exposed only to 10^{-5}M dexamethasone.

(▲) RNA from cells exposed only to 10μg/ml actinomycin D.

(●) RNA from cells pretreated for 1 hour with 10μg/ml actinomycin D and then exposed to 10^{-5}M dexamethasone (in the continued presence of actinomycin D).

of RNA metabolism, it has been difficult to approach this problem directly.

In this section I have presented data which suggest that the glucocorticoid effect on MMTV expression in GR mammary tumor cells may represent a system in which such studies are feasible. It has been shown that these cells (Ringold et. al., 1975) and other cell lines derived from mouse mammary tumors (Fine et. al., 1974; Parks et. al., 1974; Dickson et. al., 1975) spontaneously release MMTV and that the level of virus production can be augmented by treating the cells with dex. Moreover, the fact that an increase in intracellular concentration of virus-specific RNA is coordinate with the rise in virus production (Parks et. al., 1974, 1975; Ringold et. al., 1975) implies that the hormone acts at the level of RNA transcription or degradation, rather than in translation or virus assembly.

A number of indirect experiments suggest that the increased concentration of viral RNA is a primary effect of dex. Studies with inhibitors indicate that the response requires the synthesis of RNA, but not of DNA or protein. Furthermore, the RNA accumulation proceeds with only a 15 min lag after steroid addition. Thus, the induction of MMTV RNA differs from the estrogen-mediated accumulation of ovalbumin mRNA in the chick oviduct, a system in which the metabolism and translation of a specific mRNA has been well characterized (for review, see Palmiter, 1975). Even in early secondary estrogen stimulation of the oviduct, during which cellular differentiation and proliferation are minimal, ovalbumin mRNA has been reported to appear only after a lag of approximately 3 hours (Palmiter, 1975). Furthermore, the RNA increase is blocked at any stage of the response by inhibitors of protein

synthesis (Palmiter, personal communication).

The report of Young et. al. (1975) that the effect of dex on the production of MMTV is probably mediated by a specific and saturable glucocorticoid receptor has been confirmed. This receptor, identified by its ability to bind ^{3}H -dex, is similar in size, hormone affinity, and DNA-binding properties to the glucocorticoid receptors which have been characterized and defined genetically in another hormone responsive mouse cell line (Yamamoto et. al., 1974). In addition, a 500-fold excess of progesterone, a competitive inhibitor of glucocorticoid binding to receptor (Rousseau et. al., 1972), completely abolishes the dex induction of MMTV RNA.

Finally, the data are consistent with the idea that one of the effects of dex is to augment the rate of synthesis of MMTV RNA. Although the hormone may also alter the stability of MMTV RNA, the rate of the induction and the dependence on RNA synthesis suggest that stabilization of RNA alone cannot completely account for the observed accumulation of MMTV RNA. Definitive evidence that dex alters the rate of synthesis of MMTV RNA awaits the application of an assay for newly-synthesized viral RNA.

It should be emphasized that conclusions from studies utilizing metabolic inhibitors should be regarded as tentative, as the exact mechanisms and side-effects of these agents are not well understood. However, the results presented here, when taken together, suggest that GR cells are well suited for a detailed study of the mechanisms by which glucocorticoids regulate the expression of specific genes.

SECTION 3

INTRODUCTION

The results of the preceding sections suggest that the intracellular concentration of MMTV RNA is a limiting factor in the amount of MMTV produced by cultured cells derived from mammary tumors since there appears to be a good correlation between the levels of viral RNA in the cell and viral RNA released into the culture medium. Moreover, it is evident that the stimulation of virus production which occurs in cells treated with glucocorticoids is due to an accumulation of intracellular viral RNA. The hormone mediated increase in viral RNA concentration is dependent on RNA synthesis but does not require protein or DNA synthesis. The hormone response is presumably dependent on interaction of the steroid with a specific and saturable glucocorticoid receptor. Most importantly, since the accumulation of viral RNA occurs with little or no lag subsequent to addition of the hormone and is not dependent on protein synthesis it appears that this is a "primary" hormone response.

The following section presents several experiments designed to test whether the accumulation of viral RNA is due to an increase in its rate of synthesis, a decrease in its rate of degradation, or a combination of the two. In order to test these possibilities a new assay (developed by Dr. E. Stavnezer) was employed to measure the synthetic rate of MMTV RNA. The results indicate that the rate of synthesis of viral RNA is increased in hormone treated cells, but not sufficiently to account for the entire extent of the induction. Experiments which study the kinetics of degradation of steady state populations support the contention that the MMTV RNA present in hormone treated cells has

a substantially longer half-life than the viral RNA in untreated cells.

RESULTS

Assay for newly synthesized viral RNA. Two techniques for detecting newly synthesized, RNA tumor virus RNA have been described (Coffin et. al., 1974; Jacquet et. al., 1974). Both are based on the ability of ribonucleases T1 and A to degrade RNA which has not hybridized to viral DNA. The technique described by Jacquet et. al. suffers from the need to also treat the hybrids with RNase H, an enzyme which specifically degrades the RNA moiety of an RNA-DNA hybrid (Hausen and Stein, 1970). Thus, virus-specific RNA is defined as the number of counts which are sensitive to RNase T1 and A but not RNase H. The technique described by Coffin et. al. is technically demanding since the viral DNA must be extended at its 3' terminus with 40-60 dCMP residues. This "tail" serves as an affinity label allowing the DNA to be retained on a poly (I) - sephadex column. The procedures described by both of these groups are necessary since the background level of RNase A + T1 resistant RNA is too high (approximately 1% of total cell RNA) to allow detection of virus specific RNA. Since both of these techniques require the use of enzymes which are not readily available (RNase H and terminal deoxynucleotidyl transferase) I have used an assay developed in Dr. J. M. Bishop's laboratory by Dr. E. Stavnezer.

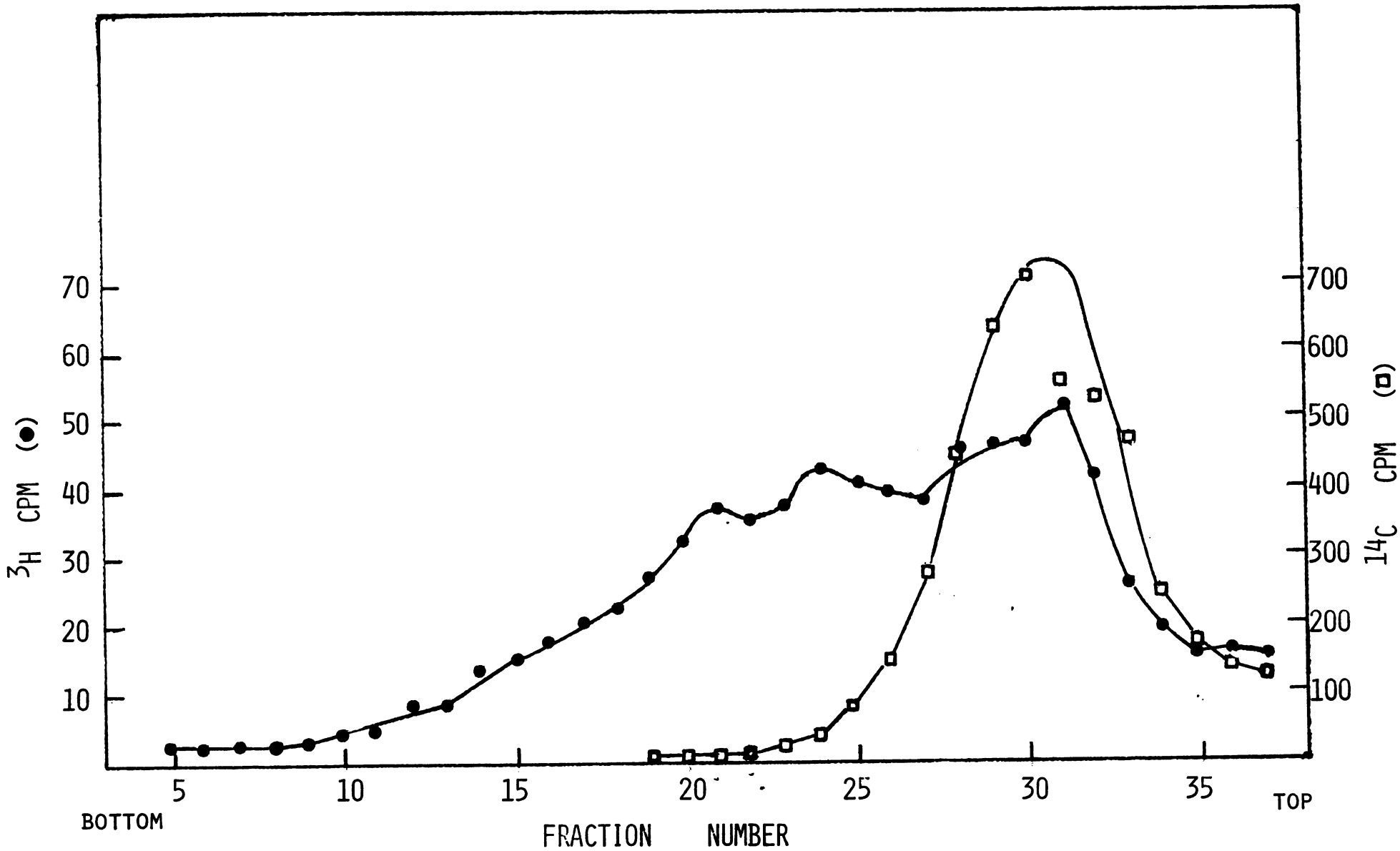
Smith et. al. (1974) described an assay which would separate DNA·RNA hybrids from RNA. They claimed that DNA·RNA hybrids, but not RNA alone, would be retained (as is double stranded DNA) on hydroxylapatite (HAP) columns at 40°C in a buffer containing 8M urea, 1% SDS, and 0.2M Na-phosphate (MUP buffer). However, on initial attempts to reproduce

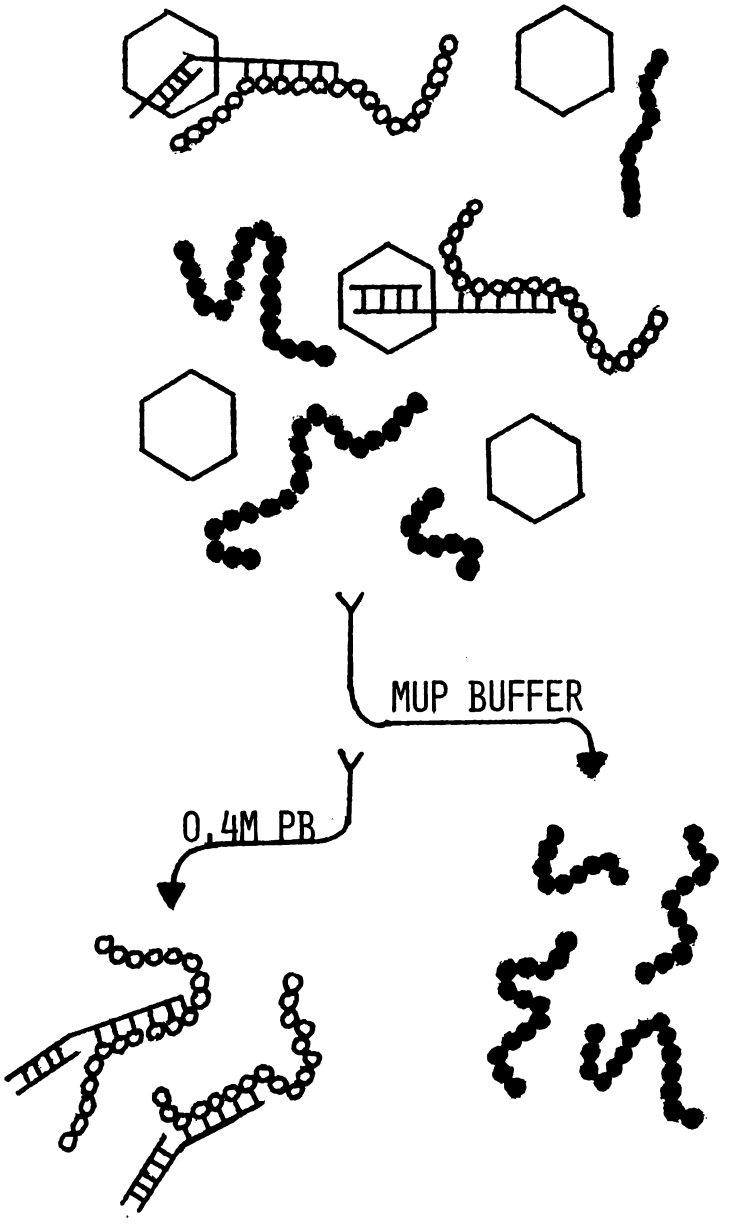
the properties of the column, Dr. Stavnazer (manuscript in prepartion) found that perfect DNA·RNA hybrids did not bind under the published procedures. However if there was a small region of double stranded DNA in the hybrid structure, the entire complex would be retained on the columns. He was subsequently able to adapt this assay to the study of avian tumor virus RNA synthesis. I have used the assay to study rates of MMTV RNA synthesis. The basis of the assay depends on the fact that viral DNA synthesized by the endogenous polymerase of detergent-activated virions (both in the presence and absence of actinomycin D) includes partial duplexes of DNA with double stranded regions and "tails" complementary to the viral genome (N. Quintrell unpublished observations). Thus when viral RNA is annealed to this undenatured DNA it can be retained on hydroxylapatite in MUP buffer. Figure 16 illustrates the properties of the assay.

In order for the assay to be sensitive, MMTV RNA should be able to hybridize efficiently to the "tailed" cDNA under MUP conditions. When increasing amounts of viral DNA are annealed to a small amount of ^{32}P MMTV 70S RNA an increasing amount of the RNA is retained on the columns, reaching a maximum of approximately 78% (Figure 17). The ratio of DNA to RNA required for maximum hybridization is approximately 50:1. Since 80-85% of the duplex DNA is resistant to the single-strand specific nuclease S1, only 15-20% of the mass of the DNA is participating in the reaction; thus the effective DNA/RNA ratio required to bind 75-80% of the ^{32}P 70S RNA is approximately 7:1. Therefore the single-stranded regions of the MMTV "tailed-duplex" DNA represent a relatively homogeneous transcript of the entire MMTV genome. This is further documented by the observation that under identical conditions

FIGURE 15

Size of MMTV "tailed-duplex" DNA in alkaline sucrose gradients. DNA was synthesized and purified as described in the experimental methods section. Approximately 1000 cpm of ^{3}H DNA (190 cpm/ng) and 6000 cpm of ^{14}C rat 5S DNA (prepared by J. Stavnezer; Stavnezer et. al., 1974) were incubated for 20' at 37° in 0.1 N NaOH, then run on a 5-20% sucrose gradient containing 0.1N NaOH, 0.9 M NaCl, and 10mM EDTA. After centrifugation at 64K rpm for 3 hours at 20°C, 5 drop fractions were collected, neutralized to pH 7, and counted in PCS counting solution.


70
60
50
40
30
20
10
^3H CPM (●)
700
600
500
400
300
200
100
^{14}C CPM (□)
5
10
15
20
25
30
35
BOTTOM
TOP
FRACTION NUMBER



MUP ASSAY FOR MMTV RNA

FIGURE 16

MUP= 0.2M PB, 8M UREA, 1% SDS

= HAP CRYSTAL

= MMTV DNA

=MMTV RNA

=NON-MMTV RNA

=PAIRED BASES

for annealing, 100% of the viral RNA can be rendered resistant to pancreatic RNase (see Appendix B).

Non-specific binding of RNA to MUP columns. Since the percentage of MMTV RNA in GR cells ranges from approximately 0.02% (-dex) to 0.6% (+dex) of cellular RNA it is necessary that the assay for pulse-labeled viral RNA have a very low background. I encountered two problems in the development of a "low-background" assay. (1) Initial experiments showed that 0.35-0.40% of GR cell ^{3}H RNA would bind to HAP under MUP conditions after incubation in the absence of cDNA. This background appears to be non-specific since only 0.5% of the bound material was capable of rebinding to HAP (Table 9).

TABLE 9

NON-SPECIFIC BINDING OF RNA TO HAP COLUMNS

Passage #	Input cpm*	Bound cpm	% Bound
1	968,000	3710	0.384%
2	3200	16	0.500%
total	968,000	16	0.0017%

*RNA from B77/3T3 cells labeled for 2 hours with 750 μCi/ml was passaged over columns of hydroxylapatite in MUP buffer as described in the experimental methods section. The RNA was not subjected to annealing conditions or exposed to MMTV DNA.

I encountered a more serious problem when I tested the ability of MMTV DNA to hybridize ^{3}H RNA from mouse cells free of MMTV RNA. For this, I employed RNA from B77-ASV transformed mouse cells (B77/3T3)

previously shown not to contain MMTV RNA sequences by annealing of labeled cDNA to unlabeled RNA (Varmus et. al., 1973a). Under standard MUP conditions (40°C) approximately 0.4% of ^{3}H B77/3T3 RNA was retained on hydroxylapatite columns after two passages. This background level was clearly unacceptable; thus, attempts were made to define the cellular RNA which bound to MMTV cDNA and to reduce its level of binding. RNA from a non-murine source was not tested; therefore I don't know whether the background annealing is species specific.

Reduction of background levels of binding. It is known that RNA tumor virus virions contain several species of low molecular weight RNA (mostly tRNA) which are cellular in origin (see review by Bishop and Varmus, 1975). If these species are represented in the cDNA then it is conceivable that they would bind. This is not the case, however, since all size classes of B77/3T3 RNA (separated in a sucrose gradient, Figure 18) were bound to MMTV cDNA and were retained on "MUP" columns (Table 10). In fact the highest percentage of binding (0.67%) was found with the largest (>30S) RNA. Although the precise nature of this RNA is unclear it appears to be poorly base paired with the MMTV cDNA. Thermal denaturation studies of this RNA (Figure 19) demonstrate that the Tm of the cell RNA·MMTV DNA hybrid is approximately 22° lower than the hybrid between MMTV 70S RNA and MMTV DNA. Thus, performing the "MUP" assay at 60° rather than 40° reduces the background 5-10 fold without significantly (~10%) lowering the efficiency of hybridization to viral RNA. Another approach to reducing background binding in many types of hybridizations is to saturate the non-specific sites with a large amount of unlabeled "competitor" RNA. In this case I used

FIGURE 17

Hybridization of MMTV RNA to "tailed duplex" DNA. ^{32}P MMTV 70S RNA (1000 cpm; approximately 0.16 ng) was annealed with increasing amounts (0.7-21 ng) of unlabeled DNA synthesized by MMTV polymerase. The percent of RNA annealed was determined by its retention on hydrosylapatite columns in MUP buffer as described in the methods section. Hybridization was at 68°C for 64 hours in 0.6M NaCl in a reaction volume of 5 μl.

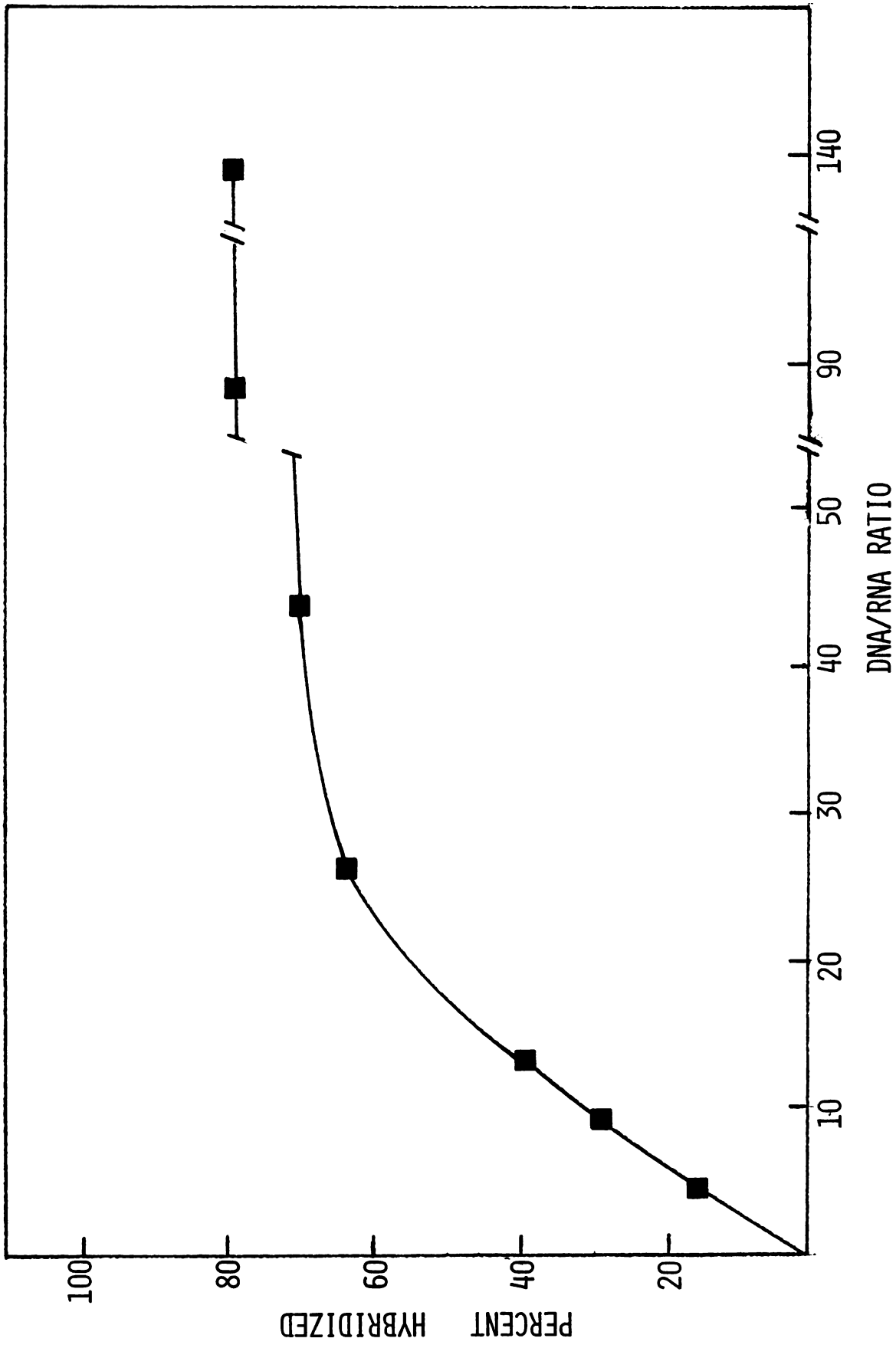

PERCENT HYBRIDIZED
100
80
60
40
20
10
20
30
40
50
90
140
DNA/RNA RATIO

FIGURE 18

Sucrose gradient centrifugation of B77/3T3 cell RNA. RNA from B77/3T3 mouse cells was sedimented in a 15-30% sucrose gradient at 64K rpm and 4°C in the SW 65 Spinco rotor. Twenty-one fractions were collected and a portion of each was counted in PCS. RNAs were pooled for hybridization to MMTV DNA (Table 20).

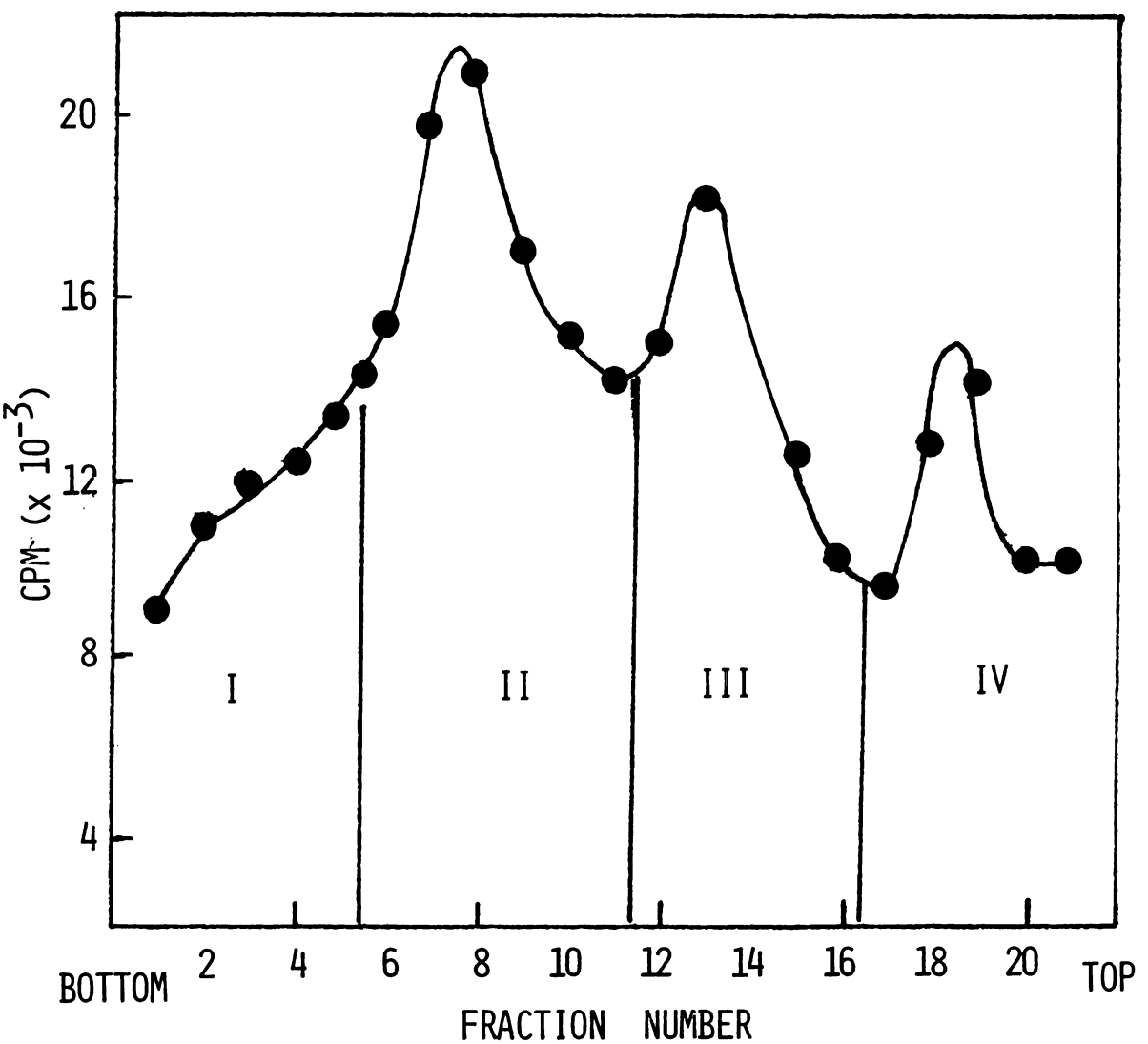

20
16
12
8
4
CPM (x 10^{-3})
I
II
III
IV
BOTTOM
2
4
6
8
10
12
14
16
18
20
TOP
FRACTION NUMBER

FIGURE 19

Thermal denaturation of MMTV DNA-RNA and MMTV DNA - B77/3T3 hybrids. Approximately 240,000 cpm ^{3}H 3T3-B77 RNA and 11,500 cpm of ^{32}P MMTV 70S RNA were hybridized with 40 ng of MMTV cDNA. The hybridization reaction was diluted into 1 ml MUP buffer and loaded onto a hydroxylapatite column at 40°C. The temperature was raised in 5° increments and maintained at each temperature for 10-15 minutes. At each step, the unbound material was collected in two 1 ml washes of the column. A third wash was discarded. Plotted are the percentage of total counts eluted from the column between 45 and 80°C. ^{3}H total counts were $\sim$ 3700 cpm and ^{32}P total counts were $\sim$ 8000.

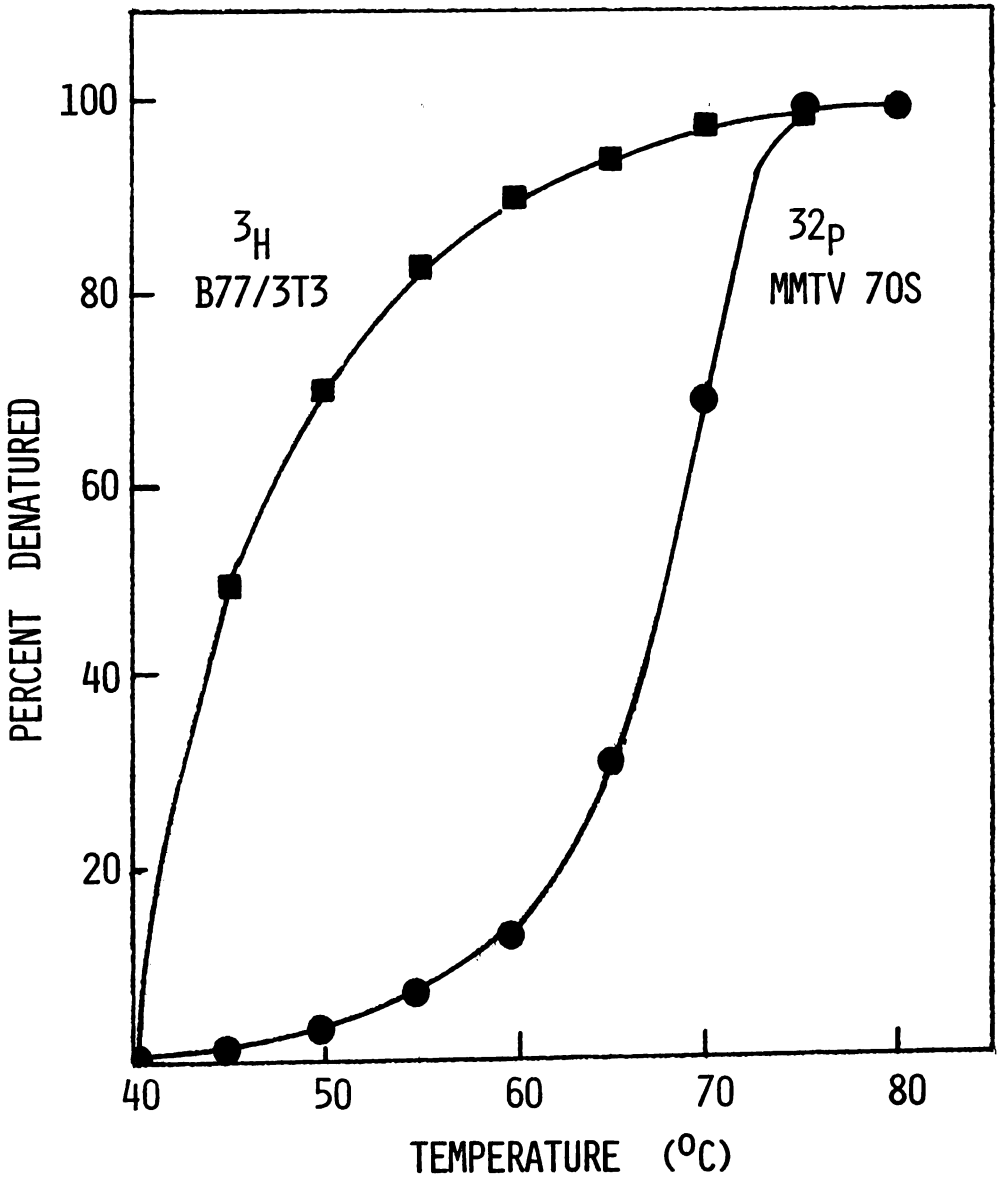

100
80
60
40
20
PERCENT DENATURED
^{3}H
B77/3T3
^{32}P
MMTV 70S
40
50
60
70
80
TEMPERATURE (^{o}C)

TABLE 10

SIZE OF UNINFECTED MOUSE CELL RNA WHICH HYBRIDIZES TO MMTV cDNA

	^{3}H combound / ^{3}H total cpm	% ^{3}H bound	% ^{32}P bound
Pool I (>30S)	462/73300	0.63	71
Pool II (20-30S)	208/56850	0.37	70
Pool III (10-20S)	297-60550	0.49	68
Pool IV (<10S)	228/79200	0.29	73
Pool II (-cDNA)*	12/52000	0.02	1

Pools of RNA from the gradient in Figure 18 were made as shown. Hybridization was performed in a volume of 10 μl for 52 hours. Approximately 25 ng of cDNA were used in each reaction. ^{32}P MMTV 70S RNA (0.15 ng) was included to monitor the efficiency of hybridization and the retention of RNA on hydroxylapatite columns in MUP buffer. The assay was performed at 40°C.

*This sample was incubated without MMTV cDNA.

L cell RNA or B77/3T3 RNA with partial success. Addition of 75 μg of L cell RNA to each hybridization reduced the background approximately 2-fold (Table 11). Moreover, it is evident that the sites on the DNA are limited since increasing the input of ^{3}H B77/3T3 RNA gives a consistently lower background. In light of this it remains puzzling that the competitor RNA effects at best a 2-3 fold reduction in the background. In any case, by eluting from the "MUP" columns at

TABLE 11

EFFECT OF COMPETITOR RNA ON THE

BINDING OF UNINFECTED CELL RNA TO MMTV DNA

^{3}H cpm input	% Bound (-compet.)	% Bound (+ compet.)*
25,000 + cDNA	0.26	0.08
" - cDNA	0.02	0
50,000 + cDNA	0.14	0.048
250,000 + cDNA	0.06	0.028
500,000 + cDNA	0.04	0.020
" - cDNA	0.009	0.005

B77/3T3 RNA was hybridized in a final volume of 15 1 for 37 hours at 68°C with approximately 40 ng of MMTV cDNA. "MUP" assays were performed at 60°C as described in the methods section.

* L cell RNA (75μg) was used as competitor.

60° and including 75-100μg of unlabeled RNA in each hybridization, the binding of RNA from cells free of MMTV RNA can be reduced to 0.02-0.06% (using approximately 1.5 x 10^5 cpm input). Although this is not ideal, several experiments have been successfully performed since the percentage of labeled RNA which is virus-specific in a short labeling period is considerably higher than its steady state concentration (see below).

Rate of synthesis of MMTV RNA in GR cells + dexamethasone. As discussed in Section 2, the dexamethasone mediated accumulation of

MMTV RNA in GR cells could be due to an increase in its rate of synthesis, a decrease in its rate of degradation, or a combination of the two. To test the rate of synthesis of MMTV RNA in treated and untreated cells I have used the MUP assay as described above. Cells were labeled for 15 or 30 minutes with 750 μCi/ml of ^{3}H -uridine; RNA was extracted and hybridized as described in the methods section. After either 15' or 30' labeling periods, the percentage of ^{3}H RNA which was virus-specific in dexamethasone-treated cells was 3-4 times greater than in untreated cells (Table 12). Dexamethasone did not

TABLE 12

RATE OF SYNTHESIS OF MMTV RNA IN

DEXAMETHASONE TREATED AND UNTREATED CELLS

EXPT. #1 (30' label)

	^{3}H $\frac{\text{cpm bound}}{\text{total cpm}}$	^{32}P $\frac{\text{cpm bound}}{\text{total cpm}}$	Corrected ^{3}H %bound (1)
GR-DEX Sample 1	$\frac{74}{66040} = .112\%$	$\frac{301}{619} = 49\%$	0.085%
Sample 2	$\frac{135}{211715} = .064\%$	$\frac{207}{614} = 34\%$	0.074%
GR + DEX(2) (48 hr) Sample 1	$\frac{170}{131490} = .129$	$\frac{211}{622} = 34\%$	0.247%
Sample 2	$\frac{286}{392530} = .073$	$\frac{88}{690} = 13\%$	0.331%
B77/3T3 Sample 1	$\frac{40}{51750} = .078$	$\frac{316}{586} = 54\%$	X
Sample 2	$\frac{67}{149650} = .045$	$\frac{331}{614} = 54\%$	X

TABLE 12 (Continued)

EXPT. #2(3) (15' label)

	^3H cpm bound / ^3H total cpm	^{32}P cpm bound / ^{32}P total cpm	Corrected ^3H %bound (1)
GR-DEX Sample 1	$\frac{70}{97950} = .081$	$\frac{581}{1060} = 55\%$	0.061%
Sample 2	$\frac{268}{476000} = .064$	$\frac{458}{991} = 46\%$	0.066%
GR +DEX (11 hr) Sample 1	$\frac{125}{88400} = .161\%$	$\frac{565}{1095} = 52\%$	0.224%
Sample 2	$\frac{325}{423025} = .088\%$	$\frac{292}{1172} = 25\%$	0.279%
B77/3T3 Sample 1	$\frac{33}{73300} = .051\%$	$\frac{629}{1070} = 59\%$	X
Sample 2	$\frac{128}{327925} = .044\%$	$\frac{690}{1157} = 60\%$	X

Cells were labeled for the indicated times with 750 μCi/ml of ^3H uridine. RNA was extracted, hybridized with MMTV DNA and assayed on hydroxylapatite (in MUP buffer) as described in the experimental methods section. Approximately 0.15 ng of ^{32}P MMTV 70S RNA, 75 μg of L cell RNA, and ^3H RNAs (as shown) were annealed with 20-30 ng of MMTV DNA for 20-30 hours.

(1) The corrected ^3H % bound was calculated as follows:

$$\frac{\%\ ^3H\ \text{GR RNA bound}}{\%\ ^{32}P\ \text{GR RNA bound}} - \frac{\%\ ^3H\ \text{B77/3T3 RNA bound}}{\%\ ^{32}P\ \text{GR RNA bound}}$$

For example: In experiment #1 GR-DEX Sample 1 was computed in the following manner: $\frac{.112}{.49} - \frac{.078}{.54} = .085\%$

TABLE 12 (Continued)

(2) dexamethasone was added for 48 hours.

(3) In experiment #2 the extent of the induction of MMTV RNA (after 11 hour treatment with dexamethasone) was measured by "C_rt" analysis. The $C_rt_{\frac{1}{2}}$ for RNA (+dex) was 20 and for RNA (-dex) 300. Therefore the steady state concentration of MMTV RNA was increased 15-fold by treatment with the hormone.

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appear to have an effect on the uptake of ^3H - uridine since the specific activities of the RNAs were comparable to those from untreated cells (9×10^4 cpm/μg). I am making the assumption that incorporation of ^3H - uridine into MMTV RNA is linear for at least 30 minutes. This seems reasonable since the estimated half-life of MMTV RNA is at least 2-1/2 hours (see following sections).

In one experiment the steady state concentrations of MMTV RNA in both cultures were determined by standard "C_rt" analysis (see Section 1). The results shown in Table 13 indicate that although there was 15-fold more MMTV RNA in the dexamethasone treated cells, the rate of synthesis of viral RNA was only stimulated 3-4 fold. This discrepancy (supported in following experiments) suggests that the accumulation of MMTV RNA may be due to a combination of effects on rates of synthesis and degradation. However, since the background in these experiments is considerable, it is possible that the absolute difference in the rate of synthesis of viral RNA between hormone treated and untreated cells is erroneous.

More convincing evidence that the rate of synthesis of MMTV RNA is increased by treatment of the cells with dexamethasone is demonstrated by the kinetic experiment shown in Figure 20. RNAs were labeled for 15 minutes at various times after the addition of dexamethasone. When the ^{3}H uridine and dexamethasone were added at the same time, the percentage of viral RNA in the labeled RNA (0.51%) was greater than in the non-hormone treated cells (0.28%). The maximal rate of MMTV RNA synthesis (0.91%) was reached within 60 minutes after addition of the hormone and was maintained at the same level 4 hours later. This experiment substantiates the conclusion made in section 2 that the increased production of MMTV RNA is a primary effect of dexamethasone. Although it is unclear why the absolute level of viral RNA is higher in this experiment than in the two shown in Table 12, dexamethasone again effects only a 3-4 fold increase in the rate of synthesis of MMTV RNA.

MMTV RNA does not decay rapidly after removal of dexamethasone. A reasonable explanation for the discrepancy between the increase in steady state concentration (10-20 fold) and rate of synthesis (3-4 fold) of MMTV RNA in dexamethasone treated GR cells is that the hormone also acts by stabilizing the viral RNA (or by inducing a more stable species of RNA than found prior to hormone treatment). This idea is supported by the relatively stable level of induced MMTV RNA after the removal of dexamethasone (Figure 21). Up to eight hours after removal of the hormone, the steady state concentration of MMTV RNA remains at the induced level and then returns to the uninduced level. Since it is very difficult to determine whether there has been a small (10-30%) decrease in viral RNA concentration during the first eight hours it is unclear

FIGURE 20

Kinetics of the effect of dexamethasone on the rate of synthesis of MMTV RNA. GR cells were labeled for 15 minutes with 750 μCi/ml ^{3}H uridine at the indicated times, RNA was extracted, hybridized, and assayed as described in the experimental methods section. The bars represent the times of labelling. All assays were performed at 60^{o}C in the presence of 75 μg cell RNA and samples were passed twice over columns of hydroxylapatite (MUP). Dexamethasone was added to a final concentration of 10^{-5}M.

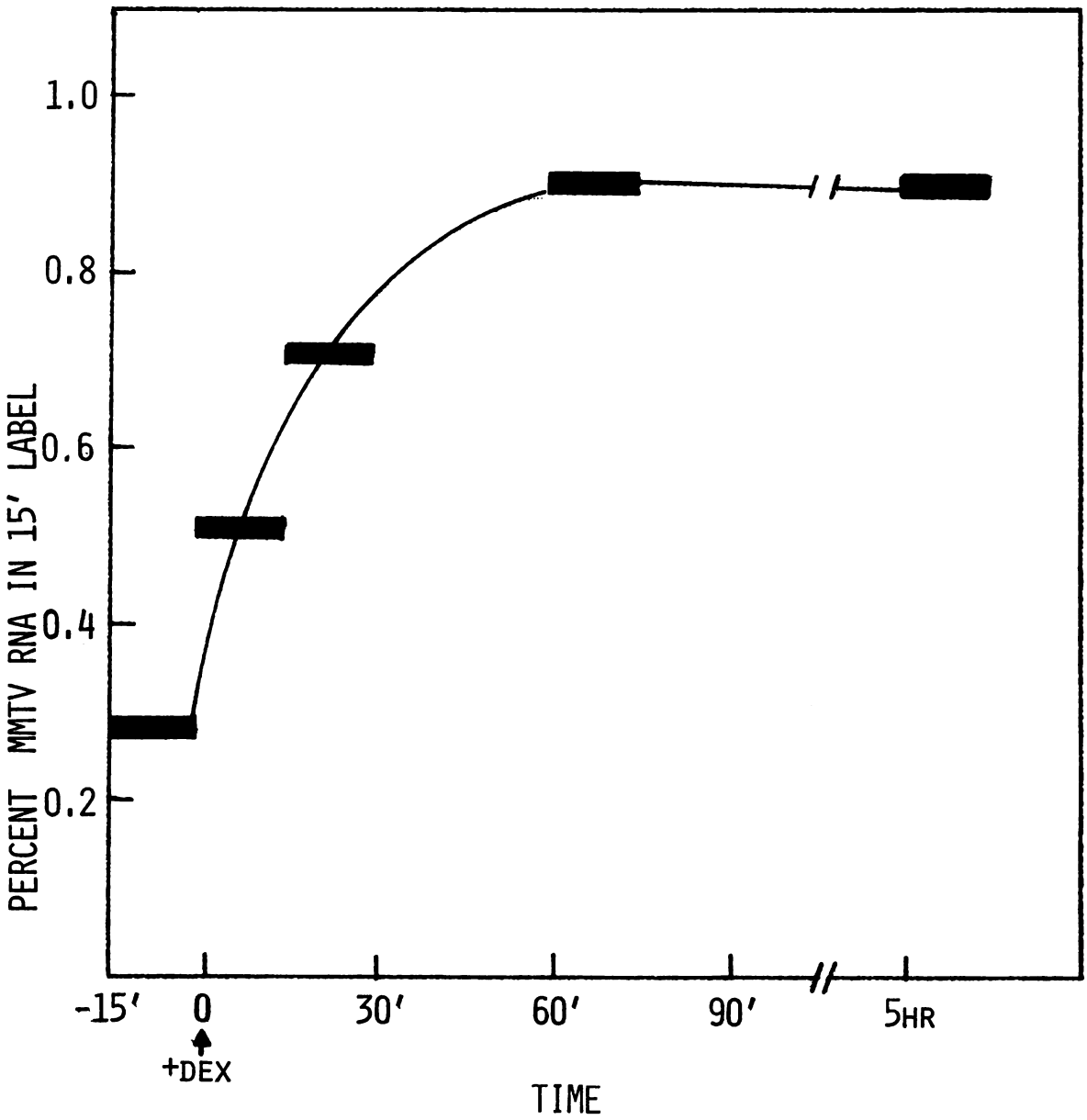
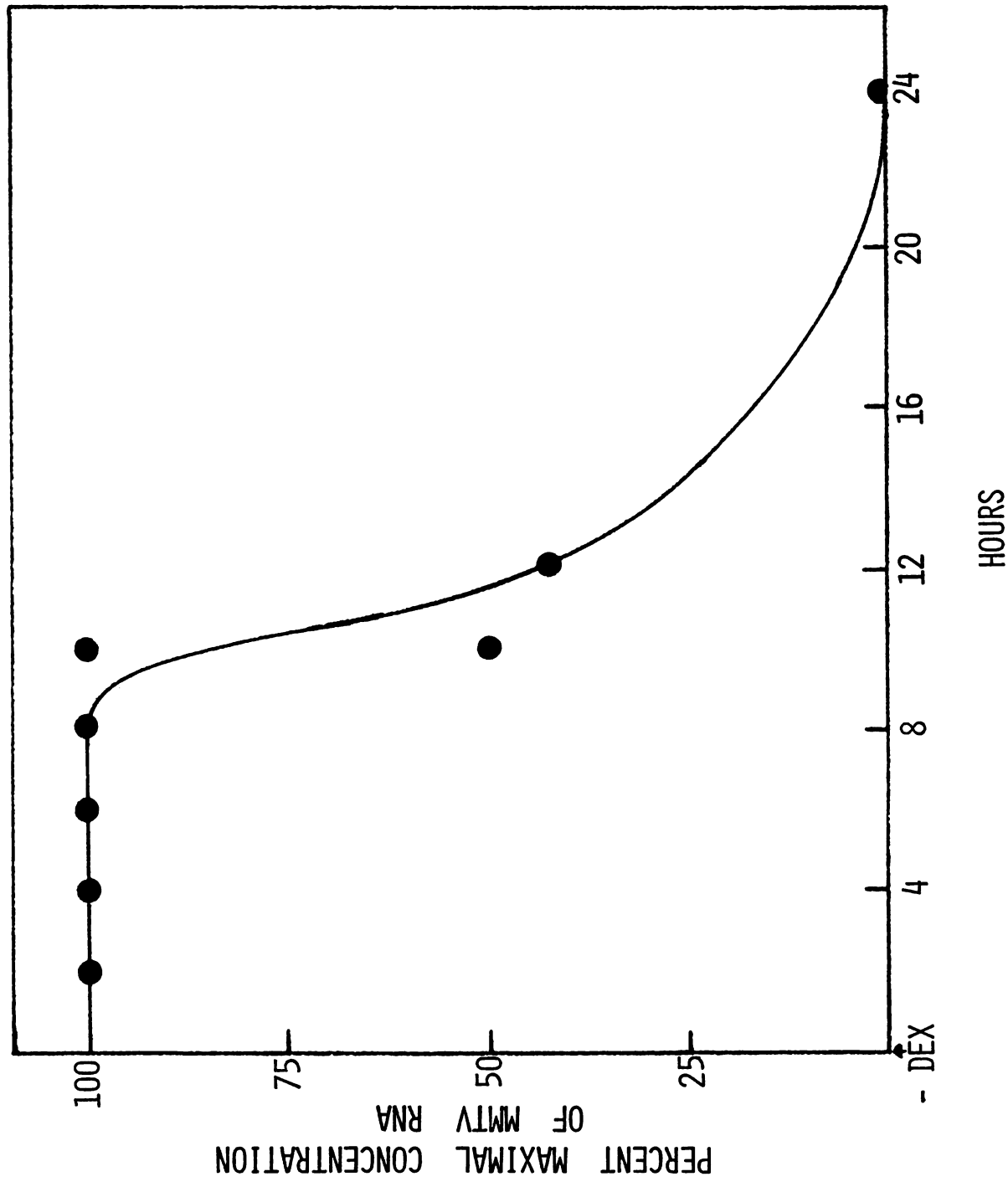

1.0
0.8
0.6
0.4
0.2
PERCENT MMTV RNA IN 15' LABEL
-15'
0
+DEX
30'
60'
90'
5HR
TIME

FIGURE 21

Deinduction of MMTV RNA. GR cells grown for 12 hours (or longer) with 10^{-7}M dexamethasone were washed five times with isotonic Tris-glucose containing 5 x 10^{-5}M progesterone, and then incubated for the indicated times in medium containing 5 x 10^{-5}M progesterone and lacking dexamethasone. RNAs were prepared as described in the methods section. Hybridizations were performed for 15-20 hours in 0.6M NaCl at 68^{o}C with 800 cpm of ^{3}H cDNA.


PERCENT MAXIMAL CONCENTRATION
OF MMTV RNA
100
75
50
25
- DEX
4
8
12
16
20
24
HOURS

whether there is a slow and progressive loss of MMTV RNA or whether there is a rapid loss after an eight hour lag period.

The deinduction experiments depend upon the efficiency with which the hormone was removed from the cells. To facilitate the reduction of effective hormone, a 500-fold excess of progesterone (a competitive inhibitor of dexamethasone action, Samuels & Tomkins, 1970) was included in the medium lacking dexamethasone. These concentrations of progesterone completely block the induction when added at the same time as the dexamethasone (Figure 11). Furthermore, progesterone added one hour after dexamethasone blocks further induction (Figure 22), although in this experiment small increments (<50%) would not be detected. An experiment performed in collaboration with Dr. Keith Yamamoto also showed that after the washing procedure less than 1% of ^{3}H labeled dexamethasone remained bound to receptors. In all of these experiments, dexamethasone was used at $10^{-7}M$, a concentration which gives about 60% of the maximum response, in order to facilitate removal of the hormone. Lastly, the rate of synthesis of MMTV RNA was measured at a time when the steady state concentration had not decreased significantly (10 hours after removal of the hormone). As shown in Table 13, the rate of synthesis had returned to the uninduced level (i.e., 1/3 to 1/4 the induced level). Thus it appears that the maintenance of induced steady-state concentrations of MMTV RNA after removal of dexamethasone is not due to an inability to wash-out the hormone. This is consistent with studies in other systems which show that the binding of steroid hormones to cells (and nuclei) is readily reversible (King and Mainwaring, 1974; Gorski and Gannon, 1976).

FIGURE 22

Ability of progesterone to block further induction after the addition of dexamethasone. GR cells were grown in the absence of dexamethasone. At time = 0, 10^{-7} M dexamethasone was added to three identical cultures and one was left without hormone. After 1 hour, RNA was extracted from one of the cultures (▲); another culture was washed and incubated with 5 x 10^{-5}M progesterone for an additional 4 hours. At the end of the treatment the cells without dex (●), the cells with dex for 5 hours (⊙), and the cells treated for 1 hour with dex and 4 hours with progesterone (□) were extracted for RNA. Hybridizations were carried out for 20 hours at 68°C in a volume of 10 μl.

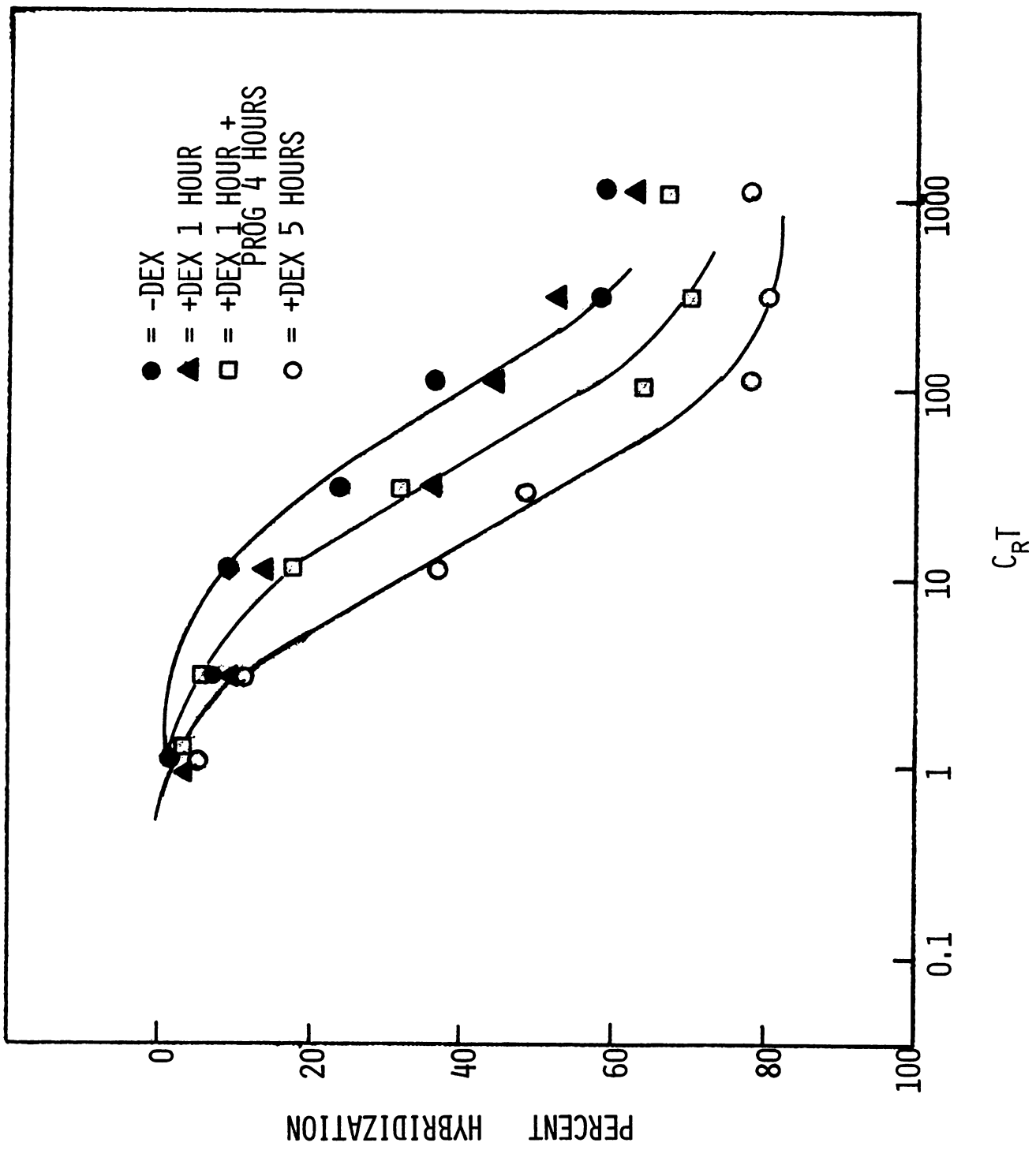

PERCENT HYBRIDIZATION
0
20
40
60
80
100
0.1
1
10
100
1000
C_RT
● = -DEX
◀ = +DEX 1 HOUR
□ = +DEX 1 HOUR + PROG 4 HOURS
○ = +DEX 5 HOURS

TABLE 13

DEINDUCTION OF MMTV RNA SYNTHESIS VS.

DEINDUCTION OF STEADY STATE MMTV RNA

Treatment	% MMTV RNA(1) in 15' label	MMTV RNA concentration (2) as % of whole cell RNA
A) -DEX	.095 - .133	0.01%
B) +DEX (22 hr)	.261 - .377	0.06%
C) +DEX (22 hr) followed by PROG (11 hr)	.129 - .131	0.06%

GR cells were grown in the absence of dexamethasone (Group A). Groups B and C were incubated with 10^{-7}M dexamethasone (sub-maximally effective concentration) for 22 hours. At that time, groups A and B were labeled for 15' with 750μCi/ml of ^{3}H uridine. Group C was washed five times with Tris-glucose containing $5X10^{-5}$M progesterone and then incubated in growth medium containing $5X10^{-5}$M progesterone (but no dex.) for an additional 11 hours. At the end of the 11 hours, Group C was labeled for 15'.

(1) The values given are for separate determinations using varying inputs of ^{3}H RNA.

(2) Steady state concentrations were determined in parallel cultures by "C_rt" analysis (see methods).

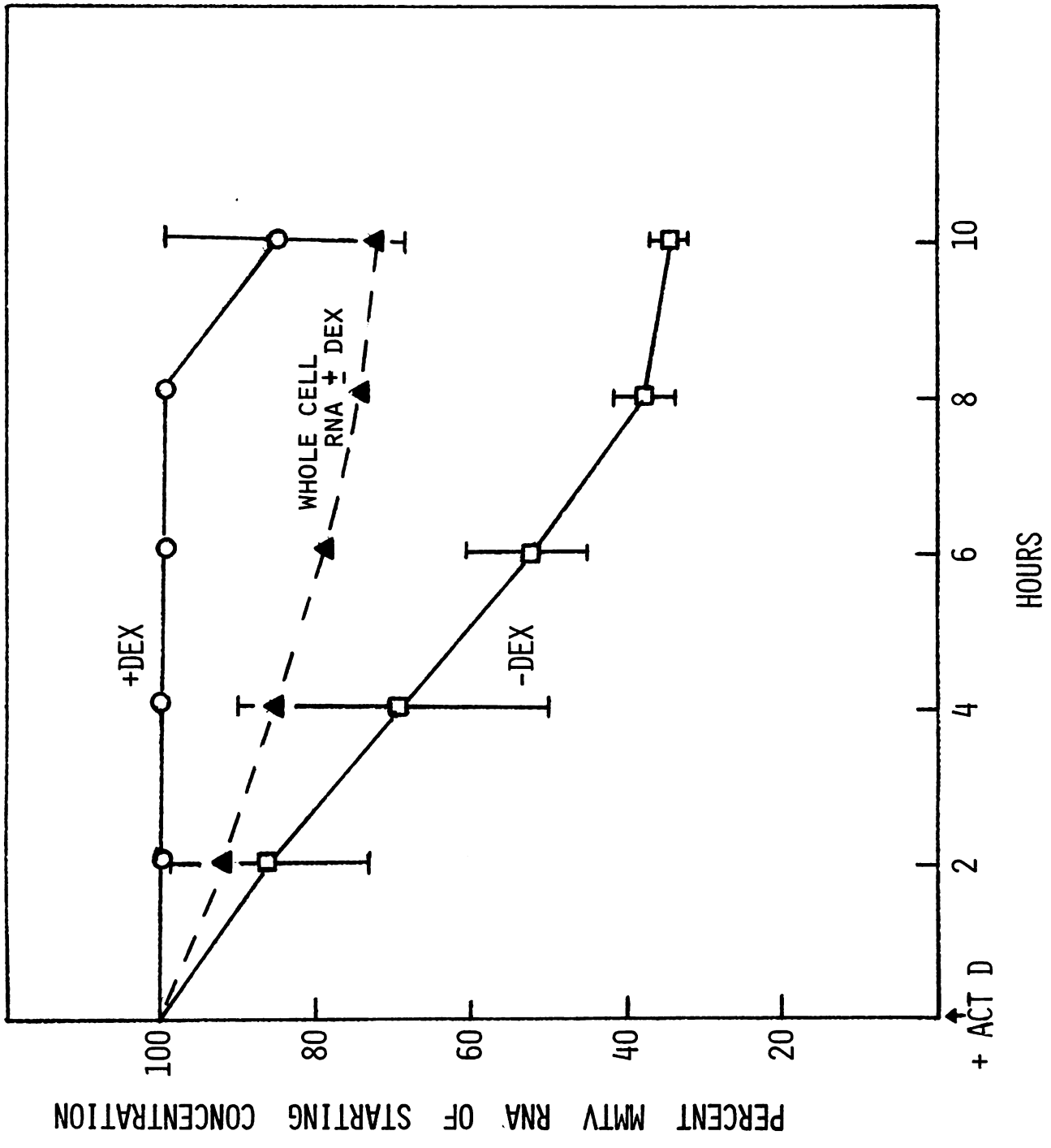
MMTV RNA decays slowly in dexamethasone induced cells treated with actinomycin D. If the MMTV RNA present in dexamethasone treated cells is in fact more stable than the constitutive RNA, it should decay more

slowly when RNA synthesis is completely inhibited. In two experiments (shown in Figure 23) this was the case. Actinomycin D (10 μg / ml) was added to cells grown for long times in the presence or absence of dexamethasone. At the indicated times, RNAs were extracted and the amount of MMTV RNA in each was determined by hybridization with ^{3}H cDNA. The results show that in non-hormone treated cells MMTV RNA decays with a half-life of approximately 6 hours, reaching 35% of its original concentration 10 hours after treatment with actinomycin D. In contrast, MMTV RNA is more stable in dexamethasone treated cells, turning over with a half-life considerably greater than 10 hours. In light of the toxicity of actinomycin D I was not able to assay time points after 10 hours; therefore I can not estimate what the half-life of MMTV RNA is in the dexamethasone treated cells. Actinomycin D was effective in the hormone treated cells as estimated from the decline of total RNA recoverable from identical cultures after treatment with the drug (Figure 23) and from its ability to inhibit the incorporation of ^{3}H - uridine into acid precipitable material (data not shown).

Intracellular localization of MMTV RNA. The data presented above demonstrate two things: (1) dexamethasone stimulates the rate of MMTV RNA synthesis within 15 minutes after its addition to GR cells and (2) the viral RNA present in hormone treated cells appears to be considerably more stable than the RNA present in uninduced cells. It is therefore conceivable that a functional difference exists between the MMTV RNA in treated and untreated cells. The first approach to answering this question was to determine the intracellular distribution of the viral RNA. If the hormone slowed the processing or transport of

FIGURE 23

Turnover of MMTV RNA after inhibition of RNA synthesis by actinomycin D. GR cells were grown in the presence (●) or absence (□) of 10^{-5}M dexamethasone for $\geq$ 48 hours. At time = 0, actinomycin D (10 μg /ml) was added to all cultures. At the indicated times RNA was extracted and the concentration of MMTV RNA was determined in each as described in the methods sections of chapters 1 and 2 (i.e., by "C_rt" analysis). The data are plotted as the relative amount of MMTV RNA present in each culture and each data point represents the average of two experiments designated by the bars (I). The absolute amount of MMTV RNA at time = 0 in the dexamethasone treated cells (●) was 10-fold greater than in the untreated cells (□). The dotted line represents the total recovery of RNA (determined by its optical density at 260 nm) from the identical cultures after treatment with actinomycin D.


PERCENT MMTV RNA OF STARTING CONCENTRATION
100
80
60
40
20
+ ACT D
2
4
6
8
10
HOURS
+DEX
-DEX
WHOLE CELL RNA ± DEX

nuclear MMTV RNA then one might expect that there would be an increase in the pool of nuclear MMTV RNA. This is not the case as seen in Figure 24. In both dexamethasone treated and untreated cells, nuclear MMTV RNA constitutes approximately 2% of the total viral RNA in the cell. The preponderance of cytoplasmic viral RNA contrasts with the situation in avian sarcoma virus infected chicken cells, in which approximately 40% of the viral RNA is found in the nucleus (Fanshier, L. and Bishop, J.M., unpublished observations).

Polyadenylation of MMTV RNA. Although the function of the polyadenylic acid (poly(A)) homopolymer found at the 3' end of most eukaryotic mRNAs and viral RNAs (Darnell et. al., 1973) is unclear, a few experiments have suggested that it may be a determining factor in the mean life-time of certain mRNAs (Huez et. al., 1974; Sheiness and Darnell, 1973; see review by Greenberg, 1975). Using oligo-dT cellulose columns to isolate poly (A) - contining RNA from GR cells, I have found that the proportion of viral RNA which is polyadenylated is unaffected by treatment with dexamethasone (Figure 25). Surprisingly, only 10% of the viral RNA in both cases is retained on oligo-dT cellulose columns (approximately 2-3% of whole cell RNA is retained after two passages). Since oligo-dT cellulose selects for RNAs that have greater than 50 adenosine residues (D. Spector, J. Stavnezer, personal communication), it is conceivable that the intracellular MMTV RNA contains short sequences of poly(A); however virion RNA has been found to contain poly(A) which is 150-200 nucleotides in length (Gillespie et. al., 1972). An alternative explanation is that the viral RNA may be partially degraded (either during the extraction or within the cell). Control experiments in which ^{32}P labeled B77-ASV,

FIGURE 24

Distribution of MMTV RNA in the cytoplasm and nucleus of GR cells (± DEX). RNA was prepared from the cytoplasm and nucleus of GR cells as described in the methods section. Hybridizations were carried out at 68°C in 0.6M NaCl for 36 hours. (●) = cytoplasmic + DEX: (■) = nuclear + DEX; (○) = cytoplasmic - DEX; (□) = nuclear - DEX. Nuclear RNA constituted 5% (+DEX) and 6.5% (-DEX) of the whole cell RNA.

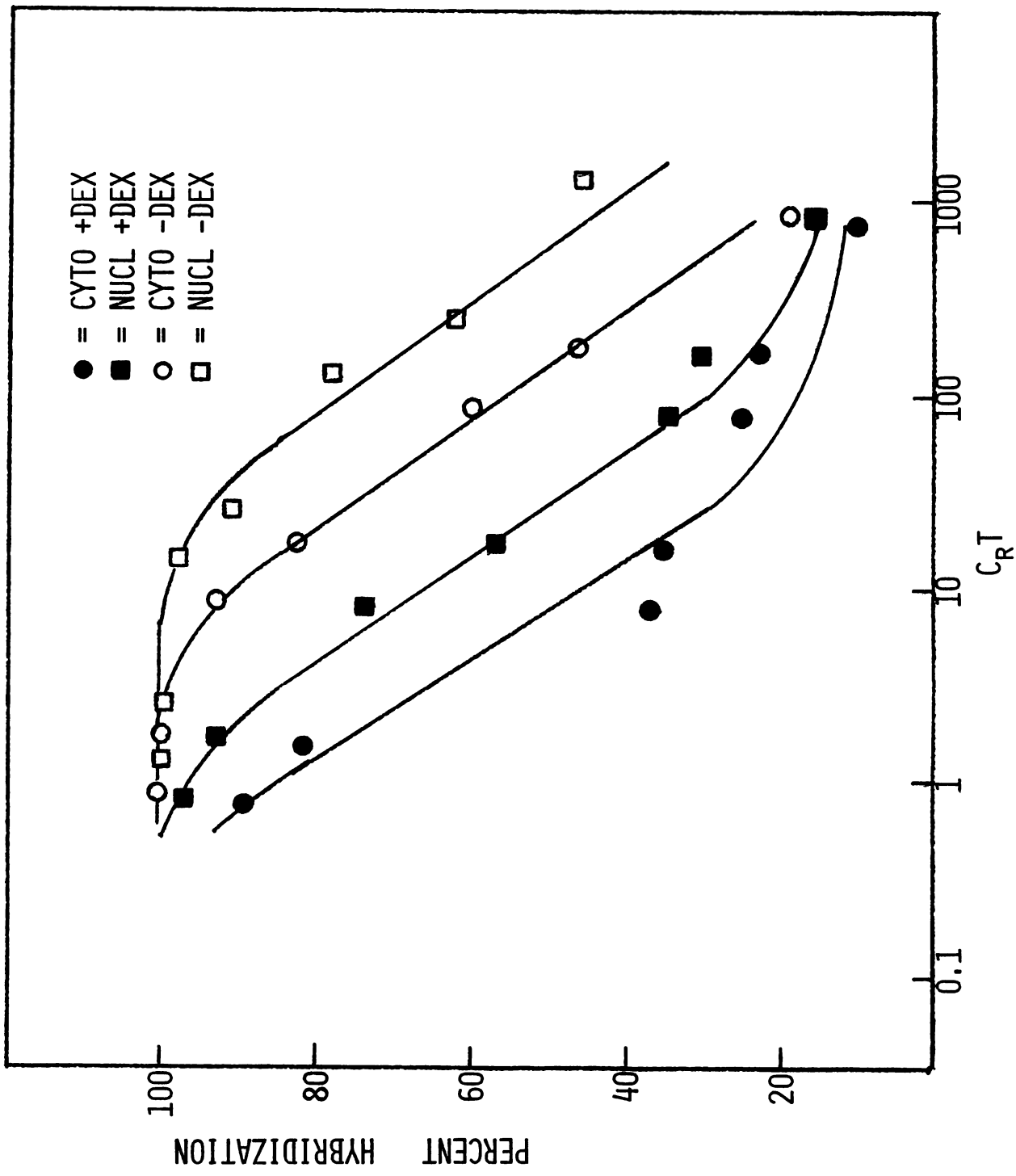
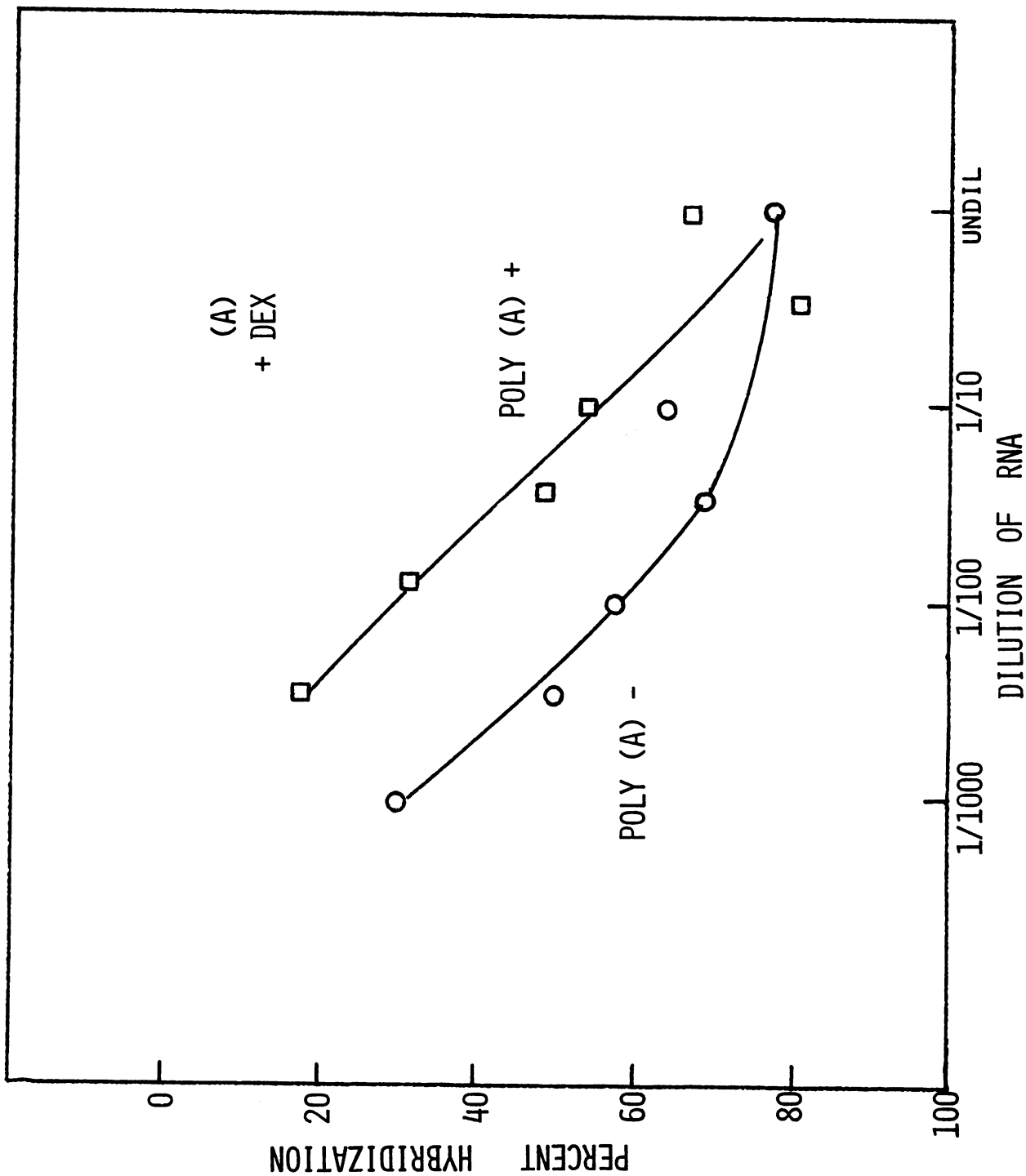
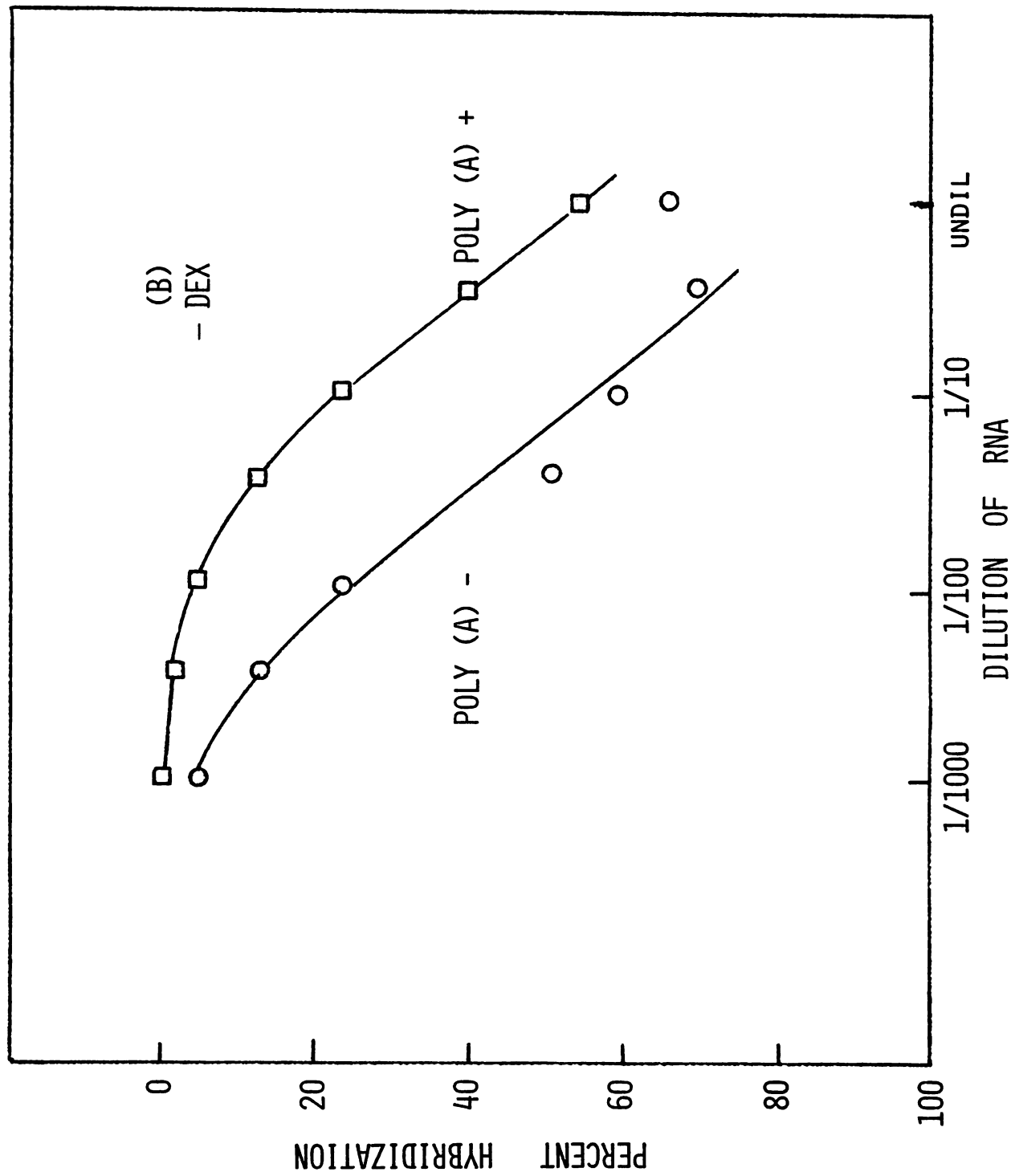

PERCENT HYBRIDIZATION
100
80
60
40
20
0.1
1
10
100
1000
C_RT
● = CYTO +DEX
■ = NUCL +DEX
○ = CYTO -DEX
□ = NUCL -DEX

FIGURE 25

Proportion of polyadenylated MMTV RNA in GR cells. RNA was prepared for fractionation on oligo-dT cellulose columns as described in the methods section. Each RNA sample was passaged twice over the columns in buffers containing 0.5% SDS. The percentages of whole cell RNA retained on the column were a) +DEX = 2.6%; b) -DEX = 2.2%. After EtOH precipitation the RNAs were resuspended in 15 μl TE buffer and equal dilutions of each were hybridized for 10 hours with 600 cpm ^{3}H cDNA.


PERCENT HYBRIDIZATION
0
20
40
60
80
100
1/1000
1/100
1/10
UNDIL
DILUTION OF RNA
POLY (A) -
POLY (A) +
(A)
+ DEX


(B)
- DEX
POLY (A) +
POLY (A) -
PERCENT HYBRIDIZATION
0
20
40
60
80
100
1/1000
1/100
1/10
UNDIL
DILUTION OF RNA

35 S RNA was added to the cells at the beginning of the extraction suggest that RNA was not being degraded during the extraction procedures. In addition, the RNAs were not denatured prior to the oligo-dT cellulose selection. Therefore, due to secondary structure, most or all of the RNA would be retained even if it contained one or two breaks. I conclude that only a small percentage (10%) of the total viral RNA in both dexamethasone-treated and untreated cells contains long poly(A) sequences.

Effect of dexamethasone on polysomal association of MMTV RNA. It is conceivable that mRNAs actively involved in protein synthesis (i.e. - polysome associated) might be more stable than those which are not (Singer and Penman, 1972). In two experiments, I found that the percentage of MMTV RNA present in polysomes (defined by its ability to be released by treatment with EDTA (Girard et. al., 1965)) was 2-3 times greater in cells treated with dexamethasone. Figure 26 shows the OD_{260} (optical density at 260 nm) tracing of the polysome gradients from cells treated with dexamethasone in the presence or absence of 25mM EDTA. The polysome profile from cells not treated with hormone was virtually identical. It is clear that the EDTA treatment was effective in releasing ribosomes from the mRNAs. Pools were made as shown, RNA was extracted, and equal amounts of each RNA were hybridized to ^{3}H cDNA as described in the experimental methods section. The results are shown in Figure 27 and the relative amounts of viral RNA in each pool are summarized in Table 14. Approximately 60% of all the MMTV RNA in hormone treated cells was present in the polysome regions of the gradient and was releasable by EDTA, whereas, in the untreated cells, only 25-30% of the viral RNA was polysome associated.

FIGURE 26

Polysome profiles from GR cells. Polysomes were prepared from cells grown in the presence and absence of dexamethasone as described in detail in the methods section. Centrifugation was for 2¼ hours at 27K in the Spinco SW 27 rotor through a 10-40% sucrose gradient. The gradients were collected from the bottom and the optical densities were monitored in a Gilford recording spectrophotometer. RNAs purified from the indicated pools were hybridized as described in Figure 27 and Table 14.

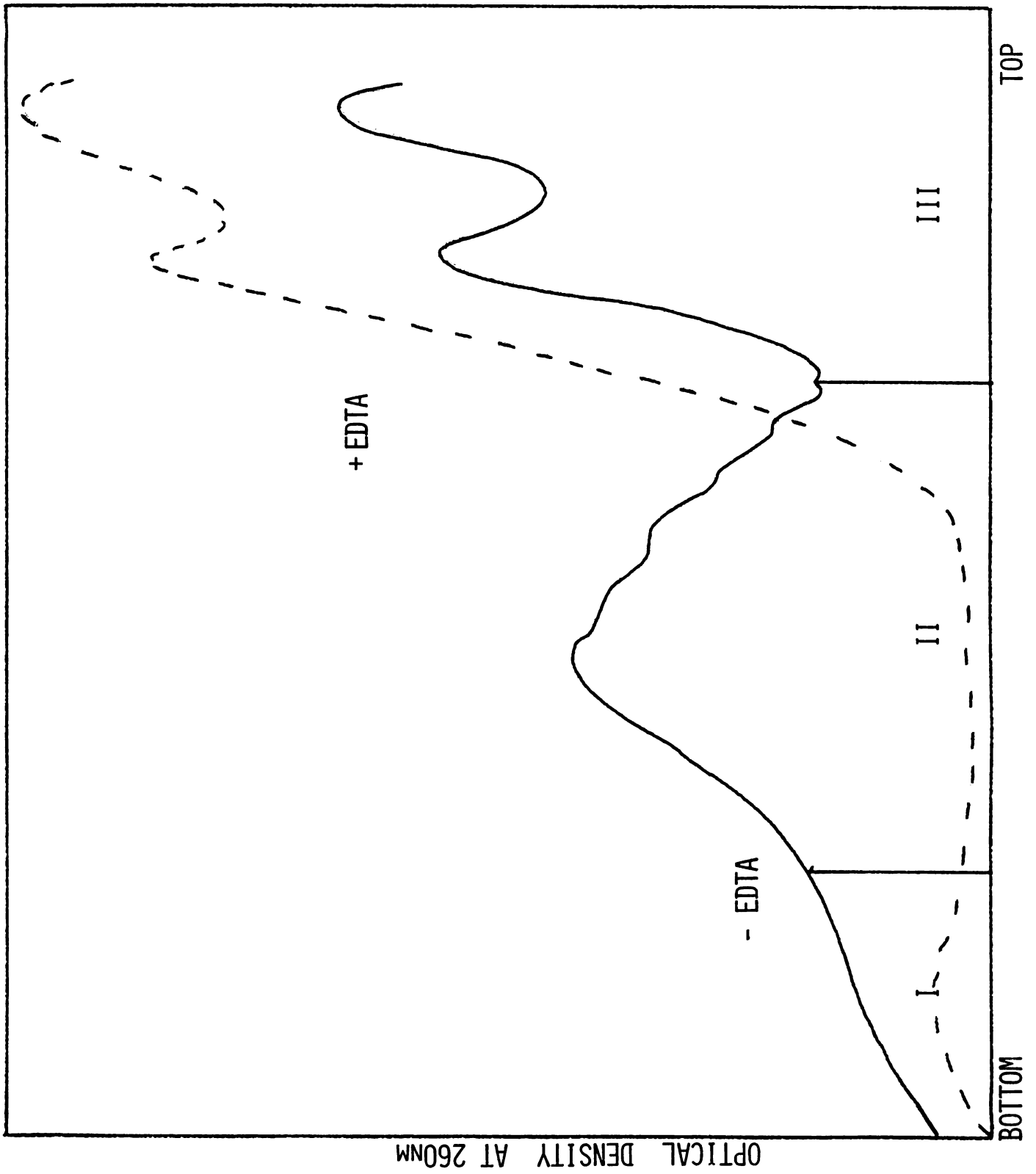
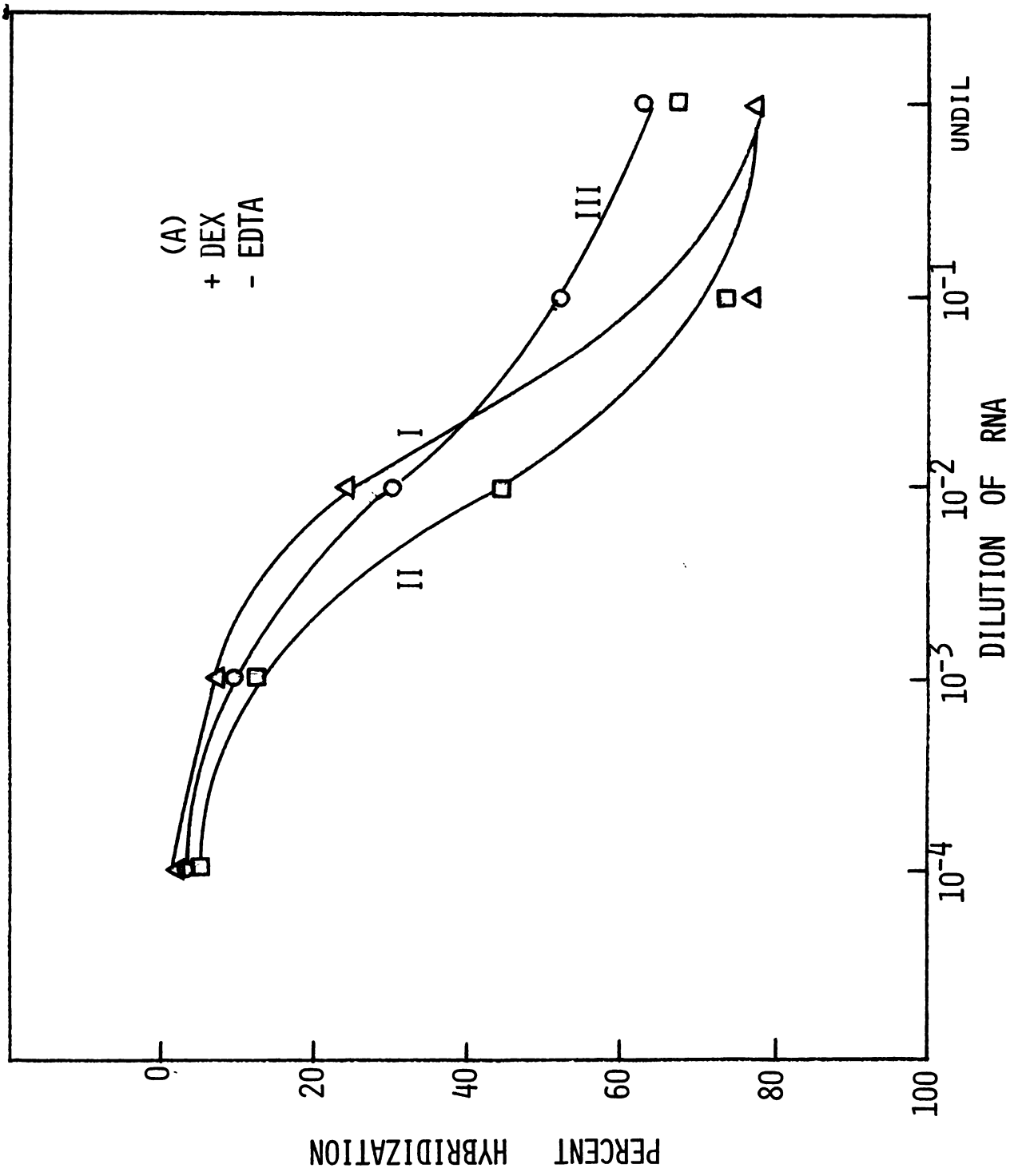
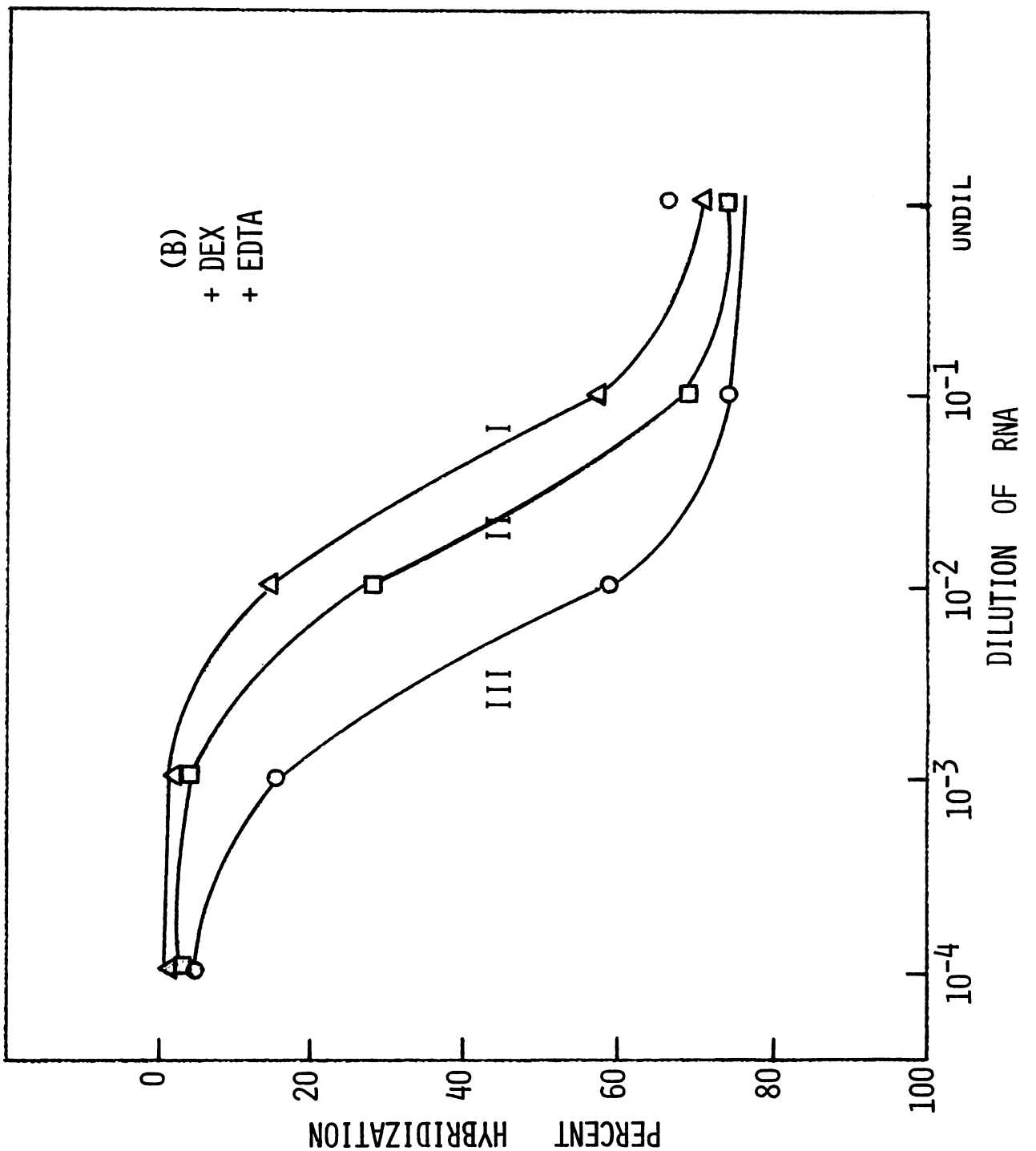
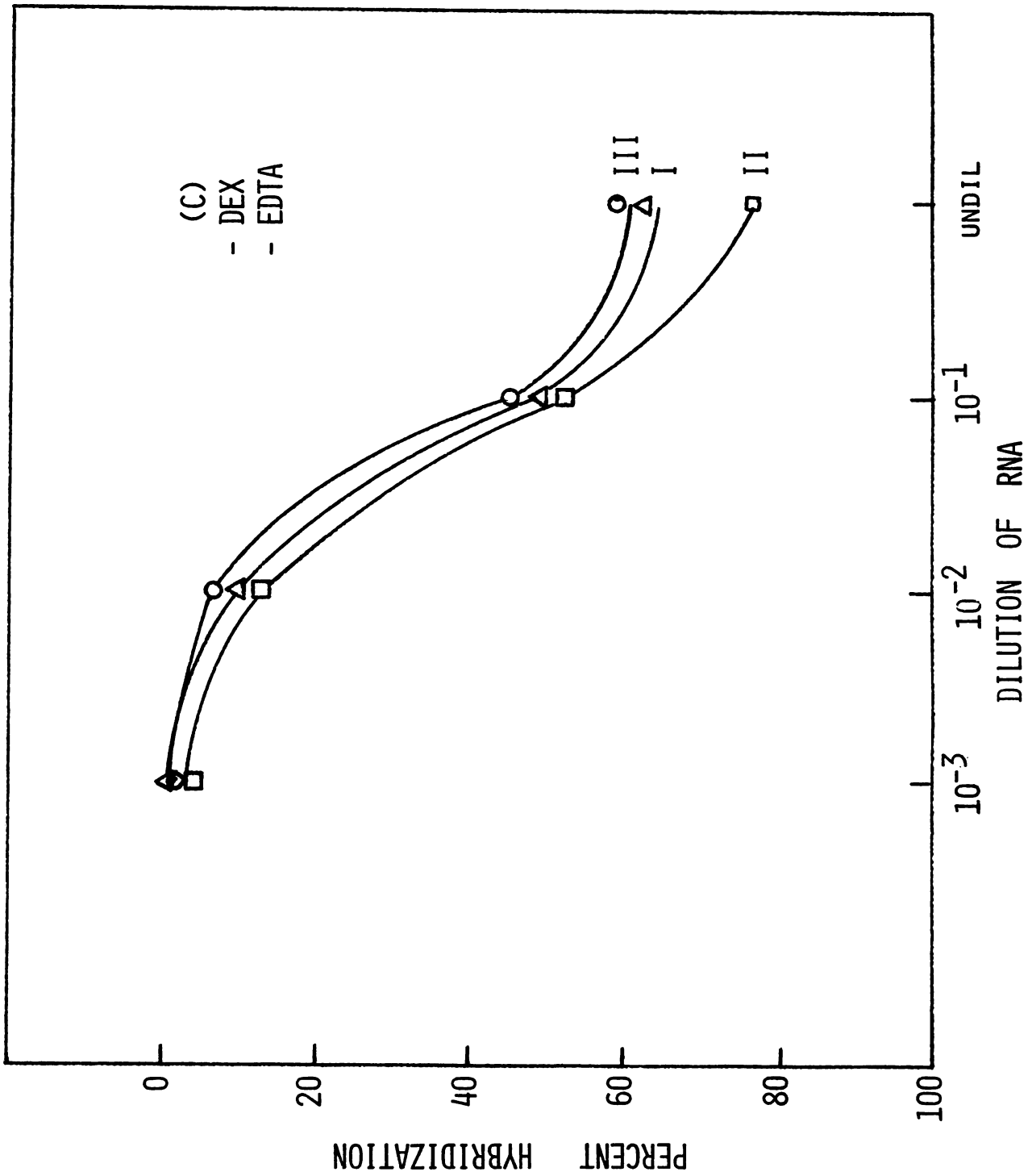

TOP
BOTTOM
III
II
I
+ EDTA
- EDTA
OPTICAL DENSITY AT 260NM

FIGURE 27

Hybridizations of RNAs from polysome gradients. Pools of RNAs were made from the polysome gradients as shown in Figure 26. After phenol extraction and EtOH precipitation, RNA from each pool was resuspended in 75μl TE. Equal dilutions of each RNA were hybridized for 24 hr (+DEX) or 42 hr (-DEX) at 68^{o}C in 0.6M NaCl.


(A)
+ DEX
- EDTA
I
II
III
PERCENT HYBRIDIZATION
0
20
40
60
80
100
10^{-4}
10^{-3}
10^{-2}
10^{-1}
UNDIL
DILUTION OF RNA


(B)
+ DEX
+ EDTA
I
II
III
PERCENT HYBRIDIZATION
0
20
40
60
80
100
10^{-4}
10^{-3}
10^{-2}
10^{-1}
UNDIL
DILUTION OF RNA


(C)
- DEX
- EDTA
III
I
II
UNDIL
10^{-1}
10^{-2}
10^{-3}
DILUTION OF RNA
PERCENT HYBRIDIZATION
0
20
40
60
80
100

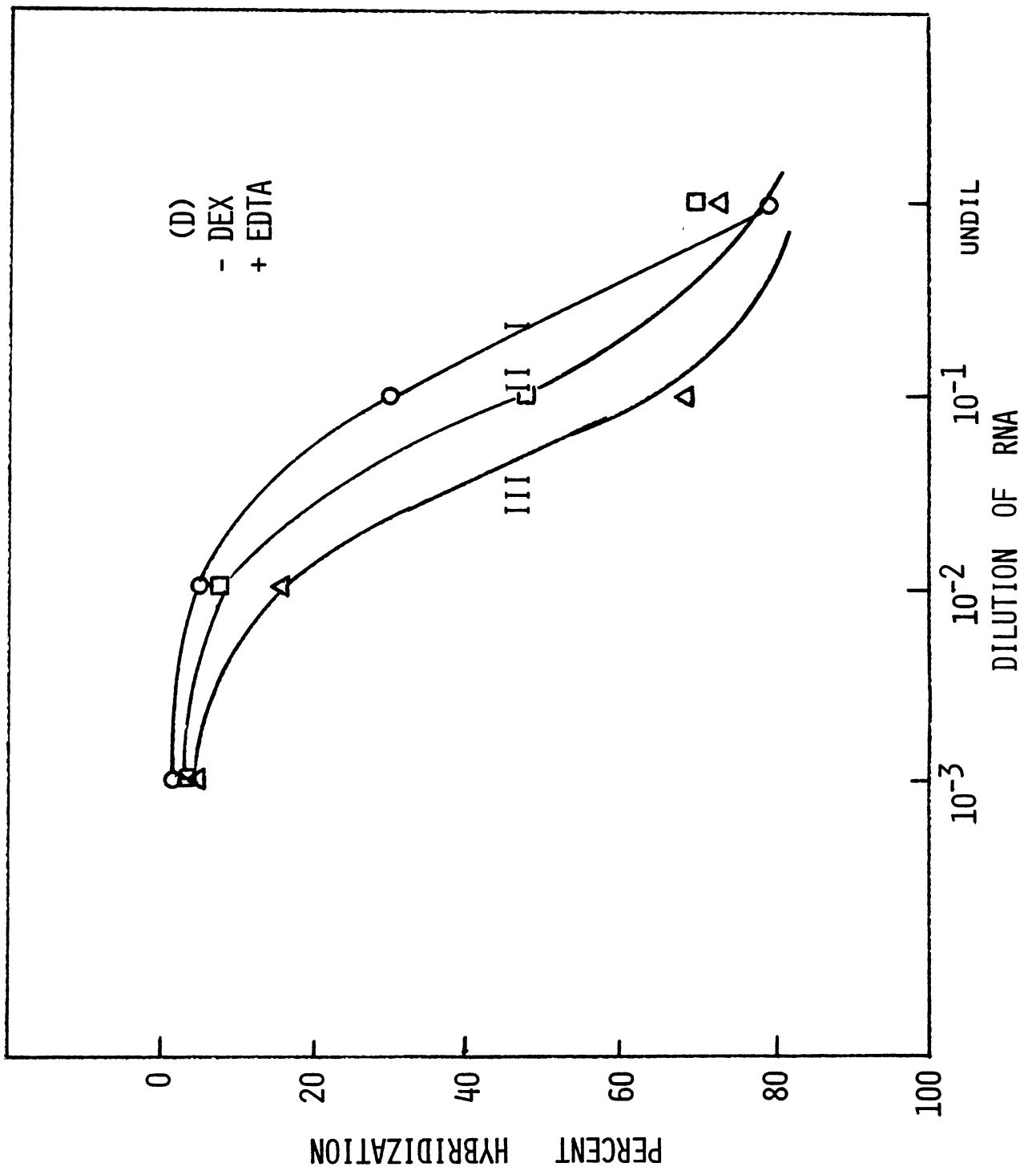

(D)
- DEX
+ EDTA
I
II
III
PERCENT HYBRIDIZATION
0
20
40
60
80
100
10^{-3}
10^{-2}
10^{-1}
UNDIL
DILUTION OF RNA

TABLE 14

POLYSOMAL ASSOCIATED MMTV RNA

	-DEX (% of MMTV RNA)			+DEX (% of MMTV RNA)		
	-EDTA	+EDTA	Δ	-EDTA	+EDTA	Δ
Pool I	33%	15%	-18	23%	10%	-13
Pool II	40%	30%	-10	67%	20%	-47
Pool III	27%	55%	+28	10%	70%	+60

RNA was extracted from the pools of polysome gradients shown in Figure 26. RNA samples were prepared and hybridized as described in the legend to Figure 27. The relative percentage of viral RNA in each pool was determined by the differences in the rate of annealing of ^{3}H MMTV cDNA to the RNAs as shown in Figure 27.

In a second experiment (not shown) 40-45% of the viral RNA in hormone treated cells and 20% of the viral RNA in untreated cells was polysome associated. It is noteworthy that a large fraction of the viral RNA in dexamethasone treated (30%) and untreated (45%) GR cells was present in the polysome region of the gradients but not affected by treatment with EDTA. This RNA presumably is in the form of ribonucleoprotein complexes which may be precursors to assembled virions. Although I draw the tentative conclusion that preferential incorporation of viral RNA into polysomes in dexamethasone treated cells is responsible for its apparent protracted lifetime, this explanation requires additional experimental validation.

Summary of the data

Clearly, one of the possible means by which viral RNA can be lost from GR cells is by the release of mature virions. In Table 15 I have tabulated several of the pertinent facts presented in Section 1 and Section 3 concerning the distribution and metabolism of MMTV RNA. Since the number of MMTV RNA molecules released per 24 hours is less than 3% of the steady state concentration of intracellular viral RNA it is evident that the production of mature virions plays an insignificant role in the half-life of the intracellular viral RNA.

TABLE 15

SUMMARY OF THE EFFECTS OF DEXAMETHASONE ON MMTV RNA

	+DEX	-DEX
# MMTV RNA molecules*/cell at steady state	5000	400
% MMTV RNA in cytoplasm	98%	98%
% MMTV RNA in nucleus	2%	2%
% MMTV RNA in polysomes	40-60%	20-25%
% polyadenylated MMTV RNA	10%	10%
# RNA molecules released as virions/ cell/24 hr	50-200	10-30
% MMTV RNA (of steady state) released as virions/24 hr	2-3%	2-3%
Approx. half-life of MMTV RNA (in Actinomycin)	> 10 hr	5-6 hr
Relative rates of synthesis of MMTV RNA	3-4	1

*One MMTV RNA molecule is defined as the equivalent of a 70S RNA molecule (approx. mol. weight = 10^7 daltons)

DISCUSSION

Is the glucocorticoid-mediated accumulation of MMTV RNA in GR cells a consequence of an increase in its rate of synthesis? The answer to this question has been the object of the experiments presented in the preceding section. The results suggest that the hormone may be acting at both the level of RNA synthesis and degradation. However, since the techniques used to arrive at this conclusion are frought with many problems I would like to devote this part of the discussion to alternate interpretations of the data. Firstly, I observed that the apparent rate of synthesis of MMTV RNA in dexamethasone treated cells was only 3-4 times greater than in untreated cells whereas the steady state levels of viral RNA were increased 15-20 fold. Since the level of labeled B77/3T3 RNA (which lacks MMTV sequences) detected by hybridization to MMTV cDNA is only 2-3 times lower than in untreated GR cells it is conceivable that the value for virus-specific RNA may be erroneous. If so, then the absolute difference between the synthetic rates of MMTV RNA in dexamethasone treated and untreated GR cells could be an underestimate. Although this is a possible argument, it is not very plausible since in a few experiments (see Figure 20) the levels of MMTV RNA synthesis in the uninduced GR cells was 4-5 fold greater than the control hybridizations with B77/3T3 RNA and yet, the induced rate remained only 3-4 times greater than the uninduced rate.

The fact that the concentration of viral RNA does not decrease rapidly upon withdrawal of the hormone (Figure 21) suggests that the viral RNA present in dexamethasone treated cells is more stable than in uninduced cells. However, it is impossible to rigorously exclude

the possibility that the hormone is incompletely removed by the washing procedure. It would be interesting if in fact the specifically-bound steroid-receptor complex remained bound for a long time (e.g., 8-10 hours) after the wash. However, based on the control experiments using progesterone (Figure 22 and Table 13) it seems that effective removal of the hormone must be complete within a very short period of time. Thus, although it is possible that the prolonged maintenance of induced levels of MMTV RNA after hormone withdrawal is a consequence of inefficient removal of the hormone, it seems more likely that there has been an effect on the rate of degradation of the viral RNA. Consistent with this interpretation, MMTV RNA decays much more rapidly in untreated cells than in dexamethasone treated cells after RNA synthesis has been shut-off with actinomycin D. It is possible that all experiments which use actinomycin D to study rates of degradation are invalid. Singer and Penman (1972,1973; also see Greenberg, 1972) have reported that actinomycin D may affect the turnover rate of several species of RNA. The drug could have similar effects upon MMTV RNA in GR cells, but it seems unlikely that there would be a preferential increase in the half-life of viral RNA in dexamethasone treated cells. One could also argue that the actinomycin D is not effectively inhibiting RNA synthesis in hormone treated cells but, based on ^{3}H - uridine incorporation and the reduction in total RNA recovery (Figure 23) from actinomycin treated cells, it seems that this is not a viable explanation. Lastly, the differences in MMTV RNA concentration which are being measured in these experiments are rather small (i.e., 3-fold at 10 hours after treatment with actinomycin). However, the same results have been seen in two separate experiments and the reduction in viral

RNA concentration in GR cells (-dex) is a time dependent function, thus I feel that the observed differences are significant.

The pathways of mRNA biosynthesis in eukaryotes are poorly understood. Likewise, very little is known about the factors which determine mRNA stability. Consequently it is very difficult to assess the significance of many of the experiments described above. In particular, it is impossible to determine whether the preferential incorporation of viral RNA into polysomes in dexamethasone-treated cells is in any way responsible for its increased stability. In addition one must postulate the RNA which is synthesized in the presence of dexamethasone is structurally different, so as to permit ribosome binding; or, one could envision that a regulatory element which promotes ribosome binding to MMTV RNA is induced by dexamethasone. The regulatory element would have to be an RNA molecule since the induction of MMTV RNA is dependent on RNA synthesis but not protein synthesis (see Section 2). There is very little precedent for either of these explanations; however, Liang and Liao (1975) have demonstrated that androgens have very rapid effects on a methionyl - $tRNA_f$ binding protein involved in the initiation of protein synthesis. Tomkins et. al. (1969) have postulated that glucocorticoids may have post-transcriptional effects mediated by molecules involved in the degradation of mRNAs. When glucocorticoids are added to HTC, rat hepatoma, cells, several enzymes including tyrosine amino-transferase (TAT) are induced. If the hormone is removed, the level of the enzyme decreases (deinduction); however, if actinomycin D is added at the same time, the enzyme levels remain high and may even increase (superinduction). Tomkins and his colleagues postulated that the production of TAT was controlled not only by its

level of messenger RNA but in addition by a labile post-transcriptional repressor which inactivates TAT mRNA (Tomkins et. al., 1969). Interestingly, when HTC cells are enucleated and the hormone is removed, TAT levels remain induced even though enzyme degradation proceeds normally. (Ivarie et. al., 1975). This argues strongly that a nuclear function, possibly the synthesis of a regulatory RNA molecule, is required for the degradation of TAT mRNA. Although the existence of such a molecule is hypothetical, there is increasing evidence that several factors (proteins and RNAs) may be involved in regulating the incorporation of specific mRNAs into polysomes (Nudel et. al., 1973; Thompson et. al., 1973). Several investigators have raised the possibility that an RNA molecule may be involved in translational control by directly affecting the initiation of protein synthesis (Craig, 1973; Goldstein and Penman, 1973; Fuhr and Natta, 1972). Recently, Heywood et. al. (1974) have isolated a 6500 dalton RNA molecule from erythroblasts and muscle which specifically inhibit the translation of heterologous mRNAs. For example tcRNAm (muscle, translational control RNA) inhibits the utilization of globin mRNA but not myosin mRNA in an in vitro protein synthesizing system. Bogdanousky et. al. (1973) have isolated a small RNA molecule from the dialysates of a reticulocyte ribosomal wash that stimulates globin synthesis. Some of my results with MMTV RNA could be partially explained by the existence of similar molecules in GR cells.

Lastly, I point out that there appears to be a discrepancy between the time required for induction of MMTV RNA ($t_{\frac{1}{2}} \sim 2\frac{1}{4}$; Figure 13) and the apparently protracted lifetime of the induced RNA. In the simplest case, the steady state concentration of RNA is determined by a zero-

order rate of synthesis and a first-order rate of decay; (i.e., $\frac{dR}{dt} = S-kR$) where R is the concentration of RNA, S is the synthetic rate, and k is the degradation rate constant. As pointed out by Berlin and Schimke (1965), the time taken to reach one-half of a new steady state level ($\frac{\ln 2}{k}$) is equal to the half-life of the induced molecule. If this were the case for MMTV RNA, then its half-life would be approximately 2-1/4 hours. The experiments presented in this section clearly illustrate that this is not the case. The turnover rate of MMTV RNA in dexamethasone treated cells as measured after treatment with actinomycin D is considerably greater than 10 hours. (Figure 23) Similarly, the decay of MMTV RNA after withdrawal of dexamethasone proceeds very slowly, reaching 50% of its starting concentration in 10-12 hours (Figure 21). Therefore, although I have not been able to construct a model (using computer simulation with the help of Dr. H. Martinez) which will accomodate all the data, I must conclude that the simple assumption of zero-order synthesis and first-order decay is not appropriate to this situation. There are other situations in which these assumptions do not hold. Perry and Kelly (1973) for instance, have shown that the messenger RNAs for histones in L cells are degraded with zero-order kinetics. Carey and Palmiter (1974) have shown that the rate of degradation of ovalbumin messenger RNA is slower in the presence of estrogen than in its absence, although simple kinetics appear to be sufficient to explain their observations (Palmiter, 1975). Hopefully, future experiments will allow me to explain the peculiar behavior of MMTV RNA in more precise mathematical terms.

Prospects for the future

How useful will the MMTV system be for answering some of the important questions about steroid hormone action? Since it is clear that dexamethasone does act (at least in part) to increase the rate of synthesis of MMTV RNA, direct tests can now be performed concerning the molecular mechanisms involved. With the increasing sophistication of *in vitro* transcription systems it may be possible to elucidate whether the hormone acts to increase initiation or elongation of RNA chains. Secondly, by using the defined receptor mutants of S49 cells described by Yamamoto et. al. (1974) it may be possible to directly study the role of the steroid receptor in the transcription process. I envision testing the ability of wild-type and variant receptors to stimulate the synthesis of MMTV RNA using GR cell chromatin or nuclei as the template. Lastly, Vaidya et. al. (1976, in press) have recently reported that cat kidney cells and mink lung cells are susceptible to infection by MMTV. Moreover, the production of virus from these cells remains sensitive to glucocorticoids. Therefore, it is quite plausible that if specific glucocorticoid receptor sites exist (as suggested by Yamamoto and Alberts, 1975), they may be contained in the MMTV proviral DNA. Since most or all of the MMTV genome can be transcribed into relatively long pieces of double stranded DNA (see Figure 15) it will be most interesting to pursue this as a means of purifying receptors and studying their interaction with DNA.

APPENDIX A

An RNA concentration of 1.6×10^{-2} μg/ml is required to reach a C_rt value of 2×10^{-2} mole-sec/liter (the $C_rt_{\frac{1}{2}}$ for viral 70S RNA) when the reaction is carried out for 20 hr at 68°C in 0.6 M NaCl. Assume I started with 35 ml of chick cell culture fluid from which B77-ASV was pelleted and viral RNA extracted. If this RNA were resuspended in 35 μl of buffer and 7 μl of the RNA solution were used in each 10 μl reaction, the V_ot would be calculated as follows:

$$V_ot = \text{(ml-equiv)} \times \text{(times of reaction)}.$$

For the example above, the B77-ASV ml equiv using undiluted RNA is:

$$7\,\mu l/35\,\mu l \times 35 \text{ ml} = 7 \text{ ml}.$$

If the reaction were carried out for 20 hr:

$$V_ot = (7 \text{ ml}) \times (20 \text{ hr}) = 140 \text{ ml-hr}.$$

From Fig. 1 we find that the $V_ot_{\frac{1}{2}}$ for the B77-ASV medium is 0.02 ml-hr. Since the reaction was performed for 20 hr in a final volume of 10 μl, the ml-equiv is 10^{-3} ml of medium. Therefore in 10^{-3} ml of medium we must have 1.6×10^{-4} μg of viral RNA. This is equivalent to 1.6×10^{-1} μg of viral RNA/ml of medium. Assuming that the molecular weight of the viral 70S RNA is 10^7 daltons, then 1.6×10^{-1} μg of RNA represents 9.6×10^9 molecules. If one particle contains one molecule of 70S RNA (Robinson et. al., 1967) then 1.6×10^{-1} μg of RNA corresponds to approximately 10^{10} virus particles.

APPENDIX B

NUCLEOTIDE SEQUENCE HOMOLOGIES AMONG MOUSE MAMMARY TUMOR VIRUSES

ABSTRACT

We have made DNA complementary to the entire genome of the mouse mammary tumor virus (MMTV) produced by GR tumor cells (MMTV-P) and we have tested the ability of RNAs from other virus strains (MMTV-L and MMTV-S) to compete with labeled RNA from MMTV-P for annealing to this DNA. RNA from these strains can compete with 96-100% of labeled RNA from MMTV-P indicating that the genomes of MMTV viruses are composed of closely related nucleotide sequences.

Several strains of mouse mammary tumor virus (MMTV) have been identified on the basis of their oncogenic potential, as well as the histopathology and growth properties of the tumors which they induce (1,2) (see Table 1). The strain of MMTV carried by GR, DD, and possibly RIII mice (MMTV-P) is highly oncogenic, induces hormone-dependent tumors (plaques), and is transmitted in the milk of infected mice. In the GR mouse, the virus is also transmitted via the gametes as an autosomal, dominant trait. The standard form of MMTV (MMTV-S) is found in the milk of C3H, A, DBA and other mouse strains. This virus is highly oncogenic, induces hormone-independent tumors and appears to be transmitted only in the milk of infected mice. A strain of MMTV with reduced oncogenicity (MMTV-L) is carried in the gametes of strains such as C3H, RIII, and some wild mice. It is often called the nodule-inducing virus (NIV) since it induces hyperplastic alveolar nodules. Several other strains of MMTV have been tentatively identified; however, their properties have not been clearly elucidated (see ref. 1). While all strains share major antigenic determinants, immunodiffusion studies have demonstrated minor antigenic differences among the coat proteins of some of the strains (3,4). However, these differences do not appear to be uniquely associated with a particular biological characteristic of the virus strains. To further elucidate the relationships among these viruses, we have performed competition hybridizations to determine the nucleotide sequence homology among the genomes of several of the MMT viruses. Similar assays in which avian RNA tumor virus reagents were employed have been described previously (5,6).

The strategy of these experiments is to anneal highly-labeled MMTV

70S RNA from one virus strain to excess amounts of MMTV-specific DNA synthesized *in vitro* and to test RNAs from other virus strains for their ability to compete with the labeled RNA for annealing to MMTV DNA. To optimize the usefulness of the test, it is necessary to anneal all or almost all of the labeled 70S RNA to the DNA synthesized by the MMTV polymerase *in vitro*. Single-stranded DNA synthesized in the presence of actinomycin D contains sequences complementary to about 60% of the nucleotide sequences of the viral genome (7). Recent experience in our laboratory with avian sarcoma viruses indicates that duplex DNA synthesized in the presence or absence of actinomycin D contains single-stranded tails complementary to the entire avian virus genome. The MMTV polymerase also generates duplex DNA with extensive single-stranded regions complementary to the viral genome (8). I asked whether such a "tailed" duplex might be a suitable reagent for our competition hybridization experiments. Labeled RNA was prepared from cultures of mammary tumor cells from the GR mouse strain. These cells produce large amounts of MMTV without detectable murine leukemia virus (9). MMTV-specific DNA was synthesized *in vitro* in the absence of actinomycin D by polymerase associated with virus produced by GR tumor cells. Duplex DNA was separated from single-stranded DNA by fractionation on hydroxyapatite, and the single stranded regions of the undenatured duplex were examined for their capacity to anneal ^{32}P 70S RNA as assessed by resistance of labeled RNA to digestion by pancreatic ribonuclease (Fig. 1). At a DNA/RNA mass ratio of approximately 50, all of the ^{32}P 70S RNA was found to be ribonuclease resistant. Since 80-85% of the cuplex DNA is resistant to the single-strand specific nuclease S1 (10), only 15-20% of the mass of the DNA

is participating in the reaction; thus the effective DNA/RNA ratio required to protect 100% of the MMTV RNA is approximately 7. We conclude that the single-stranded regions of this MMTV-specific DNA represent a relatively homogeneous transcript of the entire MMTV genome, and that the tailed duplex is a suitable reagent for competition hybridization experiments.

We tested the efficiency of the competition reaction using increasing amounts of unlabeled MMTV-P 70S RNA or GR cell RNA. Both were able to compete 98-99% of the reaction between ^{32}P MMTV-70S RNA and MMTV-P DNA (Fig. 2). Moreover, only a 3-fold excess of unlabeled viral RNA was required to compete 50% of the reaction. When the competition data are plotted semi-logarithmically, the data do not remain linear at high inputs of competitor. This may reflect a requirement for large amounts of competing RNA to saturate the small proportion of sequences over-represented in the viral DNA. In addition to being relatively efficient, the competition is specific, since viral RNA purified from the Schmidt-Ruppin strain of avian sarcoma virus does not compete at all (Table 2). Lastly, since the data for purified viral RNA and for viral RNA in GR cell RNA (determined by an independent method) are virtually identical (Fig. 2), the competition assay can measure the amount of MMTV RNA in cellular RNAs.

We have utilized the competition assay to investiage the relatedness of other MMT viruses to the virus produced by GR tumor cells (MMTV-P). Viral RNAs purified from several sources of MMTV-S and MMTV-L, were used to compete the reaction betwen GR MMTV RNA and DNA. We found (Table 2) that all MMTV RNAs tested competed 96-100% of this reaction. These data indicate that the genomes of the MMT viruses are

all composed of closely related nucleotide sequences.

Our results demonstrate that most or all of the sequences present in the GR virus (MMTV-P) are also present in the other strains of MMTV. This makes it very unlikely that we will be able to prepare nucleic acid hybridization reagents which are specific for any of the strains of MMT viruses. However, at this time we cannot determine whether MMTV-S or MMTV-L contain sequences which are not present in MMTV-P. Likewise, we have not performed thermal denaturation studies which might give an indication of the degree to which the strains of MMTV have diverged. However, since each of the strains exhibits unique biological properties (1,2) and immunological differences have been detected among the viruses (3,4), it is clear that all MMT viruses are not identical. Using the Brownlee and Sanger homochromatography technique (11), which is capable of identifying very minor changes in nucleotide sequence, we have in fact detected differences between MMTV-S and MMTV-P with respect to the products of digestion of the viral RNA by T1 ribonuclease (V. Morris et. al., manuscript in preparation).

Based on the similarities among the MMT viruses and the fact that MMTV-specific nucleotide sequences have been detected in the DNA of all mouse strains which have been tested (12,13), we believe that all strains of MMTV arose from a common progenitor. The biological differences among the strains of MMTV are therefore probably due to a limited degree of mutation and evolution of the primordial MMTV. Although several species of mice (Mus musculus, Mus caroli, Mus cervicolor) contain MMTV-specific sequences in their DNA (unpublished observation of the authors) no such sequences have been found in the most closely related genus, Rattus or in other rodents (e.g., Cricetulus, Microtus,

Peromyscus) (13, and unpublished observations of the authors). Thus, it seems very likely that MMTV was acquired by the genus Mus after its divergence from other rodents (10-15 million years ago)(14).

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TABLE 1

Characteristics of Several MMTV Strains[1]

Strain	Mouse strains	Natural route of transmission	Type of tumor	Oncogenicity
MMTV-S	C3H, A, DBA, wild mice	Milk	Hormone independent carcinomas	High
MMTV-P	DD, RIII GR	Milk Milk & Gametes	Hormone dependent carcinomas (plaques)	High
MMTV-L	C3H, RIII, wild mice	Gametes	Hyperplastic Alveolar Nodules	Low

[1] From Bentvelzen (1974) (reference #1).

TABLE 2

Virus strain	Source of Competitor RNA	% Maximum[1] Competition
P	GR 70S (T.C.)[2]	99
	GR cell (T.C.)	98
S	C3H 70S (milk)	98
	BALB/cfC3H 70S (T.C.)	98
	BALB/cfC3H (milk)	100
	BALB/cfC3H cell (T.C.)	96
	BALB/cf A 70S (milk)	100
	BALB/cf DBA 70S (milk)	98
L[3]	BALB/c infected by C3Hf (milk)	98
	BALB/c infected by wild f (milk)	98
	SR-Avian Sarcoma Virus 70S (T.C.)	0

[1]The reaction between 0.2 ng ^{32}P GR-MMTV 70S RNA and 14 ng GR-MMTV DNA was competed with saturating amounts ($\geq$ 200 ng) of purified viral 70S RNAs or cell RNAs containing MMTV RNA. Annealings were performed as described in the legend to Figure 2. The percentage of ^{32}P 70S RNA annealed was determined by its resistance to digestion by pancratic ribonuclease.

[2]T.C. = tissue culture

[3]NIV was collected from the milk of BALB/c mice that had been infected with virus from C3Hf mice by a mammary gland transplantation procedure or virus from wild mice by foster nursing as described previously (Blair, 1970 & 1971, ref. 3 & 4).

Figure 1. ^{32}P MMTV 70S RNA (1000 cpm; approximately 0.16 ng) was annealed with increasing amounts (0.7-21 ng) of unlabeled DNA synthesized by MMTV polymerase in the absence of actinomycin D. Approximately 800 ng of MMTV DNA were synthesized in a 120 ml reaction containing: virus pelleted from 7 liters of culture fluid from GR tumor cells, 10^{-4}M deoxynucleoside triphosphates, 0.3% NP-40, 3mM $MgCl_2$, 1.5% 2-mercaptoethanol, and 0.1M Tris pH 8. . The reaction was incubated at 37°C for 12 hr and then treated with sodium dodecyl sulfate (0.5% w/v) and pronase (500 μg/ml) for 1 hour. Protein was removed by extraction with phenol and RNA was removed by treatment with pancreatic ribonuclease. DNA was fractionated on hydroxylapatite columns into single and partially double-stranded components. The yield of DNA was estimated from an aliquot of the reaction in which ^{3}H-TTP (2.4×10^{-6}M) was included. Preparation of ^{32}P MMTV 70S RNA and the assay for ribonuclease resistance have been previously described (7). Hybridization was at 68°C for 64 hr in 0.6M NaCl in a reaction volume of 5 μl.

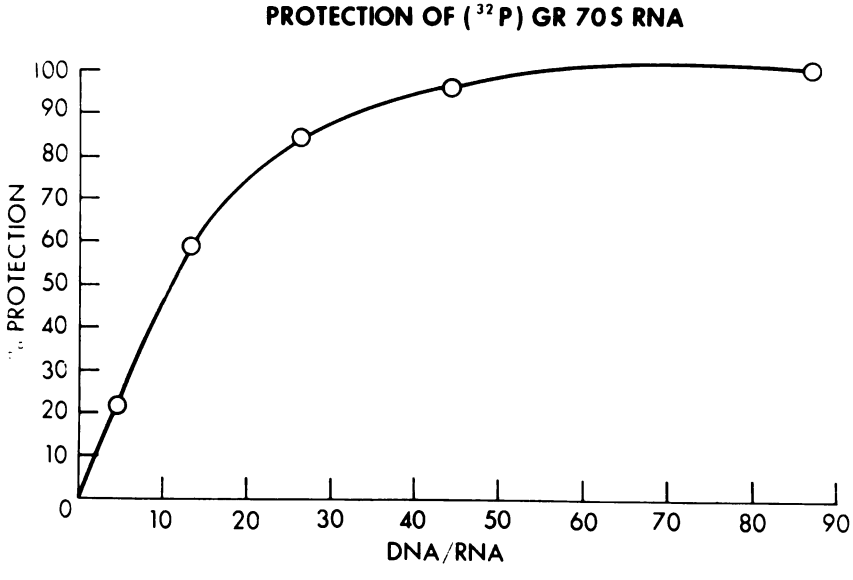
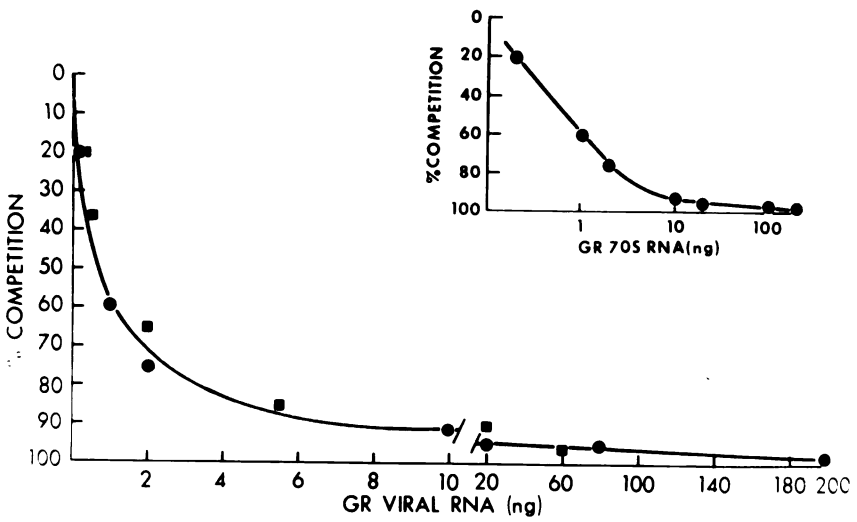


Figure 2. Approximately 0.2 ng of ^{32}P GR-MMTV 70S RNA were annealed with 14 ng of MMTV DNA in the presence of increasing amounts of: (●——●) unlabeled GR-MMTV 70S RNA or (■——■) unlabeled GR cell RNA. Annealings were performed in 0.6M NaCl at 68°C for 45 hr in a volume of 11 μl. The amount of MMTV specific RNA in the preparation of GR cell RNA was determined by molecular hybridization as described previously (7,9). In the preparation used, MMTV RNA constituted approximately 0.1% of the cellular RNA. The insert shows the data for 70S RNA plotted as a function of the logarithm of the competitor RNA.



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