PROGESTIN STIMULATION OF THE PROTOONCOGENE c-myc in T47D HUMAN BREAST CANCER CELLS

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INTRODUCTION

Breast cancer was the leading cause of death by cancers among women in the United States until 1987. Only then did lung cancers supercede it in fatalities (Vital Statistics of the United States, 1996). Whereas lung cancers have several recognized "causes", the avoidance of which minimizes one's risks dramatically, breast cancer etiology is not as clear.

Diet reportedly has some bearing on the risk factors for breast cancer, as is noted for other diseases. Nutritional studies indicate that a well-balanced diet, with emphasis on fruits and vegetables, promotes longevity. The dietary indoles in cruciferous vegetables were found to decrease cancer incidence in animal models, apparently through the induction of cytochrome P450 enzymes (Michnovicz, 1990). Other groups claim special "preventive" properties exist in diets high in fish oils (Iigo, 1997; Rose, 1997), in fiber (De Stefani, 1997; Skankar, 1991), green tea (Weisburger, 1997; Imai, 1997), soybean (Stoll, 1997; Yan, 1997), and garlic (Riggs, 1997; Pinto, 1997). Antioxidant vitamins and mineral supplements were also heralded as "therapy" (Block, 1991; Blot, 1993; Byers, 1992). Several of these food groups do have beneficial effects, reportedly lowering the risks for cancer. No given nutrient, however, has been proven either to prevent or to cure breast cancer (Am. Cancer Society Advisory Committee on Diet, Nutrition, and Cancer Prevention, 1996).

Behavior also is not the answer to a cancer-free life. Exercise is encouraged and indeed does improve one's overall health and quality of living (Shephard, 1993). Avoidance of alcohol, drugs, and tobacco is likewise accepted as advantageous, but again, is no guarantee (Marshall, 1996; Cheng, 1996). Cancers have been linked to radiation exposure (Ponnaiya, 1997), high stress, and even to low level fields of magnetic resonance, pulsed (Ubeda, 1994) or produced from overhead high voltage transmission electric lines (Theriault, 1997; Feychting, 1995), but in the populations **not** exposed, cancers still exist.

Epidemiological studies have uncovered familial links implicating genetic anomalies to

breast cancer. Through the studies of Ashkenazi Jewish women, researchers discovered that abnormalities on chromosome 17 predisposed women to breast cancer (Hall, 1990). Further work identified the gene, dubbed BRCA1, with "hot spots" of deletions/insertions at locus 17q21 (Miki, 1994). A second "breast cancer gene", BRCA2 at 13q12-13 (Wooster, 1995), when carrying germ-line mutations, increases one's risk of developing the disease to 70% by age 70 in women and increases men's lifetime risk 200-fold (Couch, 1996). Mutations affecting both alleles in either BRCA1 or BRCA2 account for nearly 50% of early-onset breast cancers in the Ashkenazi Jews (Bowcock, 1997).

Not only has genetic damage been linked to cancer through documented hereditary predispositions such as these, but also by the assignment of carcinogenicity based on mutagenic potential of substances (Ames, 1979) and from the observation of mutated chromosomes in cancer cells (Rowley, 1984). Typical mutations include translocations or inversions within chromosomes (Klein, 1983; Nowell, 1960; Nowell, 1985), deletions/insertions (Nusse, 1986), and/or abnormal amplifications of large domains (Little, 1983; Schwab, 1985). The role of retroviruses in initiating tumorigenesis and the subsequent recognition of certain cellular genes' involvement therein expanded our awareness of the genetic component of cancer (Bishop, 1983; Varmus, 1982). That faulty repair mechanisms for DNA were linked to increased cancer risk was one more indication of genetic involvement in cancer (Bishop, 1987).

The relationship between loss of self-repair in damaged DNA and cancer susceptibility is the driving force in the search for tumor-suppressor genes (TSGs). When truncation of specific alleles correlates to high incidence of cancers, as in 87% of BRCA1 and 100% of BRCA2 mutations (Couch, 1996), loss of a tumor-suppressor gene is suspected. That different regions of chromosomes house tumor-suppressor genes is commonly investigated using probes of highly polymorphic $(C\text{-}A)_n$ microsatellite repeats. Table 1 lists loci for some putative TSGs identified by this method as well as by other cytogenetic evaluations. Eventual medicinal application of transfection in which disabled genes are replaced with viable alleles is a strong contender for a

working cure for cancer (Slovak, 1996).

The complexity of factors involved in cancer, however, suggests that reinstatement of any given tumor-suppressor gene will not be adequate for curtailment of pathology of the breast. The most frequently mutated gene occurring in sporadic breast cancer is p53, a well recognized tumor-suppressor gene. One study correlated p53 gene mutations in breast tumors with poor prognosis and decreased survival times (Gudas, 1995). In another study of 192 primary breast cancers, however, there was no significant difference in survival rates for patients harboring mutated versus wild-type p53 alleles (Caleffi, 1994). The molecular interaction of growth factors, hormones, and proteins such as p53 involves too many as-of-yet undefined parameters regulating cell growth, differentiation, and apoptosis to be corrected with any single treatment.

TABLE 1

Loci for Putative TSGs	Relevance to Breast Cancer
w/i 16cM of 1q21-31	
1p32-pter	c-myc associated function
1p36	
1q41-44	
3p11-14,14-23,24-26	Loss of Heterogosity (LOH)
6q13-21	
6q23-24	Preferential association with clonal evolution of cancer
6q27	
7q31.1-q31.2	TP53 mutations associated with LOH
11q13	LOH pinpointing lost MEN-1 TSG (multiple endocrine tumor)
11p15	TH-HBB region; Harvey-Ras
11p22-23	PR gene; LOH
11q23.3	
13q12-13	BRCA2
16q22-24	BBC1 (breast basic conserved-1 gene); DPEP1 (dipeptidase-1 gene); E- and M-cadherins; metastasis association
17p13.1	TP53
17q21	BRCA1
17q25	
17	NME-1 metastasis inhibitor protein (nm23)
20q13	

(Slovak, 1996)

Mechanisms governing growth must be elucidated and ultimately manipulated in order to stem the growth of cancers. Genes directly involved in early responses to mitogens, the protooncogenes, are logical targets of investigation. These were initially discovered during the study of the life cycles of retroviruses. Protooncogenes are described as cellular counterparts to those retroviral oncogenes implicated in tumorigenesis ("proto" meaning "earliest form of" and "onco" meaning "tumor"). It is believed that viruses acquired these growth-governing genes via transduction with mutations occurring enroute that rendered them oncogenic. Of the 20 retroviral oncogenes providing models for neoplasms, 9 have recognized cellular versions (abl, erbB, ets, mos, myb, myc, H-ras, K-ras, and sis; prefix "c-" for cellular and "v-" for viral). Mutations resulting in overexpression of protooncogenes can transform established cell lines but usually not primary explants of normal cells. Exceptions to this are c-myc and c-H-ras which can transform even normal primary explants and c-src which cannot elicit neoplastic phenotypes even in cell line cultures (Bishop, 1987).

Amplification of protooncogenes is implicated in the progression of neoplasms rather than in their initiation (Winter, 1986). Of the several protooncogenes involved in carcinogenesis, c-myc at locus 8q24 is very illustrious. In Burkitt's lymphoma, the chromosome carrying c-myc has experienced a translocation mutation (Klein, 1983; Hann, 1988). In other diseases, including small cell lung cancer (SCLC) and carcinoma of the breast (Nau, 1985; Ou, 1996), amplifications in myc genes have been identified in over 20% of human tumors studied (Bishop, 1987). In one study of 185 breast cancers, the most frequent clonal gain was on chromosome 8 in which genes for c-myc and the FGF receptor gene FGFR are affected (Slovak, 1996). Overexpression of the related L-myc, initially cloned from SCLC, has been shown to induce oncogenic transformation upon its own dysregulation (Bossone, 1985). Cases of myc mutations being involved in pathogenesis of cancer are well documented in the literature, illustrating once again the complexity of one factor affecting cancer initiation and progression.

Researchers increasingly probe for clues as to what **causes** cancer. The data suggest that

lifestyle and environmental factors are implicated in up to 80% of cancers, although risk factor-associated cases of breast cancer were reportedly only 25-29% (Seidman, 1982) or at most 55% (Bruzzi, 1985). The risk factors recognized currently are age, family history, reproductive factors, and a previous history of benign breast disease. A summary of these factors and their relative risks are outlined in Table 2. The contribution of hormones to these risks is suggested by the data, and indeed, has been verified for estrogen and progesterone (Bilimoria, 1995).

TABLE 2

Summary of Breast Cancer Risk Factors

Risk Factors for Breast Cancer	Relative Contribution to Disease State	
Family History		
First-degree relative with breast cancer	1.2-3.0	
Premenopausal	3.1	
Premenopausal and bilateral	8.5-9.0	
Postmenopausal	1.5	
Postmenopausal and bilateral	4.0-5.4	
Menstrual History		
Age at menarche less than 12	1.3	
menopause greater than 55	1.5-2.0	
Pregnancy		
1st live birth from ages 25-29	1.5	
after age 30	1.9	
after age 35	2.0-3.0	
Nulliparous	3.0	
Benign Breast Diseases		
Proliferative disease	1.9	
Proliferative disease with atypical hyperplasia	4.4	
Lobular carcinoma in situ	6.9-12.0	(Bilimoria, 1995)

Estrogen and progesterone are members of the steroid family of hormones which operate through nuclear receptors. The nuclear receptor superfamily includes receptors not only for the classical sterioids, estrogen, progesterone, glucocorticoids, mineralocorticoids, and androgens, but also for retinoic acid, thyroid hormone, vitamin D_3, and the so-called orphan receptors whose ligands have not yet been identified. Steroid hormones originate from cholesterol. The cholesterol is transported in the blood by lipoproteins and enters cells where monooxygenases hydroxylate it. The sequential action of several enzymes within cells further metabolize the cholesterol-metabolite

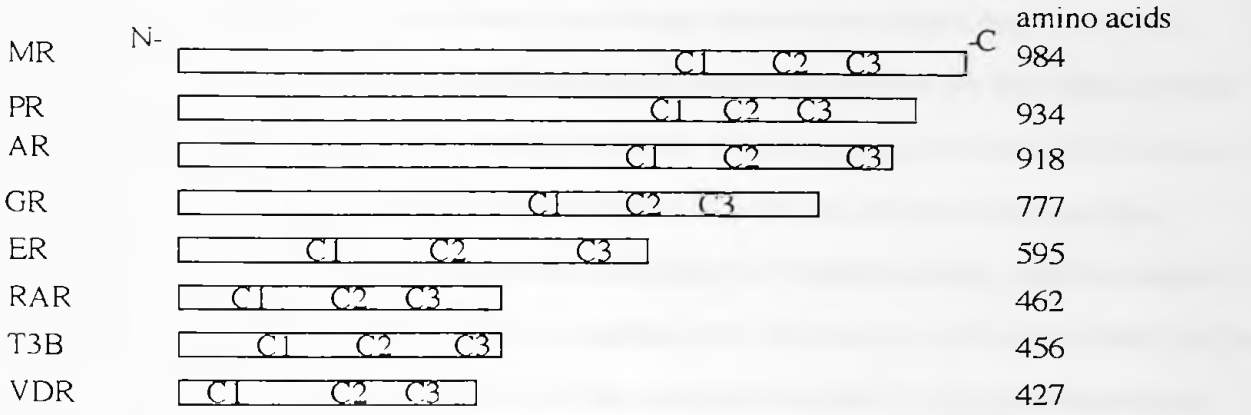
to the specific hormone which is then secreted once again into the bloodstream (Clark, 1992).

Hormones, at concentrations from the picomolar to above the nanomolar range, circulate in the plasma bound to serum proteins. Among the many serum proteins are testosterone-binding globulin (TeBG) which binds estrogen and androgens with a K_d of 10^{-10} M (Soloff, 1971) to 10^{-9} M (Griffin, 1992) and corticosteroid-binding globulin (CBG) which binds glucocorticoids and progesterone with a K_d of 10^{-9}M (Griffin, 1992). The equilibrium dissociation constant, K_d, is a measure of the tightness with which two substances bind, the lower the K_d value, the tighter the binding. A K_d of 10^{-9} M means that at nanomolar concentrations the hormone binds 50% of the globulin proteins. Being hydrophobic in nature, free hormone can diffuse across the plasma and nuclear membranes of all cells. It remains only in those cells which house receptors specific for the given hormone, these receptors having an affinity for the hormone 10-fold greater than that of serum proteins ($K_d = 10^{-10}$M).

Steroid receptors classically meet certain criteria to receive "receptor" status. Firstly, there must be a limited number of receptors per unit mass of tissue with a finite binding capacity; hence binding is saturable. Because hormones circulate at such low concentrations, receptors must exhibit high affinity for their respective ligands. High affinities for specific hormones enable receptors to discriminate among the choices of potential ligands. Although absolute stereospecificity is not achieved, receptors do display steroid specificity. Location of the receptors limits biological responses to those organs/tissues/cells which are appropriate targets. For the binding of receptors to be meaningful, there must be, of course, this biological response (Clark, 1992).

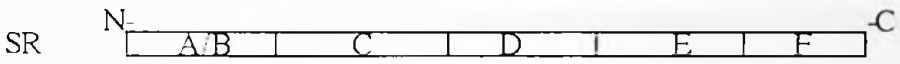
Members of the nuclear receptor superfamily have three regions of consensus homology, designated as C1, C2, and C3, shown below. C1 is the most highly conserved, housing the cysteine-rich DNA binding domain. The functional significance of the homologies in C2 and C3 have yet to be determined (Clark, 1992).

Illustration 1: The Steroid Receptor Supergene Family Homology of Consensus Domains



(Clark, 1992)

Illustration 2: Functional Domains of the Steroid Receptors



Domain A/B - transactivation function; transcription activation function-1 (TAF-1)
C - DNA binding
D - hinge region
E - ligand binding; dimerization; TAF-2
F - unknown function

The liganded receptors act primarily by modulating transcription via interaction with discrete regions of DNA termed Steroid (or Hormone) Response Elements (SRE or HRE). These SREs are enhancer elements, commonly found in the 5' upstream region of genes, but are effective regardless of position or orientation. In general, SREs are imperfect hexanucleotide palindromes separated by spacers of differing lengths. The palindromic nature of SREs suggests that receptors interact with these elements as functional dimers. The transcriptional response to steroids is controlled by much more than mere binding of the hormone-receptor complex to its SRE. In fact, the full role of hormones in gene transcription has yet to be resolved (Carr, 1992).

Much controversy exists concerning absolute requirement for ligand involvement, hormone-

receptor interaction with transcriptional machinery, receptor function, and mechanism(s) of gene activation. For years the exact mechanism by which the hormone bound to its receptor arrived in the nucleus was unsettled. At one point it was thought that the unoccupied receptors were exclusively cytoplasmic. It was thought that chaperone proteins such as the heat shock proteins hsp90 and hsp70 were bound to the receptors, thereby effecting a conformation of the receptor conducive to ligand binding. Upon receptor binding of hormone, the heat shock proteins dissociated, the liganded receptor underwent "activation" or "transformation", and the complex was transported to the nucleus by unknown mechanisms. This theory, commonly called The Two-Step Mechanism, had several variations, but the consensus was that (1) the hormone induced conversion of the native cytoplasmic receptor protein to a biochemically active form that then (2) reacted with target genes (Hansen, 1988). Experimental evidence for this was the observance of liganded receptors' presence in the nuclear fraction versus the cytoplasmic localization of unliganded receptors in cells following homogenization and differential centrifugation (Gorski, 1968; Jensen, 1968). Re-examination of the cellular location of unoccupied steroid receptors began with the demonstration of high aporeceptor yields in the nuclear fraction following ultracentrifugation of highly concentrated homogenates (Sheridan, 1979; Welshons, 1984).

This re-evaluation continued with the development of new investigative techniques. Production of monoclonal antibodies for steroid receptors and the advent of immunocytochemistry allowed the issue of cellular localization to be reexamined. Using non-disruptive in situ hybridization, researchers could demonstrate that both the free and occupied receptors were localized to the nucleus for all steroids but the glucocorticoids (Gorski, 1976; King, 1984). The glucocorticoid receptor (GR) apparently maintains an equilibrium between the cytoplasm and nucleus (Griffin, 1992).

A limitation of immunocytochemical identification of receptor localization has been illustrated, however, with the discovery of membrane-bound estrogen receptors operating through nongenomic estrogen-dependent pathways in pituitary tumor cells (Pappas, 1995). Epitopes on

the receptors against which the antibodies were generated may not always be available on the various forms of receptors possibly existing in the cell. Visualization of the location of antibodies with conventional fluorescence microscopy also was hampered by the spatial noise and flare intrinsic to the device (Pappas, 1995). Evidence that steroids interact with other proteins in the membrane has been reported for estrogen (Smith, 1989), glucocorticoids (Gametchu, 1993), and progestin metabolites (Majewska, 1992). Whether or not these interactions are mediated by conventional or modified steroid receptors has not been unequivocally determined.

When steroid hormones act to alter transcription rates of targeted genes, what mechanisms are employed? Apparently, nuclear localization of receptors is not hormone-dependent. It was postulated that hormone binding caused conformational changes in receptors necessary for the required dimerization of receptors preresquisite to their binding to the HREs. Furthermore, it was supposed that liganded receptors have increased affinity for HREs. These issues have been investigated extensively for the estrogen-estrogen receptor complex.

In one study it was shown that the unliganded, or aporeceptor, for estrogen existed as a phosphoprotein. After hormone treatment, the receptor was hyperphosphorylated and became biologically active. Hyperphosphorylation of the estrogen receptor (ER) was correlated with increased nuclear retention and DNA binding (Denton, 1992). Another study demonstrated that the aporeceptor bound the estrogen response element (ERE) weakly, and it was only upon addition of estradiol that the ER dimerized and tightly bound the ERE (Kumar, 1988). Nonliganded ER was held loosely in the nucleus, easily extracted upon homogenization in hypotonic buffers (Welshons, 1984). With the addition of hormone, however, the ER tightly bound the chromatin, requiring a salt concentration of 0.4M and higher to extract (Gorski, 1976). Using recombinant techniques with the human estrogen receptor (hER), Notides and co-workers demonstrated that the hER exhibits positive cooperativity for estradiol binding, and at physiological ER concentrations the estrogen receptor exists as a dimer (Obourn, 1993).

These findings are contrasted to those of Katzenellenbogen and co-workers in which

similiar levels of ER and RNA polymerase II were associated with chromatin regardless of pre-exposure to ligand (Wrenn, 1990). The unliganded ER altered gene transcription by binding promoter regions. This ligand-independent transactivation was possibly due to the transactivation function-1 (TAF-1) of the ER. Although ligands were not necessary for ER-ERE interaction, they were thought to aid in stabilizing the ER within the cells in lieu of heat shock proteins (hsp). Their data suggested the interaction of ER with heat shock proteins was an in vitro artifact as more than half the ERs were not bound by inhibitory proteins (e.g. hsp90) in whole cells. Ligands may also stabilize ER dimerization, thus effecting increased DNA binding (Reese, 1992).

Other workers likewise report ER's ability to bind ERE oligonucleotides in the absence of ligand or other proteins (Curtis, 1992; Furlow, 1993; Klein-Hitpass, 1989). Dimerization of the estrogen receptor was not directly induced by estradiol (Furlow, 1993), nor was ER dimerization necessary for interaction of the receptor with ERE oligonucleotides (Medici, 1991). A single mutation destroying the dyad symmetry of the 13-basepair consensus ERE (GGTCAnnnTGACC) did not prevent ER interaction, although this interaction was limited to monomers (Medici, 1991). This supports a physiological role for GGTCA half-element pairs in estrogen-induced activation of the chicken ovalbumin gene (Tora, 1988).

Evidence from Gorski and co-workers supports the model that hormone is not required for ER-ERE interaction. Furthermore, in contrast to previous reports, their data suggest that homodimer formation is not only unnecessary, but also does not occur as the active ER form. High affinity binding of the ER to a consensus vitellogenin estrogen response element (vit ERE) exhibits a stoichiometry of 1 mole ER to 1 mole vit ERE, suggesting the active form is a monomer. Estrogen is seen as an inducer of protein-protein, but not protein-DNA, interactions (Furlow, 1993).

Examined at a thermodynamic level, the interaction of the estrogen receptor with an estrogen-responsive DNA sequence was found to display similiar dissociation rate constants (k_{-1}) and equilibrium dissociation constants (K_d) regardless of whether ER was ligand-free or bound by

agonist or antagonist. Curtis and Korach concur with Furlow et al. in that the ligand-binding domain of the ER does not exert a regulatory effect at the level of sequence-specific DNA binding (Curtis, 1992). Even the assumption that an estrogen response reflects relative ERE occupancy of the activated ER may not be fully correct. Webb and co-workers found that EREs were occupied to a very small extent (less than 10%) under normal conditions. The capacity of the cell then greatly exceeds the ER concentration normally found in estrogen-responsive cells, i.e. 1-8 x 10^4 ER/cell. This raises the possibility that estrogen induction is controlled by other parameters, such as the further binding of the receptor to some limiting factor required for full receptor activation. The ER-ERE complexes may be inactive in vivo in the absence of such a factor (Webb, 1992).

An alternate possibility is that the ERE is not always involved in ER-mediated transcription as was noted for the ovalbumin gene in which transcriptional activation occurred indirectly via interaction of ER with the fos-jun complex (Gaub, 1990). Transcription of the human glycoprotein hormone α-subunit gene is negatively regulated by estrogen in the absence of high affinity binding sites/EREs for the ER. Sequences from the 5' flanking region of this gene when transfected into heterologous cell lines likewise fail to bind ER but confer estrogen responsiveness in the form of transcriptional suppression (Keri, 1991). These incidences of ERE-independent estrogen regulation of transcription, however, seem to be more the exception than the general rule. It is well documented that ER-ERE binding serves to enhance gene activation. That several other factors act in conjunction with this continues to be investigated.

The intermolecular engagement of ERs by other proteins has been observed to occur upon activation of the receptor. Formation of these large complexes was found to be ligand-dependent, inhibited by molybdate, and occurred even in the presence of RNase, DNase, and 0.4M KCl. Proteolysis destroyed the complexes which had defined stoichiometry for their large, fixed molecular size. Clearly these complexes involved the receptor with other proteins, but the identity of these proteins had not yet been determined (Nelson, 1989).

Work to identify and characterize those accessory receptor-associated proteins involved in ER-DNA interactions employed immuno-, steroid-, and site-specific DNA-affinity chromatography techniques. Proteins of 70 and 55 kilodaltons (kDa) were in all three column eluates. Proteins of 45- and 48-kDA were eluted in ER-specific affinity columns. The 70-kDa protein was identified as hsp70 and the 55-kDa protein as a member of the protein disulfide isomerase (PDI) family, but the identity of both the 45- and 48-kDa species was not resolved (Landell, 1994). Follow-up work by Landell and co-workers demostrated that the four protein combination altered kinetics of ER-DNA interaction, improving ER's ability to bind vitERE. These ER-associated proteins also induced higher order ER-DNA complexes which significantly distorted DNA conformation (Landell, 1997).

Whether or not these proteins are absolutely essential for high affinity ERE binding is still unresolved. One report claims hsp70 is required for maximal ERE binding when using highly purified recombinant human ER (Greene, 1994). This finding has been contested by other investigators. Although hsp70 was found associated with partially purified calf uterine ER, it apparently was not responsible for increased ERE binding affinity, ligand retention, or total amount of ER-ERE complexes formed. Furthermore, hsp70 did not affect the ER ligand binding domain conformation once the estrogen receptor was activated and occupied by ligand (Klinge, 1997).

Another manner in which estrogen and/or its receptor regulates transcription may be in facilitating the disruption of chromatin structure in the vicinity of the promoter to allow transcriptional machinery access to deoxyribonucleotides. It has been shown that chromatin templates around which nucleosomes are assembled are refractory to transcription (Schlissel, 1984; Knezetic, 1986). Alleviation of the suppressive nature of nucleosomes on regulatory regions such as promoters can occur upon addition of transcription factors before or during nucleosome assembly (Workman, 1988). Disruption of chromatin structure over the promoter was reported to be rapidly induced by hormone. DNase I-hypersensitivity, a measure of chromatin disruption,

was not merely a consequence of receptor binding of DNA, but was greatly enhanced by the presence of transactivation factors. In fact, unless there were multiple steroid receptor dimers binding adjacent SREs, no nucleosome displacement was observed (Pham, 1991).

The involvement of transcription factors in steroid induction of transcription should not be minimized. Accessibility of these factors to the promoter greatly influences the observed results in studies investigating regulation by chromatin. Differences between conclusions from in vitro and in vivo experiments may well be due to the level at which reconstitution is achieved with respect to auxiliary factors. The nuclear receptor co-activator (NCoA) gene family includes SRC-1/NCoA-1, TIF-2/GRIP-1, CBP, p160, and p/CIP which complexes with a significant portion of CBP in the cell. Both p/CIP and NCoA-1 are required for proper functioning of the nuclear receptors for retinoic acid, estrogen, progesterone, and the thyroid hormone (Torchia, 1997).

High levels of histone acetylation of associated DNA disrupt chromatin structure, making it more susceptible to DNase I digestion (Vidali, 1978; Nelson, 1978). Sodium butyrate, an inhibitor of histone deacetylase enzymes (Boffa, 1978; Sealy, 1978) causes extensive hyperacetylation of histones (Riggs, 1977) but was not found to affect overall rates of in vitro transcription in isolated nuclei (Mathis, 1978; Moore, 1978), although this says nothing about the comparative transcription rates of individual genes. The dosage of butyrate commonly used (5-10mM) may have affected observed results. Bartsch et al. demonstrated that butyrate concentrations in excess of 4mM had inhibitory effects on basal and hormonally induced gene transcription, whereas maximal stimulation occurred at 0.5-1mM (Bartsch, 1996). Later work using the steroid retinoic acid (RA) provided evidence for hormone-induced alterations of chromatin structure that enabled accessibility within the promoter and was correlated with transcriptional activation. The chromatin structure in and around the promoter underwent dynamic, reversible changes without globally affecting nucleosomal organization (Bhattacharyya, 1997). Other work has indicated an involvement of liganded ER in facilitating the opening of chromatin to transcriptional machinery in the presence of the steroid receptor coactivator-1 (SRC-1). SRC-1 is

believed to act in coordinating nuclear receptors' amino- and carboxyl-terminal transcripton activation functions (TAF-1 and -2, respectively) for full activation of the receptor (McInerney, 1996). Identification of the steroid receptor coactivator-1 as a histone acetyltransferse (Spencer, 1997) further substantiates steroid involvement in gene activation at multiple levels.

Although controversy continues to exist as to the exact mechanisms by which hormones act in regulating gene expression, it is undisputed that estrogen and progesterone affect the growth and development of mammary tissues. In fetal life proliferating epithelial cells form approximately two dozen short cords which become mammary ducts connected to the nipple. Alveolar structures derive from the blind end of these ducts later in gestation. With the removal of placental estrogen and progesterone, however, growth of the breast halts. Mammary development resumes shortly before onset of menarche due to the increased secretion of ovarian estrogen. The ducts lengthen and branch, and their terminal ends bud. Estrogen itself is not sufficient for induction of ductal growth, but acts in concert with anterior pituitary hormones. Only in the presence of growth hormone and prolactin does estrogen promote ductal development. Lobuloaveolar develpment is controlled by progesterone and prolactin, although concomitant or preceeding estrogenic stimulation is also required for full progestin activation (Frantz, 1992). The interplay between these two steroid hormones can be antagonistic or stimulatory, depending on the particular cellular environment (Gronemeyer, 1991; Kraus, 1995). This cross-talk between estrogen and progesterone has added to the controversy surrounding their respective roles as mitogens in mammary cells.

The progesterone receptor has multiple isoforms, PR-A, PR-B, and, putatively, PR-C (Wei, 1990), all arising from the same gene, locus 11q13 (Law, 1988), but differing in size due to variations in transcription start sites. Data from the labs of Gronemeyer, Chambon, and Horwitz suggest PR isoforms A and B are translated from different mRNAs (Kastner, 1990). This is supported by the identification of multiple species of PR mRNA in normal human endometrium and the breast cancer cell lines T47D and MCF-7 ranging from 2.8 kb to 11.4 kb (Read, 1988).

Whereas PR-B originates from the first AUG codon, PR-A's start codon is located downstream, but in-frame, at AUG 165. PR-C utilizes a methionine at codon 595 in exon 2 as its start codon. The N-terminally truncated PR-C lacks the first DNA-binding zinc finger of PR but contains the second zinc finger, the hinge region, and the hormone-binding domain (Wei, 1990). Additional progesterone receptor mRNAs not encoding B- or A-receptors have also been isolated which could be translated into PR-C. In vitro translated PR-C was found to act functionally in the presence of PR-A and -B and may provide another means of modulating transcriptional activity of the progesterone receptor (Wei, 1996).

Investigators have examined variations in estrogen and progesterone receptor content as a function of age, menstrual, and menopausal status in breast cancer patients. Surprisingly, fluctuations in steroid receptor content seen throughout age and menstrual cycling were not correlated with blood levels of the steroid hormones in normal subjects. Within the carcinoma groups, however, associations were observed between ER and PR content and blood steroid hormone levels in the luteal phase of the menstrual cycle and within postmenopausal women above 59 years of age. A decrease in ER in mid-cycle versus perimenstrual cycles was noted. Middle-aged (45-59 yrs.) carcinoma groups also were associated with decreased PR content and ER function. In those carcinoma groups experiencing the lowest hormone levels and highest ER content, the ER levels were found to decrease as the estrogen or ratio of estrogen-to-progesterone blood levels rose. This was in contrast to carcinoma groups with high progesterone levels in which PR content increased with increasing ratio of estrogen-to-progesterone blood levels (Nikolic-Vukosavljevic, 1996).

Another study involving 33 premenopausal women noted changes in ER and PR content in uterine tissue during the cycle with maximal concentrations of both receptors occurring in mid- to late proliferative phase. Whereas ER declined throughout the secretory phase, PR had complex dyssynchronous fluctuations during the same phase. In the glandular epithelium, PR content decreased dramatically. In the stroma and myometrium, however, a significant amount of PR was

maintained. Alterations of receptor content as evidenced here may be one method of regulation in steroid-dependent growth and differentiation in target tissues (Lessey, 1988).

Estrogenic control of progesterone receptor transcription is yet another manner in which the hormone environment has an impact on tissue growth and differentiation in hormone responsive cells. In the rat PR gene there are multiple, widely spaced EREs capable of weak but detectable ER-ERE binding (Kraus, 1994). Estrogen treatment stimulates PR synthesis alone or synergistically with serum components insulin and the insulin-like growth factor-1 (IGF-I) (Katzenellenbogen, 1990). This increase in PR levels is due to estrogenic stimulation of the rate of synthesis rather than affecting PR's rate of degradation (Nardulli, 1988). The estrogen receptor's transcription activation function-1 (TAF-1) is not required for estrogen regulation of PR gene transcription, although TAF-2 apparently is needed (Savouret, 1994).

Induction of the progesterone receptor gene can also be accomplished in hormone-free conditions with the addition of agents which increase intracellular cyclic 3', 5'-adenosine monophosphate (cAMP) levels. Maximal enhancement of PR levels was seen upon treatment with micromolar concentrations of 8-bromo-cAMP (8-Br-cAMP), and this stimulation was negated by subsequent addition of the cyclic nucleotide-dependent protein kinase inhibitor H8 or the protein kinase A inhibitor PKI (Aronica, 1991). The effect of 8-Br-cAMP on ligand-independent transactivation was more pronounced for the human PR A isoform (hPR-A) than for the B isoform (hPR-B). Furthermore, 8-Br-cAMP treatment with or without synthetic progestin R5020 down-regulated levels of hPR-B in COS-1 monkey kidney epithelial cells with only marginal effects on hPR-A levels. This suggests phosphorylation may differentially regulate activity of PR isoforms which themselves apparently operate as distinct regulators of differentiation and proliferation (Kazmi, 1993).

An increase in PR expression can manifest itself as increased transactivation as evidenced by a 3-4-fold increase in PR-mediated gene transcription upon dual treatments with progestins and 8-Br-cAMP or okadaic acid. Again this stimulation was abrogated with co-treatment of H8.

Although progestins cause a 2-fold increase in phosphorylation of receptor isoforms hPR-A and B, neither 8-Br-cAMP, okadaic acid, nor H8 changed the total ^{32}P labeling of PR nor were responsible for phosphorylation of receptor resulting in a slower migrating species on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) mobility assays. Apparently cAMP-dependent phosphorylations affecting PR transcriptional efficiency involve other integral proteins or are on key sites of the PR and are not represented as a change in total phosphorylation (Beck, 1992).

What role phosphorylation of PR plays in transactivation of the receptor is under considerable investigation. In one study in which hPR was overexpressed in Spodoptera frugiperda (Sf9) insect cells using the recombinant baculovirus system, it was determined that hormone-induced PR phosphorylation was not responsible for PR upshifts on SDS/PAGE mobility assays, nor did it impact on receptor-DNA binding (Christensen, 1991). Data from Horwitz and co-workers supported Christensen's finding that hormone-induced phosphorylation was not necessary for nuclear binding. They reported that within 15 minutes of translation both PR isoforms could undergo transformation and bind DNA with accompanying secondary phosphorylation. Horwitz et al. did, however, provide evidence that the slower, hormone-induced phosphorylation step was responsible for migration differences for the 114-, 117-, and 120-kDa PR-B species (Sheridan, 1989).

Phosphoamino acid analysis of PR from the human breast cancer cell line T47D revealed phosphorylation occurred on serine, but not on tyrosine or threonine residues. 10nM progesterone treatment increased PR phosphorylation 2-fold in contrast to treatment with epidermal growth factor (EGF), the Protein Kinase C stimulant 12-O-tetradecanoylphorbol-13-acetate (TPA), or dibutyryl cAMP, which did not significantly affect phosphorylation of PR (Rao, 1987). Phosphorylation of serine residues in the chicken oviduct PR was found to be important in regulating both basal and hormone-induced activity. Basal activity occurred when Ser-211 and Ser-260 were 20% phosphorylated and Ser-530 dephosphorylated. Hormone treatment resulted in a

1.5- to 2-fold increase in phosphorylation of Ser-211 and -260 and a greater than 33% phosphorylation of Ser-530 (Bai, 1994). The cAMP-dependent protein kinase is known to phosphorylate Ser-528, and the catalytic subunit of this kinase mimicked the hormonally-induced increases of serine phosphorylation (Denner, 1990).

This increase in serine phoshorylation is in agreement with studies of O'Malley and co-workers in which chicken PR phosphorylation increased on 3 sites in response to hormone, causing a change in receptor mobility on SDS/PAGE gels. This phosphorylation, however, was strictly dependent on double stranded DNA and was thought to be accomplished by a DNA-activated protein kinase similar or identical to one isolated from nuclei of HeLa cells, a human cervical carcinoma cell line. This series of DNA-dependent phosphorylations may be required for PR activation, by-passing hormone requirements as noted in in vitro transcription assays (Weigel, 1992).

Additional sites of serine phosphorylation were noted in human PR and their function tested in 4 sets of serine to alanine substitution mutants. Mutation of 3 sites, Ser-190 at the amino-terminus of PR-A, a cluster of serine residues just upstream of the DNA-binding domain (DBD), or Ser-676 in the hinge region in the B-upstream segment (BUS) inhibited transcription by 20-50% in a promoter- and cell-specific manner. Mutation of 10 serine clusters located in regions common to PR-A and PR-B and mutations of 6 serines peculiar to BUS, however, did not diminish the transactivation function of either the full-length hPR-B or the construct BUS-DBD-NLS in which the TAF-3 function of the B-upstream segment (BUS) was fused to the DNA binding domain (DBD) and the nuclear localization signal (NLS) of hPR. These data suggest that phosphorylation does not affect transactivation mediated by TAF-1 or TAF-3, but may act on the transcriptional machinery distinguishing the A and B isoforms of hPR (Takimoto, 1996).

Intrigued by the presence of multiple isoforms for the human progesterone receptor, researchers have sought to delineate specific functions for these proteins. It appears that hPR-B behaves as a typical transcriptional activator of progestin-inducible genes, whereas hPR-A can

function as a transcriptional inhibitor or activator, depending on the context. hPR-A was shown to repress hER-mediated transcriptional activity in a manner largely dependent on absolute expression levels of hPR-A rather than hER (Wen, 1994). The A isoform hPR protein can also inhibit hPR-B-induced gene activation. In promoter and cell contexts where hPR-A was inactive, it functioned as a strong trans-dominant repressor of hPR-B. Although hPR-A did not repress vitamin D receptor function, it did inhibit transcription induction by classical steroids glucocorticoid, androgen, and mineralocorticoid receptors. The trans-dominant effects of hPR-A were also observed using a DNA-binding defective hPR-A mutant (Vegeto, 1993). PR-A repression apparently, then, does not require direct interaction with chromatin, but as in the case of hER-mediated repression, involves noncompetitive interaction of the receptor with distinct contact sites or separate cellular targets. Using two breast cancer cell lines independently expressing only B- or A-receptors, Miller et al. demonstrated that R5020-stimulation of flavin-containing monooxygenase 5 (FMO5) occurred only in PR-B cells with A-receptors again being inhibitory (Miller, 1997).

Differential effects of hPR-A and -B on estradiol-dependent transcription were also reported by Chalbos and co-workers. Treatment with the synthetic progestin R5020 potently suppressed estradiol-induced levels of pS2 and cathepsin D mRNA, but unlike Wen's report, occurred only in cells expressing hPR-B. PR-B transrepression was dependent on promoter context, but PR-A was ineffective regardless of the reporter construct used. When both isoforms were equally present, PR-B was dominant, maintaining >70% inhibition. Unlike hPR-A-mediated repression, hPR-B apparently acts on the estrogen receptor to repress its TAF-2 activity (Chalbos, 1994).

hPR-A and -B also behave differently when occupied by progesterone antagonists. In HeLa cells co-transfected with a PRE-tk-CAT reporter and hPR-B expression vectors, strong transcriptional activity occurred upon PR activation with either agonist (R5020) or antagonist (RU486, ZK112993, or ZK98299). Antagonist-induced transcription was PRE-independent but did require a functional DNA-binding domain (DBD) on hPR-B. hPR-A was not activated when

occupied by antagonists. Furthermore, when co-expressed with hPR-B, the A-isoform worked in a dominant fashion to annul the inappropriate activation of hPR-B by antagonists (Tung, 1993).

One explanation for these different isoform-dependent behaviors may be the additional transactivation function, TAF-3, located in hPR-B's B-upstream segment (BUS). Similar to antagonist-activated PR-B activity, BUS-DBD-NLS constructs require an intact hPR DBD to strongly and autonomously activate transcription. In a promoter- and cell-dependent manner, TAF-3 can activate transcription autonomously or synergistically act with TAF-1 and TAF-2. It is the autonomous TAF-3 function that may be responsible for antiprogestin-occupied hPR-B's stimulation of gene expression (Sartorius, 1994).

Differences in phosphorylation of hPR-A and -B were also questioned as possible means of receptor regulation. In hormone-free conditions 8-Br-cAMP was found to amplify hPR-A-mediated transactivation more strongly than that of hPR-B's. 8-Br-cAMP +/- R5020 down-regulated hPR-B levels in COS-1 cells while only marginally affecting hPR-A content. Apparently, then, phosphorylation can indeed affect differentially the activity of these two isoforms (Kazmi, 1993).

In "normal" PRE-dependent transactivation it was questioned as to whether or not differences also occurred in the interaction of A- and B-receptors with the chromatin. Edwards and co-workers reported equal enhancement of PR-DNA binding by the high mobility group chromatin protein HMG-1. Because HMG-1 recognizes distorted DNA structures and can cause further distortion by bending DNA, it is possible that HMG-1 enhancement of PR binding occurs via structural alterations in target DNA (Prendergast, 1994).

Follow-up work by Prendergast et al. on the ability of hPR-A and -B to bend target DNA revealed many similiarities. Both PR isoforms induced substantial distortions with directional bends toward the major groove of the DNA helix. The apparent bend centers were similiarly located a few basepairs from the middle of the PRE. Both the calculated distortion angles and directed bend angles, however, were greater for hPR-B than for -A. The greater transcription

activation potential of hPR-B may be linked, then, to its more pronounced effect on DNA structure (Prendergast, 1996).

That liganded PR acts in remodeling chromatin during transcriptional activation is supported by the observation that the coactivators SRC-1 and CBP are required for PR transactivation. SRC-1 and CBP have histone acetyltransferase (HAT) activity, bind to themselves, and recruit other HAT factors to target promoters. Inhibition of histone deacetylase strongly pontentiated PR-mediated transcription (Jenster, 1997). Together these data suggest liganded PR acts ultimately, as does the ER, to de-repress chromatin and facilitate assembly and stabilization of the transcriptional machinery.

Understanding the molecular dynamics of gene activation provides the basis for comprehending cell growth, differentiation, normal cell death, and ultimately, even their dysregulation as seen in cancer. One proto-oncogene intimately involved in all four parameters is c-myc. Mishaps with expression of this proto-oncogene have been reported in Burkitt's lymphoma (Hann, 1988; Klein, 1983), small cell lung cancer (SCLC) (Nau, 1985; Ou, 1996), and in a preponderance of primary breast cancer tumors (Varmus, 1982). So, what is this proto-oncogene's function? How does it work? How is it regulated? And, could steroid hormones affect its behavior?

The human c-myc gene encodes two functional polypeptides, Myc-1 (439 amino acids (aa) with an apparent molecular weight, M_r, of approximately 65,000) and Myc-2 (453 aa, M_r of approximately 68,000). These proteins do not arise from post-translational interconversions, but are derived from alternative translational initiations at exons 1 and 2 of myc mRNA. (Hann, 1988) A third polypeptide, mycHEX1, only 188 aa in length and of unknown function, arises from a nonconventional start codon further upstream in exon 1 which was previously thought not to be translated (Gazin, 1986; Dedieu, 1988). Myc-1 and -2 contain domains common to transcription factors, namely the leuzine zipper (LZ) (Landschulz, 1988) and helix-loop-helix (H-L-H) domains (Murre, 1989), a helical stretch of basic amino accids termed the basic region (BR) (Agre, 1989),

and a nonapeptide nuclear localization signal (NLS) (Dang, 1988). The H-L-H and LZ motifs are required for Myc dimerization with Myc's partner protein Max ("myc Associated X"), whereas the BR of Myc is required for the Myc-Max heterodimers to bind DNA (Blackwood, 1991).

Although Myc is involved in cell cycle regulation during G_o-G_1 transition and entry into S phase, its mRNA expression is not restricted to any phase, but rather, is maintained in continuously proliferating cells (Thompson, 1985). Levels of Myc protein rise rapidly within the first 2 hours of G_1 upon mitogenic stimulation, are maintained throughout G_1, and then return to basal levels for the remainder of cell cycle progression. Thompson and co-workers have reported that c-myc mRNA transcription remains constant throughout the cell cycle, even in density-arrested cells supplied with serum growth factors (Thompson, 1985). Not only is the synthesis of Myc protein maintained during all cell cycle phases, but the half-life and modification of c-myc proteins likewise remain constant both in normal and transformed cells (Hann, 1985). mRNA levels naturally do decline upon removal of serum and upon cells reaching confluency. Dean and co-workers have reported that this effect on steady state mRNA levels occurs without affecting the level of transcription, suggesting post-transcriptional control also exists (Dean, 1986a). The c-myc gene is down-regulated after growth ceases and with the onset of differentiation (Spencer, 1991).

Platelet-derived, epidermal, fibroblast, and transforming growth factors (PDGF, EGF, FGF, TGF-α, and TGF-β (in mink lung epithelial cells)) promote transcriptional initiation of c-myc in various cellular contexts. (Kelly, 1983; Cutry, 1989; Skouteris, 1992; Sutherland, 1992; Paterson, 1995; Kim, 1991) Insulin and IGF-1 (Gai, 1989; Banskota, 1989), IL-2, phytohemagglutinin (PHA) and 12-O-tetracedanoyl phorbol-13-acetate (TPA), as well as ionomycin (Lindsten, 1988; Reed, 1985; Reed, 1986), Concanavalin A, lipopolysaccharide (Kelly, 1983), pregnant mare serum gonadotropin (PMSG) (Delidow, 1990), erythropoietin (Li, 1996), dsRNA (Mundschau, 1995), adrenocorticotropin hormone (ACTH), dibutyryl cAMP (in adrenal tissues), and the protein synthesis inhibitor cycloheximide (Liu, 1996) also promote c-myc

expression.

Other growth factors, TGF-β (Pietenpol, 1990; Paterson, 1995) and the tumor necrosis factor-α (TNF-α), plus dibutyryl cAMP (in HL-60 cells) (Kronke, 1987) down-regulate c-myc via inhibition of transcriptional initiation. Blocks to transcriptional elongation is another way in which reagents limit Myc protein production. Substances operating via this route include retinoic acid (Ou, 1996), 1,25-dihydroxy vitamin D_3 (Hulla, 1995; Simpson, 1987), dimethylsulfoxide (DMSO), a potent inducer of granulocytic differentiation (Eick, 1986), and PKC inducers 1,2-dioctanoylglycerol and phorbol 12,13-dibutyrate (Salehi, 1988).

Multiple levels of regulation of c-myc expression tightly control the amount of the gene product Myc. Myc protein expression is regulated at the level of transcriptional initiation and elongation, posttranscriptionally by different portions of the c-myc transcript affecting message stability, at the level of translation, and by post-translational modifications as well (Marcu, 1992). The function of Myc protein is only beginning to be elucidated.

The Myc protein acts as a sequence-specific transcription factor when bound as a heterodimer to Max, a basic region, helix-loop-helix, leucine zipper [(BR)H-L-H(LZ)] protein of 160 amino acids (Blackwell, 1990). Myc's ability to function also as a DNA replication factor was suggested by experiments in which treatment with c-myc antisense oligonucleotides prevented entry of cells into the S-phase of the cell cycle (Heikkila, 1987). Binding of the Myc/Max protein complex to a 7bp sequence in the regulatory region upstream of the first exon of the c-myc gene was shown to be indispensable to both maximal ori and enhancer activities (Ariga, 1989), again suggesting a role for Myc in regulation of DNA replication.

Evidence for Myc's role as a transcription factor binding specifically to CACGTG sequences in the DNA (Blackwell, 1990) came with demonstration of Myc/Max binding to the 5' upstream region of the dihydrofolate reductase gene (DHFR). DHFR is a key enzyme in folate metabolism supplying the cells with dTTPs necessary for DNA synthesis (Mai, 1994). Myc can

also suppress genes as was noted in its repression of CCAAT/enhancer-binding protein α-gene (C/EBPα) expression through interactions with the core promoter region (Antonson, 1995).

Another way in which Myc protein may impact on growth in breast cancer cells is via interaction with other transcriptional regulators including Yin-Yang-1 (YY1) which itself behaves as transcriptional activator, repressor, or initiator depending on cellular context. Binding of Myc protein to YY1 completely inhibited YY1's repression of thymidine kinase in reporter constructs (Shrivastava, 1993). Myc has also been shown to inhibit activity of TFII, another transcriptional initiator (Roy, 1993). Myc can also bind the TATA-binding protein (TBP) of the TFIID transcription initiation complex (Maheswaran, 1994), thereby enabling Myc regulation of growth processes at the level of the basal transcriptional machinery.

The Myc protein can bind a regulatory region upstream of c-myc's own exon 1 to positively regulate its own expression (Ariga, 1989). It is also regulated in a cell- and promoter-specific fashion by other oncogene products. Early transforming proteins of the polyoma virus cause increases in c-myc transcripts (Zullo, 1987), as does c-myb expression (Evans, 1990; Cogswell, 1993). v-abl in a myeloid cell line upregulated c-myc transcriptional initiation (Birchenal-Roberts, 1996), but in a fibroblast line was responsible for amplified c-myc loci with concommitant transcriptional blockage resulting in reduction in Myc protein (Nepveu, 1985). c-Abl is a nonreceptor protein tyrosine kinase which increases expression of c-myc reporter constructs, likely via interaciton with other DNA binding proteins (Arcinas, 1994). In mice adrenal glands, kidneys, and splenic B cells, but not in heart, liver, thymus, brain, and lung, c-Abl can directly transactivate c-myc transcription (Wong, 1995). Other tyrosine kinases, fms, src, and trk, cooperate in promoting constitutive myc expression (Cleveland, 1989). v-raf and fos/jun complexes, however, negatively regulate myc (Zullo, 1988; Hay, 1989). The rel-oncogene-related family of NF-κB factors have differential effects on the c-myc promoter. Classical NF-κB potently activated c-myc transcription unless co-expressed with v-rel. v-rel itself or chicken c-rel

did not affect transcription whereas murine c-rel had a slight stimulatory effect (La Rosa, 1994). Conflicts in reports over myc regulation by TGF-β, dibutyryl cAMP, and v-abl, plus others not noted here indicate that cellular physiology impacts greatly on signaling pathways responsible for growth regulation.

Steroid hormones estrogen, progesterone, glucocorticoids, and retinoic acid, as noted previously, also affect c-myc regulation. Growth inhibitory effects of glucocorticoids in lymphoid cells involve down-regulation of myc (Rhee, 1995) at the level of transcriptional initiation (Forsthoefel, 1987). In glucocorticoid-treated fibroblast cells, however, inhibition of proliferation did not involve regulation of either c-myc mRNA or protein (Frost, 1994). These experiments demonstrate the importance of cellular context for the role of c-myc in regulation of cell proliferation.

Stimulation of growth in the human breast cancer cell line MCF-7 by estrogen is supported by rapid induction of c-myc, independent of protein synthesis (van der Burg, 1989). Estrogenic stimulation in MCF-7 cells of the myc gene is exclusively at the level of transcription (Dubik, 1988). Although a consensus ERE was not identified, a 116-bp region encompassing the TATA box of the P2 promoter was found necessary for estrogen-mediated regulation of c-myc (Dubik, 1992). In normal human breast epithelial (HBE) cells, 10nM estradiol elicited a biphasic increase in c-myc mRNA levels with the first peak at 30 minutes and the second at 2 hours post-treatment. This stimulation again was found to be at the transcriptional level (Leygue, 1995). In male Wistar rats administration of estradiol resulted in increased c-myc mRNA levels in their anterior pituitary glands. Occurring with estogen-induced c-myc stimulation was transcription of the prolactin gene (Szijan, 1992).

The effect of progestins on c-myc expression in the breast cancer cell line T47D is complex. Initially, synthetic progestins were reported to cause a rapid but transient induction of c-myc, a transient acceleration of cells already in G_1 of the cell cycle through its cycle of replication,

and cellular arrest in the following cycle with subsequent growth inhibition (Musgrove, 1991). Treatment of T47D cells with progestin medroxyprogesterone acetate caused a 2-fold increase of c-myc mRNA levels, followed by a decrease, and then partial recovery (Wong, 1991). Both these groups, however, reported that progestin inhibited cell growth. A possible mechanism whereby the progesterone receptor itself could repress the c-myc promoter has been postulated for the avian oviduct system. A 54-bp receptor-binding factor (RBF)-binding element located between dual matrix-associated regions of c-myc's P2 promoter provides a unique chromatin/nuclear matrix structure which can bind the PR (Lauber, 1997). Occupation of this β-sheet structure could cause DNA conformational changes so as to disrupt normal processing by transcriptional machinery.

Although progesterone was reported to be repressive in the growth of breast cancer cells by numerous investigators in previous decades (Horwitz, 1985; Chalbos, 1982; Lippman, 1976), data from Moore and co-workers and others demonstrated stimulation in these cell lines (Hissom, 1989; Moore, 1991; Manni, 1987; Robinson, 1987; Longman, 1987). A major contribution to reconciliation of these conflicting reports was Katzenellenbogen's discovery of estrogenic properties of phenol red, a pH indicator routinely included in cell culture media (Berthois, 1988). Variation in experimental conditions, in the quantity and quality of serum's constituents, growth factors, trace hormones, and as-of-yet still undefined components of signaling pathways, is increasingly recognized as critical in defining cellular context, and ultimately, the observed response (Moore, 1981; Welshons, 1992). The modification of Horwitz's stance on progestins' relation to breast cancer growth exemplifies the evolution of the research community in grappling with the many elusive factors affecting growth regulation (Groshong, 1997).

Since the mid-80's Moore and co-workers have rigorously maintained that progestins could and did behave mitogenically in T47D breast cancer cells (Hissom, 1987; Hissom, 1989; Bowden, 1989). In trying to unravel the mechanism(s) by which progestins stimulate growth, these workers have examined various elements that might be involved in this response. Correlative increases in lactate dehydrogenase (LDH) activity upon progestin treatment were examined

(Hagley, 1987) but were found to be dependent on RNA and ongoing protein synthesis, and thus were not due to direct gene activation by this hormone. This was also true for the dose-dependent elevation of thymidine kinase activity witnessed upon progestin stimulation in T47D cells (Moore, 1991). A possible gene target for progesterone-mediated growth stimulation would be a protooncogene involved in immediate early responses to mitogen such as c-myc.

We have hypothesized that progestins elicit a growth response, in part, due to direct activation of the c-myc gene. To test this hypothesis a series of experiments was proposed in which we examined levels of c-myc mRNA following treatment with the synthetic progestin R5020. They are as follows.

T47D cells were treated with R5020 at various concentrations to determine optimal dosage and for various time intervals to pinpoint the treatment time for maximal response.

Co-treatment with the antiprogestin RU486 was done to assess more clearly whether or not stimulation of c-myc was likely operating through PR-mediated pathways. Earlier, Moore and co-workers had uncovered concentration-dependent growth agonist activity of RU486, establishing RU486's dual potential as antagonist/agonist. (Bowden, 1989) In our experiments the concentration of RU486 was kept well below the growth agonist-associated concentration.

The possibilty that progestins stimulated c-myc via a nonspecific steroid mechanism was addressed by comparing c-myc induction by members of each of the five classes of steroid hormone at a physiologically relevant concentration of 10 nM. This experiment also allowed us to determine what other hormones might stimulate myc under our experimental conditions. Dubik and Shiu had reported estrogen stimulation of c-myc in the ER-positive cell line MCF-7 (Dubik, 1988), and reports conflicted over glucocorticoids' ability to transactive the c-myc gene (Forsthoefel, 1987; Frost, 1994). These hormones have not been tested for their effect on c-myc gene expression in T47D cells.

To determine whether or not the synthesis of new proteins was required to elicit progestin-mediated changes in c-myc mRNA levels, pre- and co-treatment with the protein synthesis inhibitor

cycloheximide (CHX) was performed. Preliminary experiments were conducted to determine the cycloheximide dosage, as well as the duration of exposure and treatment regimen, required to inhibit at least 90% of protein synthesis. Viability of cells was initially checked at a much higher dose of CHX than what was eventually used in the actual experiments. This check, however, allowed us to verify that any reduction in protein synthesis observed was not simply due to cell death.

Increases in mRNA levels can be due to enhanced transcription, reduced degradation, or a combination thereof. Stabilization of transcripts results in a longer half-life for the message and is a common mechanism by which mitogens operate. To investigate if R5020 was indeed protecting c-myc mRNA from degradation, transcription was halted for various lengths of time in both control and hormone-treated cells with the addition of the inhibitor actinomycin D after 1 hour of R5020/control exposure. By blocking elongation, actinomycin D eliminated production of any new transcripts, so that the relative levels of c-myc mRNA over time were indices of message decay. Information from the graph of these data was used to determine c-myc mRNA's half-life in both control and R5020-treated cells. A change in mRNA stability could be responsible for observed differences in mRNA levels, but would not preclude hormonal enhancement of transcription.

To specifically address progestin activation of transcription, nuclear run-on assays are needed. In this assay the nuclei of treated cells are isolated. Within these nuclei are the genes that have been transactivated and a limited number of pre-mature mRNA transcripts that have yet to be exported to the cytoplasm. Elongation of already initiated transcripts is the only source of quantifiable c-myc levels. The nuclei are treated with a cocktail in which one ribonucleotide is radioactively labeled, thereby providing a tag for freshly transcribed mRNA species. This mRNA is isolated and quantitated as detailed in Materials and Methods. Any differences in newly synthesized mRNA levels from control versus R5020-treated cells would reflect effects on the rate of transcription. This experiment was conducted but did not yield conclusive results.

MATERIALS AND METHODS

Preparation of all solutions/reagents are detailed in the Appendix. The manufacturers of reagents, supplies, and equipment are also listed therein.

Cell Culture:

T47D wild type cells were obtained from the American Type Culture Collection. They were grown in Gibco-BRL growth medium (see below) in polypropylene tissue culture flasks ($75cm^2$ - $175cm^2$) with canted necks in air containing 5% CO_2 at 37°C. Cells were harvested by replacing the growth medium with "splitting solution", i.e. Hank's balanced salt solution without calcium and magnesium but with 1mM ethylenediamine tetraacetate (EDTA), incubating 10 minutes at 37°C, agitating the cells free, and centrifuging the cells to store as a pellet at -85°C. Cells were passaged weekly upon reaching a confluency of 60-70% using the "splitting solution" as above, replacing 15% of the cells into the same culture flask. After a total of 5 passages, a fresh culture flask was used.

RNA Isolation:

Total cellular RNA was isolated using a slightly modified version of Chomczynski and Sacchi's Single Step method (Chomczynski, 1987). Cell pellets, stored in 50ml disposable centrifuge tubes, were retrieved from the -85°C freezer, placed on ice, and maintained thereon throughout the benchtop procedure. Solution D (1 ml/10^7 cells) was added to the frozen pellets and vortexed at full speed for one minute. Care was taken to ensure that the cell pellet was completely dissolved at this point. 2M sodium acetate (NaOAc) pH 4.0 (0.1 ml/10^7 cells) was added, and the 50ml centrifuge tube was inverted 5 times to ensure mixing. Water-saturated phenol (1.0 ml/10^7 cells) was then added, and the mixture was again inverted 5 times. Next, 49:1 chloroform:isoamyl alcohol (0.2 ml/10^7 cells) was added and the mixture shaken vigorously for 10

seconds. The cells were then cooled on ice for 30 minutes during which time the cell mixtures were transferred to clean autoclaved 15ml Corex tubes. The Corex tubes were centrifuged at 10,000 x g (9100 rpm in JA-20 rotor, Beckman J21-C centrifuge) for 20 minutes at 4°C. The upper aqueous layer was transferred with sterile Pasteur pipets into clean autoclaved 15ml Corex tubes. An equal volume of isopropanol was added, vortexed gently to mix, and then placed at -20°C with the tubes covered with parafilm for an incubation of not less than 1 hour. Typically the RNA was allowed to precipitate for 4-6 hours before proceeding. Following the incubation, the Corex tubes were centrifuged at 10,000 x g for 20 minutes at 4°C. The pellet was dissolved in Solution D (1.0 ml/10^7 cells). One volume of isopropanol was added, mixed, and the tube returned to -20°C for an incubation of at least one hour. When the volume of Solution D was less than 1 ml, the resuspended pellet was transferred to autoclaved 2ml microcentrifuge tubes and precipitated with 1 volume of isopropanol. This allowed for greater ease of handling. Often the precipitation was allowed to proceed overnight. The tubes were then centrifuged at 4°C for 10 minutes at 10,000 x g for Corex tubes or 15,000 x g for 2ml microcentrifuge tubes. The pellets were resuspended in ice-cold 75% ethanol (1.0 ml/10^7 cells), spun again as above, and the pellet was dried in vacuo in a desiccator (Corex tubes) or in the Savant Speed-Vac SC110 (microcentrifuge tubes). Care was taken not to over-dry the pellet as this would make resuspension very difficult. The final RNA pellet was resuspended in 0.1% diethyl pyrocarbonate-treated water (DEPC-H_2O) to approximate a concentration of 3.3 mg/ml or higher.

RNA concentration was determined by absorbance at 260nm (A_{260}) in a 1cm pathlength cuvette where concentration equals (A_{260}) (40 µg/ml) (total sample volume in cuvette, ml) (volume of RNA , µl, added to the sample volume)$^{-1}$. The RNA was aliquotted into 20-25 µg samples for gel electrophoretic separation. These were used immediately or precipitated with 0.1 volume of 3M NaOAc pH 5.2, plus 2.2 volumes of absolute ethanol and stored at -20°C.

RNA Dot Blots:

Prior to use, the Bio-Rad Bio-Dot Microfiltation Apparatus was cleaned with 0.1M NaOH, rinsed with deionized distilled water then with DEPC-H_2O, and air-dried in the sterile hood. The nylon membrane was cut to the size of the manifold, 9 x 12 cm^2, and soaked in 10X SSC for at least 10 minutes. The apparatus was assembled per Bio-Rad instructions. Wells were filled with 100 µl 10X SSC and left undisturbed while the RNA samples were prepared.

RNA samples were denatured with addition of 3 volumes of RNA Denaturing Solution (see below), incubated at 65°C for 15 minutes, and quick-chilled on ice. Two volumes of ice-cold 10X SSC were added to the RNA. Samples were centrifuged briefly to collect contents.

Vacuum was applied to the Bio-Dot apparatus until the 10X SSC was pulled through. The RNA samples were loaded, any empty wells receiving 10X SSC, and the vacuum reapplied. All wells were then filled with 10X SSC, 0.735ml volume, and suctioned through. This was repeated once. The apparatus was then dismantled. The wet nylon membrane was marked with pencil to identify the RNA samples, placed on Whatman 3MM filter paper to air-dry for at least one hour, and UV cross-linked at 1200Hz on model UVP CL-1000 cross-linker. Membranes were stored sandwiched between pieces of 3MM Whatman paper, wrapped in aluminum foil, and placed in a lab bench drawer.

RNA Gel Electrophoresis:

The gel, 100 ml of 1% agarose in 2.2M formaldehyde, 1X MOPS buffer solution, was poured into a BRL Horizontal 11.4 gel box and allowed to polymerize at least 30 minutes before covering with 750 ml 1X MOPS buffer.

RNA samples were prepared in autoclaved 1.5ml microcentrifuge tubes, 20 µg RNA in 20 µl final volume of deionized formamide (50% v/v), 2.2M formaldehyde, 1X MOPS buffer. An RNA ladder (2 µl) and 10-20 µg of representative sample RNA were prepared as above to be run

in lanes 1 and 2 on every gel. All RNA samples were denatured at 65°C for 5 minutes and quick-chilled on ice. Prechilled Gel Loading Buffer (2µl) was added to each sample, mixed by pipeting, and the samples were briefly centrifuged to collect sample prior to loading onto the gel.

Electrophoresis was accomplished by applying 70 volts to the 14 cm gel (5 volts/cm) until the dye front had migrated 2/3 down the gel. The gel was rinsed 10 minutes with shaking in 200 ml of 0.1% DEPC-H_2O. Lanes 1 and 2 containing the RNA ladder and the representative sample were cut from the gel, soaked 30 minutes in ethidium bromide solution (5 µg/ml), placed in 200-300 ml deionized water to destain for 6-12 hours, and then viewed at 300nm on the Fotodyne UV light box to verify RNA integrity and location of the 18S and 28S rRNA. The rest of the gel was rinsed an additional 45 minutes in 10X SSC with shaking prior to transfer to a nylon membrane. Transfer in 10-20X SSC was accomplished either by downward or upward capillary action (see below). After transfer was complete, the wet nylon membrane was marked with a pencil to identify the preparation and the location of sample lanes. The nylon membrane was air-dried 1 hour, then UV-cross-linked at 1200 Hz on model UVP CL-1000 cross-linker. The membrane could be stored sandwiched between 3MM Whatman paper, protected in aluminum foil wrap in the lab bench drawer, or utilized immediately in Northern blot hybridization.

Downward capillary transfer:

A cellulose sponge purchased from local vendors was prepared for usage in Northern blotting as follows. The sponge was rinsed 15 minutes in tap water with repeated wringing to ensure maximum purging of manufacturer's chemicals. It was then boiled in deionized water for 20 minutes, followed by 2 minutes of rigorous rinsing in deionized water. The sponge was then soaked in 300 ml of water for 20 minutes, removed and wrung dry, and then left to air dry atop a plastic test tube rack.

In a shallow plastic dish a stack of paper towels was placed, 2-3 cm high, folded in a rectangle. On top of the paper towels was placed 3MM Whatman filter, cut with the same

dimensions as the nylon membrane, presoaked in 10X SSC. Directly centered on top of the blotting paper was a nylon membrane, glossy side down, cut with dimensions slightly larger than the gel's and presoaked in 10X SSC for at least 10 minutes. The agarose gel was gently applied to the top of the nylon and covered with two 3MM Whatman filters, presoaked in 10X SSC and of the same size as the gel. Any bubbles were removed by rolling a glass stirring rod along the surface after each layer was applied. Plastic wrap framed the top blotting paper, extending to cover the edges of the plastic dish, to provide a barrier against undirected saturation of the transfer apparatus. Finally, a cellulose sponge, thoroughly rinsed of all chemical reagents, then saturated in 10X SSC, was centered on top of the plastic wrap-framed blotting apparatus and covered itself with a sheet of plastic wrap to minimize evaporation (Zhou, 1994). Transfer was allowed to proceed for a minimum of 6 hours, but frequently overnight.

Upward capillary transfer:

A blotting support was constructed in a plastic tray (10 x 12 x 2.25 inches) in which 700-900 ml of 20X SSC were placed. A plastic pipet tip holder, used as a "stage", was centered in the tray and covered with a wick constructed of three 3MM Whatman filters, 14 cm wide (the length of the gel) and 13 inches long. Centered on top of the wick, saturated with 20X SSC, was the nylon Hybond-N membrane, cut exactly the same size as the gel and itself pre-saturated with 20X SSC. Three pieces of 3MM Whatman filter paper, same dimensions as the gel and saturated in 20X SSC, were placed directly on top of the membrane. Air bubbles were again removed after each layer was applied by rolling a glass stirring rod along the surface. On top of Whatman blotting paper a 5 cm stack of paper towels was placed and weighed down with a glass plate on which additional weights were positioned. The entire apparatus was then covered with plastic wrap. Transfer proceeded for 12-16 hours.

Silanization of hybridization bottles:

A 1% (v/v) solution of dimethyldichlorosilane in benzene was placed in the hybridization bottles and heated to 60°C in the hood. The tubes were rinsed in the dimethyldichlorosilane

benzene solution and then dried in an oven at 60°C. Rinsing and drying was repeated once.

Northern blot analysis:

Fresh prehybridization solution, 175 µl/cm^2 of nylon, was prepared for each membrane and incubated with the nylon membrane in pre-treated silanized hybridization bottles in a Robbins Scientific Hybridization Apparatus. The hybridization bottles were rotated to ensure adequate exposure of the membrane to the solution. Air bubbles were squeezed from the membrane-glass interface with a rubber policeman on long glass stir rod. After the incubation temperature reached the steady hybridization temperature of 42°C, 20 µg/ml denatured salmon sperm DNA was added to the prehybridization solution (see below). Prehybridization continued for at least 4 hours. Denatured ^{32}P-labeled cDNA probe for either c-myc or glyceraldehyde phosphate dehydrogenase was added to the prehybridization mixture and incubation at 42°C continued for an additional 16-30 hours.

The nylon membrane was subjected to a series of washes to remove non-specific binding of the labeled probe to the nylon. Radioactive solutions were poured into a designated radioactive waste container and disposed of according to NRC guidelines. First the nylon membrane was rinsed briefly with 50 ml of 2X SSPE, 0.1% SDS at room temperature, then soaked 10 minutes in 150 ml of the same solution. The second wash was again at room temperature, 200 ml of 2X SSPE, 0.1% SDS, for 10 minutes. The third wash was at 65°C for 15 minutes in 200ml of 1X SSPE, 0.1% SDS. The fourth and fifth washes were 10 minutes each at 65°C in 200ml of 0.1X SSPE, 0.1% SDS.

The nylon membrane was blotted on Kim-wipes to remove excess wash solution and then sealed in plastic "seal-a-meal" bags with an American International Electric impulse sealer at a temperature setting of 2-3. Care was taken to ensure that the membrane remained moist and that all air bubbles were removed from the "seal-a-meal" bag. The sealed nylon membrane was then

loaded into an autoradiogram cassette in the dark-room with an intensifying screen and a sheet of unexposed x-ray film on either side. The membrane was secured to the bottom x-ray film with tape to prevent its movement during exposure. The entire cassette was wrapped in aluminum foil and placed at -85^{0}C to develop. The time needed for adequate development of the x-ray film was estimated according to the cDNA probe used and the strength of the signal from the nylon as measured with a Geiger counter. Exposure time varied from 8 hours to 3 days or more. The exposed x-ray film was developed, and the intensity of the exposure of the autoradiogram was analyzed densitometrically.

Each nylon membrane was stripped of the first probe and reprobed with the second probe to identify mRNA levels of c-myc and of the non-progestin-stimulated mRNA glyceraldehyde-3-phosphate dehydrogenase (GPDH). Stripping was accomplished by incubating the membrane twice for 15 minutes each in boiling hot 0.1X SSPE, 0.1% SDS. The nylon membrane was stored moist in a "seal-a-meal" bag at 4^{0}C.

Acquisition of cDNA probes:

The probe for c-myc was graciously provided by Dr. Shelly Finver of the Microbiology Department, Marshall University School of Medicine, WV as the plasmid pRyc7.4 which includes a 1016 basepair (bp) cDNA fragment (60% of exon 2 and the entire exon 3) of the human c-myc gene inserted into the Pst1 restriction site of plasmid pBR322. This was amplified in the Escherichia coli strain DH5-α. The probe for GPDH was given by another member of Marshall University's Department of Microbiology, Dr. Donald Primerano as pRGPDH. The rat GPDH cDNA fragment, 1233 bp in length, is inserted at the Pst 1 site of pBR322 to produce pRGPDH, which was then transfected into the E. coli strain NM522. Both of the probes were used as the short, excised cDNA fragments rather than the entire plasmids. Preparation of NM522 competent cells and transformation were accomplished by a fellow graduate student, Cathy Sole, and the transformed NM522 stock cells given to me under Dr. Primerano's direction. Stock colonies of

both DH5-α and NM522 were stored in 50% glycerol at -85°C.

Active cultures of these transformed bacteria were streaked onto agar plates of Luria-Bertani (LB) broth containing 12.5 μg/ml tetracycline-HCl, incubated at 37°C for 10-12 hrs, and then stored at 4°C. Colonies thereon were viable for 4-6 weeks. Individual colonies of at least 1.5mm in size were selected randomly to be grown for isolation of the plasmid DNAs. These DH5-α or NM522 colonies were cultured in LB medium supplemented with 12.5 μg/ml tetracycline-HCl (LB+tet) at 37°C with agitation for 12-16 hours in Ehrlenmyer flasks. Because tetracycline is light-sensitive, all medium in which it was included was protected from light. Several methods were used to isolate the plasmid DNA, including polyethylene glycol (PEG) preparations, Promega Wizard and Magic Midi- and Mini-preps, and QIAGene plasmid kits. The basic procedure called for lysis of the bacterial cells followed by neutralization and centrifugation to remove genomic DNA. Procedures for isolation of plasmid DNA differed in the marketed kits, some employing affinity resins in their final purification step. The manufacturers' guidelines were followed.

PEG plasmid isolation:

Transformed E. coli were grown in 200 ml sterile LB+tet in an autoclaved 1 liter Ehrlenmyer flask (E-flask) as described above. The cells were then centrifuged in 250ml Nalgene bottles at 5000 x g (6K rpm, GSA rotor) for 5 minutes at 4°C and the supernatant discarded into a bleach-waste receptacle. The pellets were resuspended on ice in 3 ml of freshly prepared ice-cold lysis buffer (see below) with an inverted 10ml sterile serological disposable pipet. Frothing was carefully avoided. The resuspension was transferred to fresh sterile 50ml disposable centrifuge tubes and set in ice-water for 10 minutes. Six milliliters of Solution II (0.2M NaOH, 1% SDS) were added and mixed by inversion, and the tube returned to ice for 10 minutes. Four milliliters of the neutralization solution, 3M sodium acetate, pH 4.8, were added and mixed by hard shaking for 2 minutes after which the tube was placed on ice again for 10 minutes. Proteins and genomic

DNA were centrifuged out of solution at 11,000 x g (9.6K rpm in JA-20 rotor, Beckman centrifuge) for 10 minutes at 4°C. The supernatant was transferred to fresh sterile 50ml disposable centrifuge tubes and centrifuged as above to guarantee complete avoidance of the white precipitate of proteins and genomic DNA. RNase A (50 µg) was added to the supernatant, and the mixture incubated for 20 minutes in a 37°C water-bath. One volume of 1:1 TE-phenol/chloroform, pH 7.5-8.0, was added and vortexed for 5 minutes to extract plasmid DNA. This was centrifuged at 2000 x g (3K rpm, JA-20 rotor) for 3 minutes at 4°C. The upper aqueous layer was transferred to a fresh sterile 50ml tube, one volume of 24:1 chloroform/isoamyl alcohol added, and the mixture vortexed 5 minutes. This was centrifuged as above. The upper aqueous phase was transferred to an autoclaved 30ml Corex tube, mixed with one volume of isopropanol, and placed at -20°C for at least 30 minutes. If precipitation failed to occur with addition of the isopropanol, 0.1 volume of 3M NaOAc pH 4.8 was also mixed to the solution prior to incubation at -20°C. Afterwards, the preparation was spun at 11,000 x g for 10 minutes at 4°C. The pellet was totally dissolved in 0.4 ml sterile water. Next, 0.1 ml 4M NaCl was added and mixed. This was followed by the addition and mixture of 0.5 ml 13% PEG (w/v). The suspension was placed in ice-water for 1 hour, after which it was centrifuged at 10,000 x g for 10 minutes at 4°C. The pellet was resuspended in 1 ml ice-cold 70% ethanol, transferred to an autoclaved microcentrifuge tube, and dried for approximately 15 minutes at low temperature in the Speed-Vac or until the pellet was no longer wet but not completely dehydrated. The plasmid DNA was then resuspended in low TE and stored at 4°C. Storage at -20°C necessitated precipitation with 0.1 volume of 3M NaOAc, pH 4.8, and 2.2 volumes of ethanol.

Labeling cDNA probes:

cDNA probes were labeled with radioactive ^{32}P (Amersham Redivue) using random priming kits marketed by Promega or Ambion. Nonincorporated nucleotides were removed from the labeling mixture by passing the mixture over a G-50 Sephadex column. One microliter of the

eluate was mixed with 249 µl of water for evaluation of percent radioactive incorporation. Five microliters of this 1:250 dilution was spotted onto a glass fiber filter disc, washed in ice-cold 10% trichloroacetic acid (TCA)-1% sodium phosphate solution to precipitate labeled DNA, and then rinsed three times with 95% ethanol at room temperature. The filter was allowed to air dry, immersed in 5 ml scintillation fluid, and counted in the scintillation counter. The formula used to calculate percent incorporation was provided by the manufacturers.

The G-50 Sephadex column was prepared on the day of use in a 1cc tuberculin syringe. The plunger was removed and glass wool was used to plug the bottom. G-50 Sephadex slurry (see below) was pipetted into the upright syringe, taking care to avoid introduction of air bubbles. The syringe was centrifuged in the clinical centrifuge at 500 X g for 5 minutes to pack down the Sephadex. Additional Sephadex was added and packed until the column was nearly full. Space for a 150 µl volume was left vacant at the top. The column was then equilibrated with 100 µl equilibration buffer (see below), centrifuging at 500 X g for 5 minutes..

Experiments:

Growth medium was modified to exclude phenol red, and the fetal bovine serum (FBS) was treated with dextran-coated charcoal (see below) once in initial experiments and twice in later experiments as this was found to give better results. Cells were plated into culture flasks (75 or 150cm^2) so that they covered approximately 15% of the surface area (approximately 1 million cells per 75cm^2) and allowed to grow to 20-30% confluency with at least one medium change before treatment commenced. Hormones were added in absolute ethanol, and an equivalent volume of ethanol were added to control flasks. Approximately 6 million cells per sample were treated at given concentrations for the times indicated below. Certain experiments had a pre-treatment regimen in which the phenol red-free, double charcoal-stripped serum containing medium was replaced with growth medium that was serum-free, insulin-free, and phenol red-free (SF/IF/PRFM) for 4 hours prior to and during treatment. At the conclusion of the experiment,

cells were harvested, placed in 50ml disposable centrifuge tubes, centrifuged at 1000 rpm in the Damon/IEC HN-SII centrifuge at room temperature, and stored at -85^{0}C.

R5020 Dose Response:

T47D cells (5.4 x 10^{7}) were seeded in 48 T-75 culture flasks and grown in CS^{2}-FBS medium until reaching 20-30% confluency. Medium was changed at least once. On the day of treatment, cells were pre-treated 4 hours with 14 ml serum-free, insulin-free, phenol red-free medium (SF/IF/PRFM) and grouped into 8 different paired treatment groups: control plus 10^{-12}M R5020 to 10^{-6}M R5020. Cells were treated for 1 hour with R5020 or vehicle and then harvested. Ethanol at a final concentration of 0.1% was present in all treatments. Each treatment was done in duplicate.

R5020 Time Course:

T47D cells (9 x10^{7}) were seeded in 46 T-150 flasks in CS-FBS medium and grown until reaching 20-30% confluency with at least one change of medium. On the day of treatment, flasks were paired so that each treatment group had approximately the same number of cells. The best matched 44 flasks were used for the experiment. Treatments were done in duplicate.

In the initial experiment, time points for treatment with 10^{-8}M R5020 were chosen as 5 min., 15 min., 30 min., 1 hour, 2 hours, 6 hours, and 24 hours. Vehicle alone (0.1% ethanol) was also administered for time points 5 min., 1 hour, 6 hours, and 24 hours. At the end of treatment times of 1 hour or less, the flasks were immediately placed on ice to prevent further cellular activity and the cells harvested with ice-cold splitting solution and rubber policemen. For treatment times greater than 1 hour, cells were harvested in 37^{0}C splitting solution with agitation and a serological pipet.

Subsequent experiments evaluating optimal treatment times for R5020 were conducted so that at each time point, 5 minutes through 6 hours, cells were treated with either 10^{-8}M R5020 or

vehicle (0.1% EtOH). Harvesting procedures were identical to those outlined above.

Effect of Antiprogestin RU486:

T47D cells (3 x 10^7) were seeded into 25 T-75 culture flasks or into 17 T-150 flasks and grown in CS^2FBS medium until reaching a confluency between 20 and 30%. Medium was changed at least once. Flasks were grouped, pairs of triplicate T-75's or duplicate T-150's, and were incubated for 2 hours with one of the following treatments: 10^{-9}M R5020 + 10^{-5}% ethanol (EtOH), 10^{-10} M RU486 + 10^{-4}% EtOH, 10^{-9} M R5020 + 10^{-10} M RU486, or 1.1x 10^{-4}% EtOH in 10% CS^2-FBS medium. Final concentrations were 1.1 x 10^{-4}% EtOH in all treatments.

Hormone Specificity:

T47D cells (5 X 10^7 cells) were divided into 44 T-75 flasks. Alternately, approximately 68 X 10^6 cells were split among 30 T-150 flasks. Cells were grown in CS^2-FBS medium until 20-30% confluent with at least one change of medium. T-75 flasks were grouped into pairs of triplicates and T-150's into pairs of two for 7 different 1 hour treatments, control or 10^{-8}M hormones: aldosterone, dexamethasone, estradiol, progesterone, R5020, or testosterone. Cells were incubated 4 hours in serum-free, insulin-free, phenol red-free medium (SF/IF/PRFM) prior to treatment. Hormone or EtOH was dissolved and dispensed in SF/IF/PRFM for these experiments. The final ethanol concentration was 0.1% for all treatments.

Cycloheximide viability determination:

Six-well tissue culture plates were seeded at 15% confluency, 1.5 X 10^{-5} cells per well, in 10% CS^2-FBS medium and grown to 25-35% confluency with at least one medium change. Two wells were combined for each of the three treatments: (I) 1mM cycloheximide in 0.1% EtOH + 0.1% ethanol, (II) 1mM cycloheximide in 0.1% EtOH + 10^{-8}M R5020 in 0.1% EtOH, or (III) 0.2% ethanol as the control. Cells for treatments I and II were treated 4 hours with cycloheximide in 0.1% EtOH, while control received 0.1% ethanol for the same 4 hours. Following pre-treatment with cycloheximide, the cells were exposed to either an additional 0.1% EtOH for

treatments I and III or to 10^{-8}M R5020 in 0.1% EtOH for 1 hour for treatment II. The final EtOH concentration was 0.2% for all treatments. At the end of this hour all medium was removed, and 2 ml of 0.4% trypan blue solution were added to each well. The cells were incubated at room temperature for 5 minutes with the trypan blue, after which the stain was removed and the cells examined under the microscope for uptake of trypan blue, an indicator of cell death (Ausubel, 1995). Four fields of view were randomly selected for each well. The total number of cells were counted, both those blue with the stain and those still clear. The numbers for each view were combined for a given well. Percent viability was calculated as 100% minus [(number of blue cells) divided by (number of total cells) X 100%] for each well. The percent viability was averaged for the two wells of each of the given treatments.

Cycloheximide dosage:

Six-well culture plates were seeded at 10% confluency, 9 X 10^4 cells per well, in serum-free, phenol red-free medium (SF/PRFM) and grown to 25-30% confluency. Cells were then treated for 30 minutes with either additional SF/PRFM (for control) or various concentrations of cycloheximide in SF/PRFM: 0.5 µg/ml, 1 µg/ml, 5 µg/ml, and 10 µg/ml. Three wells were used for each treatment. After 30 minutes, 50 µl of ^{3}H-leucine, 1 mCi/ml, were added to each well and incubation continued for 4 hours. Tritiated waste was removed to radioactive waste containers. One milliliter of cold phosphate buffered saline (PBS) was added to each well, incubated for 2 minutes, then removed to radioactive waste. The plates were placed on ice, and a series of cold 10% TCA washes were performed for 20 minutes. The plates were then washed with room temperature methanol for 5 minutes. The plates were air dried. One-half of a milliliter of 0.3N NaOH in 1% SDS was added to each well and the plates rocked gently to ensure that the entire bottom surface was covered with solution. The plates sat at room temperature for 30 minutes. Contents of the wells were collected, added to 5 ml scintillation fluid, and counted. Values were averaged for each treatment group.

R5020 and Cycloheximide Effect:

T47D cells (4.8 X 10^7) were resuspended in 25 ml of regular BME growth medium and split into 25 T-150 culture flasks, giving each flask a starting confluency of 13-15%. Cells were grown to 20-25% confluency with at least one change of medium. On the day of the experiment the medium was replaced with medium lacking serum, insulin, or phenol red (SF/IF/PRFM) for 4 hours. Duplicate sets of paired flasks were treated with either vehicle (0.1% EtOH), 15 μg/ml cycloheximide + 0.1% EtOH, 10^{-8}M R5020 in 0.1% EtOH, or cycloheximide plus R5020 for 1 hour in SF/IF/PRFM. The final ethanol concentration in each flask was 0.1%. After treatment, flasks were kept upright at 4^0C until harvested to minimize prolonged reaction to the reagents.

Determination of c-myc mRNA Half-life with Actinomycin D:

T47D cells (1.5 x 10^8) were split into 48 T-150 culture flasks in 10% CS^2-FBS medium and grown to 20-30% confluency with one change of medium. On the day of the experiment, the medium was changed to SF/IF/PRFM in which the cells were incubated for 4 hours. Cells were then treated for 1 hour with either 10^{-8}M R5020 in 0.1% EtOH or with 0.1% EtOH alone. At the end of the hour, control and R5020 flasks in duplicate pairs were treated with 1μg/ml actinomycin D for time periods of 0 minutes, 15, 30, 45, 60, or 90 minutes.

After the mRNA for each sample had been isolated and quantitated densitometrically, a graph of the log of [Initial c-myc mRNA levels]/[c-myc mRNA levels] versus actinomycin D treatment time yields a plot from which the slope of the line can be determined. That slope is equal to k, the first order rate constant for degradation of c-myc mRNA. The half-life, $t_{1/2}$, can be calculated from the equation $t_{1/2} = 0.693/k$.

Nuclear Run On Assay:

1. Preparation of Membrane Filter:

cDNA samples, 12 μg each of plasmids pBR322, pRyc 7.4, and pRGPDH, were linearized with Pst I restriction endonuclease digestion in Promega's 1X Buffer H for 1 hour at

37°C per manufacturer's protocol. Plasmid samples were then heated 5 minutes at 95°C, quick chilled on ice, and mixed with 1 volume of chilled 20X SSC. Five microgram aliquots of the plasmids were fixed onto the membrane in parallel lines.

II. Treatment of Cells:

Thirty T-150 culture flasks of T47D cells were grown to 20-30% confluency in 10% FBS growth medium. On the day of the experiment, medium was replaced with serum-free, insulin-free, phenol red-free medium (SF/IF/PRFM) for 4 hours, followed by treatment for 1 hour with either 10^{-8}M R5020 in 0.1% EtOH or 0.1% EtOH alone. Cells were harvested, centrifuged at 500 x g for 10 minutes at room temperature, and the pellets frozen at -85°C.

III. Preparation of Nuclei:

Cells were retrieved from the freezer and placed on ice to thaw. The cell pellet was loosened by gently vortexing for 5 seconds. NP-40 lysis buffer A (4 ml) was added to the pellet while gently vortexing. After the lysis buffer was added, the cells were vortexed an additional 10 seconds at half maximal speed and then placed on ice. One drop of lysed cells was resuspended in 200 µl of 1:1 PBS /Harris hematoxylin stain and examined on a hemocytometer to ensure cells were lysed and nuclei free of cytoplasmic material. To determine the total number of nuclei retrieved in control- and R5020-treatments, three 10 µl aliquots were taken, diluted 10-fold, and counted on a hemocytometer. The nuclei were centrifuged for 5 minutes at 500 x g at 4°C and the supernatant discarded. The pellet was resuspended in 4 ml NP-40 lysis buffer A as above, centrifuged again at 500 x g, and the pellet of nuclei resuspended in 200 µl of glycerol storage buffer (see below) with gentle vortexing. Resuspension was difficult to accomplish, requiring several short bursts of vortexing. Nuclei were kept chilled on ice between vortexing episodes. The original protocol called for freezing the nuclei at -135°C at this point, to be stored until the

next step, and also intended that nuclei be counted here. I deviated from this protocol because I did not feel I could pre-treat, treat, harvest, lyse, count, and isolate nuclei successfully in one day.

IV. Nuclear Run-on Transcription:

Samples, 200 µl each of Control- and R5020-treated nuclei, were transferred to separate plastic 15ml disposable centrifuge tubes. 2X Reaction Buffer (200 µl) with nucleotides plus 10 µl of 10 mCi/ml [α^{32}P] UTP were added to the nuclei and incubated for 30 minutes at 30^{0}C with shaking in the Gyrorotor water bath.

V. RNA Isolation and Quantitation:

The nuclei were recovered and RNA isolated as described above except that radioactive wastes were monitored and disposed of according to NRC guidelines. Each RNA sample was dissolved in 1 ml TES solution in 15ml tubes and shaken for 30 minutes at 30^{0}C in the Gyrorotor water bath to ensure RNA was completely dissolved. A 5 µl aliquot of each sample was spotted onto Whatman GF/F glass fiber filters in duplicate and radioactivity counted in 5 ml scintillation fluid. The samples were diluted as appropriate in TES buffer so that Control- and R5020-treated samples had equal radioactivity. Ideally, samples were to be 5 X 10^6 cpm/ml, but samples used here had 10-fold less radioactive than desired.

VI. Hybridization of RNA to DNA on filters:

The strips of nylon membrane on which the DNA had been applied were coiled and placed in two separate 5 ml plastic scintillation vials, labeled "Control" or "R5020", and pre-hybridization solution added to cover the strips. The mixture was warmed to 65^{0}C, 20 µg/ml denatured sonicated non-homologous Salmon Sperm DNA was added, and incubation continued for 4 hours in the Gyrorotor water bath with shaking. Radiolabeled RNA (1 ml), control and R5020, was added to the appropriate vials and incubation continued for 36 hours.

After incubation, the nylon strips were washed twice at room temperature for 10 minutes in 2X SSPE, 0.1% SDS, once at 65^{0}C in 1X SSPE, 0.1% SDS, and again at 65^{0}C for 10

minutes in 0.1X SSPE, 0.1% SDS. The membrane strips were placed in seal-a-meal plastic and subjected to autoradiography at -85°C. Upon development of the autoradiogram, no signal was detected for control samples even though equal amounts of labeled mRNA had been added to each nylon membrane strip.

Possible explanations for the difference between intensity of control versus R5020 signals are (1) cDNAs were not equally affixed to the two nylon strips, (2) less nuclei were obtained for the control treatment, and (3) dramatically fewer c-myc transcripts were initiated/elongated in control cells and thus were below detection limits. Signal from the R5020 sample itself was so faint that the film had to be left for several weeks at -70°C to develop.

The most important change to make to improve the experiment will be to isolate the nuclei immediately upon harvesting the cells. Another place for improvement could be in the fixation of cDNA to the nylon membrane. Rather than removing the nylon from the dot blot apparatus prior to denaturation, one could add the denaturing solution to the sample wells for the prescribed 5 minutes, remove it by vacuum, apply the neutralization solution for 1 minute, remove that solution, and then dismantle the apparatus. The manufacturer of Hybond-N agreed that this would be an appropriate method.

RESULTS

Figure 1 shows the maximum response of R5020 in stimulating production of c-myc mRNA in T47D breast cancer cells occurs at 10 nM. This is within the physiological range of serum concentration for progesterone, reportedly 6-64 nM during the luteal phase of menstruation (Wilson, 1992).

In Figure 2 the time course for c-myc activation by progestins demonstrates that induction of myc transcription is rapid, reaching maximal mRNA levels at 1 hour. An increase in mRNA levels has been observed in our lab as early as 5 minutes, although such was not the case for the experiment illustrated in Figure 2. Nearly a 2-fold increase, however, was observed at 15 minutes with stimulation continuing to climb, peaking at approximately 4-fold in 1 hour. More typically, maximal stimulation ranged from 2- to 3-fold, but in all cases occurred with 1 hour of R5020 treatment.

As shown in Figure 3, RU486 reduces c-myc mRNA levels to control values when administered in conjunction with R5020. RU486 itself, at the concentration used, does not affect c-myc mRNA expression either positively or negatively. R5020, on the other hand, stimulates c-myc roughly 2-fold. This level of stimulation was statistically different from that observed with all other treatments, using the Student-Newman-Kuels analysis of variance test ($p = 0.0021$). No other treatments were significantly different from one another.

The consensus steroid response element recognized by the progesterone receptor [GGTACA-NNN-TGTTCT] is also recognized by receptors for glucocorticoids, mineralocorticoids, and androgens (Clark, 1992). To test if these other steroids would utilize c-myc's putative response element and also affect transcription of the gene, we treated cells with representatives from these other classes of steroids. As seen in Figure 4, dexamethasone, a synthetic glucocorticoid, aldosterone, a mineralocorticoid, and testosterone, an androgen, in

comparison only weakly enhanced c-myc levels. As had been demonstrated in other cell lines, estrogen greatly stimulated c-myc, being the most potent mitogen tested here. Treatment with progesterone elicited a strong stimulation of nearly the same magnitude as R5020. This pattern of stimulation (Estr. > R5020 > Prog. > Dex ≅Test.≅ Ald.) was repeated in two additional experiments, verifying hormone-specific stimulation of c-myc. Whereas stimulation by estrogen and the progestins was statistically significant, transcriptional enhancement by the other steroids was not consistently demonstrated.

At this point all evidence is supporting our hypothesis that enhanced expression of c-myc is part of the mechanism by which progestins promote growth of T47D breast cancer cells, and that this effect is a primary one, at the level of transcription. To explore the alternative explanation that progestins were upregulating c-myc secondary to production of other proteins, cycloheximide experiments were conducted. This particular inhibitor of protein synthesis operates by "freezing" ribosomes on polysomes, thus effectively blocking initiation and elongation (Vasquez, 1979). Unfortunately, cycloheximide itself has been reported by other researchers to cause increases in c-myc mRNA via de-repression (Kelly, 1983; van der Burg, 1989). When protein synthesis ceases, the production of any labile repressors of c-myc also is stopped. Observed increases in c-myc mRNA by cycloheximide may be due to de-repression of its synthesis. Being a synthetic reagent, cycloheximide may also be affecting other cellular pocesses in addition to its action on ribosomes. Its total impact on the cell has not been determined.

T47D cell viability was maintained in medium containing as much as 1 mM cycloheximide (data not shown). In Figure 5 it was demonstrated that protein synthesis was inhibited 90% or more by cycloheximide doses of 10 and 20 μg/ml in cells that had been grown for 2 or 5 days in 10% double charcoal-stripped fetal bovine serum (CS^2FBS) medium. No significant difference in the degree of inhibition was observed between 1 and 4 hours of cycloheximide exposure. Unfortunately, the impact of these conditions on superinduction of c-myc by cycloheximide was

not tested.

Researchers routinely use cycloheximide as the inhibitor of choice but do so over a range of concentrations and exposure times. Being aware of the importance that cellular environment may have on cyclohexim de's subcelluar interactions, scientists have also experimented with different pretreatment protocols. To establish which concentration, duration, and pretreatment would best suit protein sythesis inhibition in our T47D cells, I varied each of the parameters and evaluated their impact on protein sythesis as measured by relative incorporation of tritiated leucine (^{3}H-Leu) into newly synthesized protein. The results of these three experiments are graphed in Figure 5.

No significant differences in percent inhibition were noted for treatments of 10 versus 20 µg/ml of cycloheximide. The impact of pre-incubating cells in CS^{2}FBS medium for 2 versus 5 days was not significant. The reduced ^{3}H-Leu incorporation noted in cells that had been treated for 5 days in CS^{2}FBS medium and then exposed to medium or cycloheximide and ^{3}H-Leu for 1 hour was likely due to a limitation in time allowed for the incorporation of ^{3}H-Leu, 1 vs. 4 hours. When comparing ^{3}H-Leu incorporation for treatments with equal exposure to the labeled amino acid, control cells with 2 days of CS^{2}FBS pretreatment had 33% less incorporation of ^{3}H-Leu than cells pretreated for 5 days in CS^{2}FBS medium. This may have been because there were more cells in the 5-day treatment. I had not adequately controlled for this. In any case, 10-20 µg/ml cycloheximide reduced protein synthesis 90% or more for every case. In terms of percent reduction in translation, there was also no significant advantage of prolonged exposure to cycloheximide over an hour.

Since all the experiments conducted thus far had had a 5-day exposure to CS^{2}FBS medium prior to initiation of experimentation and I was unable to determine the effect of cell number on these results, I continued to use the 5-day pretreatment regimen, although it might prove worthwhile to investigate this more thoroughly . A cycloheximide dose midway between concentrations tested, 15 µg/ml, was chosen for use in subsequent experiments because this was

closer to the dosage utilized by Mauvis-Jarvis when demonstrating that estrogen's stimulation of c-myc was not dependent on synthesis of new proteins (Leygue, 1995).

In an effort to reduce further basal transcription in the cells, medium was replaced with that lacking known c-myc stimulants serum, insulin, and estrogen (in the form of phenol red) for 4 hours before and during the one hour of treatment. Cells were treated with (1) cycloheximide, (2) R5020, (3) cycloheximide + R5020, or (4) vehicle. Results of this experiment are found in Figure 6. As was reported in other systems, cycloheximide stimulated an increase in c-myc mRNA levels. This stimulation was slightly less than that of R5020's. Concommitant treatment with cycloheximide and R5020 showed an additive increase in mRNA, but this increase was not statistically different from the effect of cycloheximide alone as determined by the Student-Newman-Kuels multiple comparison procedure, the p-value being 0.062. Cycloheximide did not interfere with R5020-stimulation of c-myc, but considering the inhibitor's own contribution to enhancing mRNA levels, one cannot definitely conclude that progestins stimulate in the absence of ongoing protein synthesis.

Although dual cycloheximide-R5020 treatments routinely showed stimulation over that of either agent's alone, the addition was always only marginally more than cycloheximde's stimulation. Figure 7 illustrates the mean of 3 independent experiments of duplicate treatments. R5020, CHX (cycloheximide), and CHX + R (plus R5020) all are significantly different from controls but not from one another.

The fact that cycloheximde stimulated c-myc mRNA production to the maximum that we had observed with R5020 may indicate that this is the ultimate level that c-myc can be stimulated under our experimental conditions. Even though this experiment could not aid in supporting our hypothesis, these data at least did not suggest its negation. Perhaps different results would have been obtained if another inhibitor with a more defined mode of action were used, such as ricin which inhibits elongation by specific N-glycosidase action on native 28S rRNA (Adams, 1992). If mRNA levels rise only as a consequence of myc's de-repression, however, changing inhibitors

would not help. Considering that c-myc mRNA increases rapidly upon hormonal stimulation (Figure 2), it is highly unlikely that a new protein is synthesized and elicits its own response in the time frame observed. Overall, our data suggest that progestin stimulation of c-myc mRNA levels probably does not require new protein synthesis.

In order to determine whether the increase in c-myc mRNA is due in part to progestin stabilization of the message, experiments with the RNA synthesis inhibitor actinomycin D were done. The parallel lines presented in Figure 8 inform us that R5020 does not affect the rate of c-myc mRNA degradation. The slopes of these lines, -0.0296, were calculated by the method of least squares. The rate constant of the first-order reaction (k) whereby mRNA is being degraded is equal to this slope. Using the formula $t_{1/2} = 0.693(k)^{-1}$, the half-life for c-myc in T47D cells in our conditions is about 23 minutes. This agrees with half-life determinations for c-myc from other investigators (Dubik, 1988; Leygue, 1995; Dean, 1986).

Although enhancing the half-life of c-myc message would not preclude R5020's transcriptional involvement in promoting increases in c-myc mRNA, it would complicate the determination of progestin's role in c-myc stimulation. As it turned out, R5020 does not alter mRNA stability and thus, is more likely operating as originally hypothesized, i.e. by increasing the rate of transcription, a primary effect.

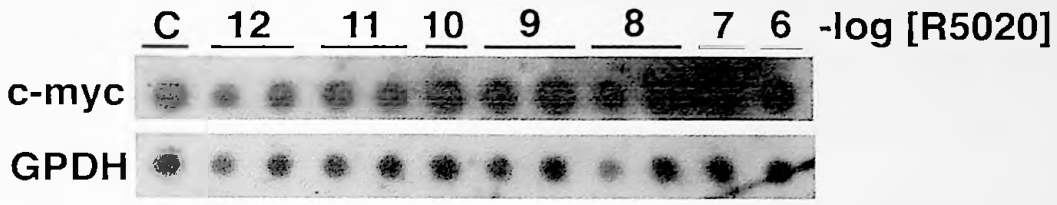
The experiment that will answer this question directly is the nuclear run-on assay which has so far been inconclusive. The data described above, however, predict that nuclear run-on assays will show that progestins stimulate the rate of c-myc transcription.

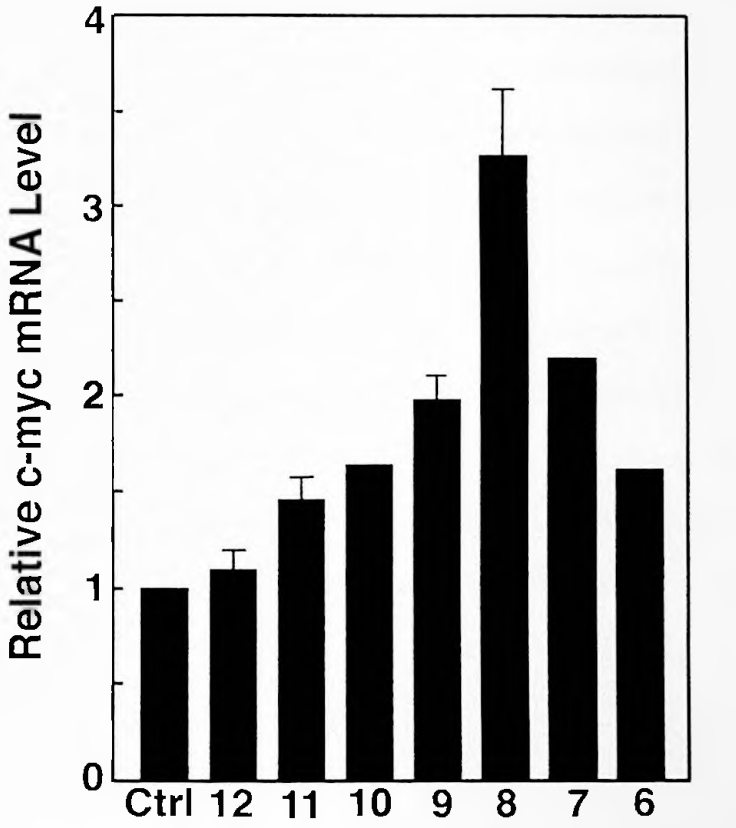
FIGURES AND LEGENDS

FIGURE 1: Dose-dependent Stimulation of c-myc mRNA Levels

Cells were plated at about 15% confluency in plastic T-150 flasks in phenol red-free medium containing 10% double charcoal-stripped fetal bovine serum (CS^{2}FBS). They were maintained 5 days in CS^{2}FBS medium with one change of medium. On the day of the experiment, cells between 20 and 30% confluency were pretreated 4 hours with serum-free, insulin-free, phenol red-free medium (SF/IF/PRFM). Cells were then exposed, in SF/IF/PRFM, for 1 hour to vehicle (0.1% ethanol) or R5020 in 0.1% ethanol at concentrations of 10^{-12} M to 10^{-6} M. Each treatment was performed in duplicate.

RNA was isolated from treated cells, and in this experiment, applied to a nylon membrane using a dot blot apparatus. Nylon blots were probed sequentially with ^{32}P-labeled glyceraldehyde phosphate dehydrogenase (GPDH) and c-myc probes. Autoradiograms were analyzed densitometrically. Levels of c-myc were normalized against GPDH levels and expressed relative to control. This experiment was repeated twice, once as above and once using northern blot analysis. The results were essentially the same.





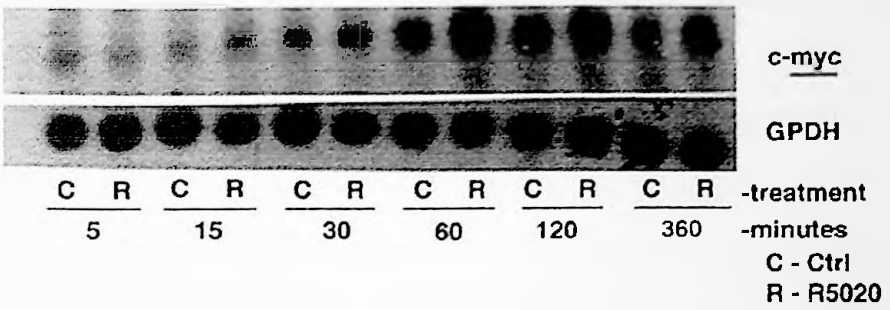
Error bars = range of values

FIGURE 2: Time-dependent Stimulation of c-myc mRNA Levels

T47D cells were seeded at 13-15% confluency in T-75 plastic flasks in medium containing 10% double charcoal-stripped fetal bovine serum (CS^2FBS) and grown for 5 days with one change of medium. On the day of treatment, cells between 20-30% confluency were supplied with fresh CS^2FBS medium in which vehicle (0.1% ethanol) **[C]** or 10^{-8} M R5020 **[R]** was included. Cells were treated for 5, 15, 30, 60, 120, and 360 minutes. For treatment times of 1 hour or less, flasks were chilled in the interim between cessation of treatment and initiation of harvesting to preclude further cellular processes. Each treatment was done in duplicate.

RNA was isolated from cells and electrophoresed on a denaturing 1% agarose gel. Northern blots were probed sequentially with ^{32}P-labeled c-myc and glyceraldehyde phosphate dehydrogenase (GPDH) cDNAs. Autoradiograms were analyzed densitometrically. Levels of c-myc were normalized with GPDH levels and expressed relative to control.

The representative of three experiments is shown. The amplitude of stimulation varied, but the pattern of time-dependent stimulation was identical among experiments. The plateau noted between 15 and 30 minutes of R5020 treatment in Figure 2 was unique to this experiment. In other experiments, mRNA levels increased from 15 to 30 minutes. In all experiments, the mRNA levels at 1 and 2 hours of R5020 treatment were statistically different from control.


c-myc
GPDH
C R C R C R C R C R C R
-treatment
5 15 30 60 120 360
-minutes
C - Ctrl
R - R5020

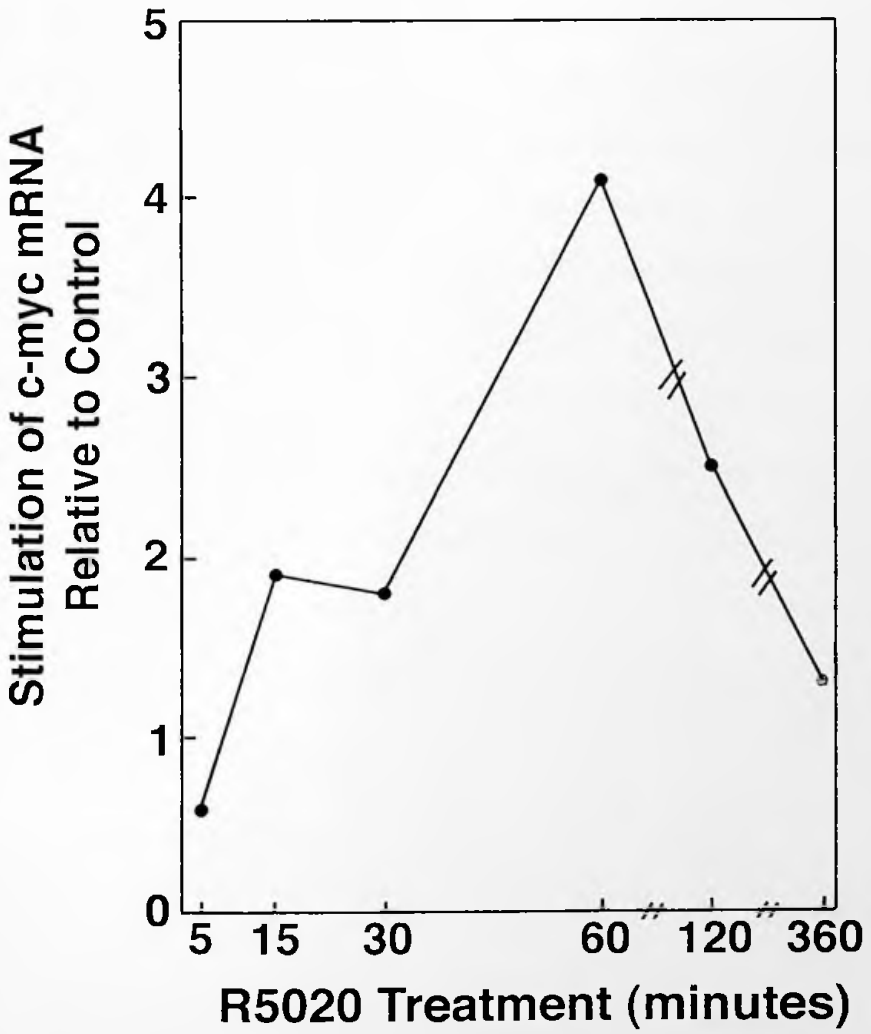

Stimulation of c-myc mRNA
Relative to Control
0
1
2
3
4
5
5 15 30 60 120 360
R5020 Treatment (minutes)

FIGURE 3: The Effect of RU486 ON R5020 Stimulation of c-myc mRNA Levels

Cells were plated in T-175 plastic flasks in phenol red-free medium containing 10% charcoal-stripped fetal bovine serum (CSFBS) and grown 5 days with one change of medium. Fresh medium was supplied on the day of treatment. Cells between 20 and 30% confluency were treated for 2 hours either with ethanol vehicle alone **[Ctrl]**, 10^{-9} M R5020 **[R5020]**, 10^{-10} M RU486 **[RU486]**, or both **[R + RU]**. All sets contained 0.11% ethanol. Treatments were done in triplicate.

Because RU486 has been reported to behave as an agonist at higher concentrations, a 100 pM dose was used here. The concentration of R5020, optimal at 10 nM in stimulating c-myc mRNA levels, was reduced to 1 nM in order that the concentrations of R5020 and RU486 remained comparable. Because this experiment was conducted prior to the time-dependency experiments, cells were treated for 2 hours, less than the optimal treatment time. As noted in Figure 2, significant stimulation of c-myc mRNA by R5020, however, is evident still at 2 hours.

RNA was isolated and quantitated as in Figure 2. Data represent the mean of 4 separate experiments and were statistically evaluated using the Student-Newman-Kuels Multiple Comparison analysis. Error bars indicate Standard Error of the Mean [S.E.M.]. *: R5020 treatment was statistically different ($p=0.002$) from all other treatments. φ: No other treatments were significantly different from one another.

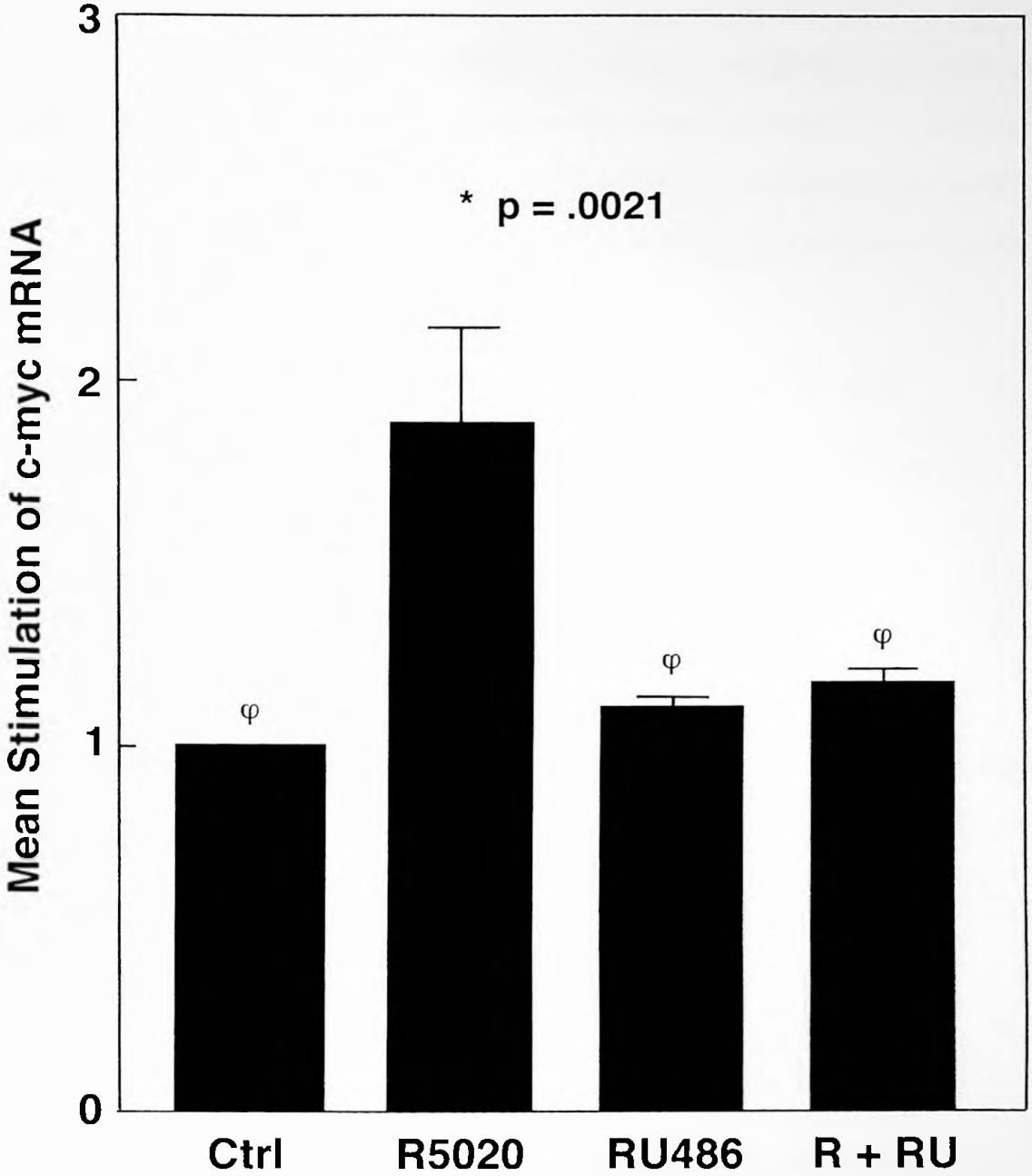

3
2
1
0
Mean Stimulation of c-myc mRNA
* p = .0021
φ
φ
φ
Ctrl
R5020
RU486
R + RU

FIGURE 4: Hormone Specificity: Stimulation of c-myc mRNA

Cells were grown for 5 days in CS-FBS medium with one change of medium. 4 hours prior to the experiment the medium was replaced with serum-free, insulin-free, phenol red-free medium (SF/IF/PRFM). Cells between 20 and 30% confluent were treated in SF/IF/PRFM for 1 hour with vehicle (0.1% ethanol) [C] or the following hormones at a 10^{-8} M concentration in 0.1% ethanol.

A - Aldosterone

C - Control

D - Dexamethasone

E - Estrogen

P - Progesterone

R - R5020

T - Testosterone

Treatments were done in duplicate and the experiment repeated 3 times. RNA was isolated and analyzed as in Figure 2. A representative northern blot and corresponding quantitation of densitometric scans are shown here.

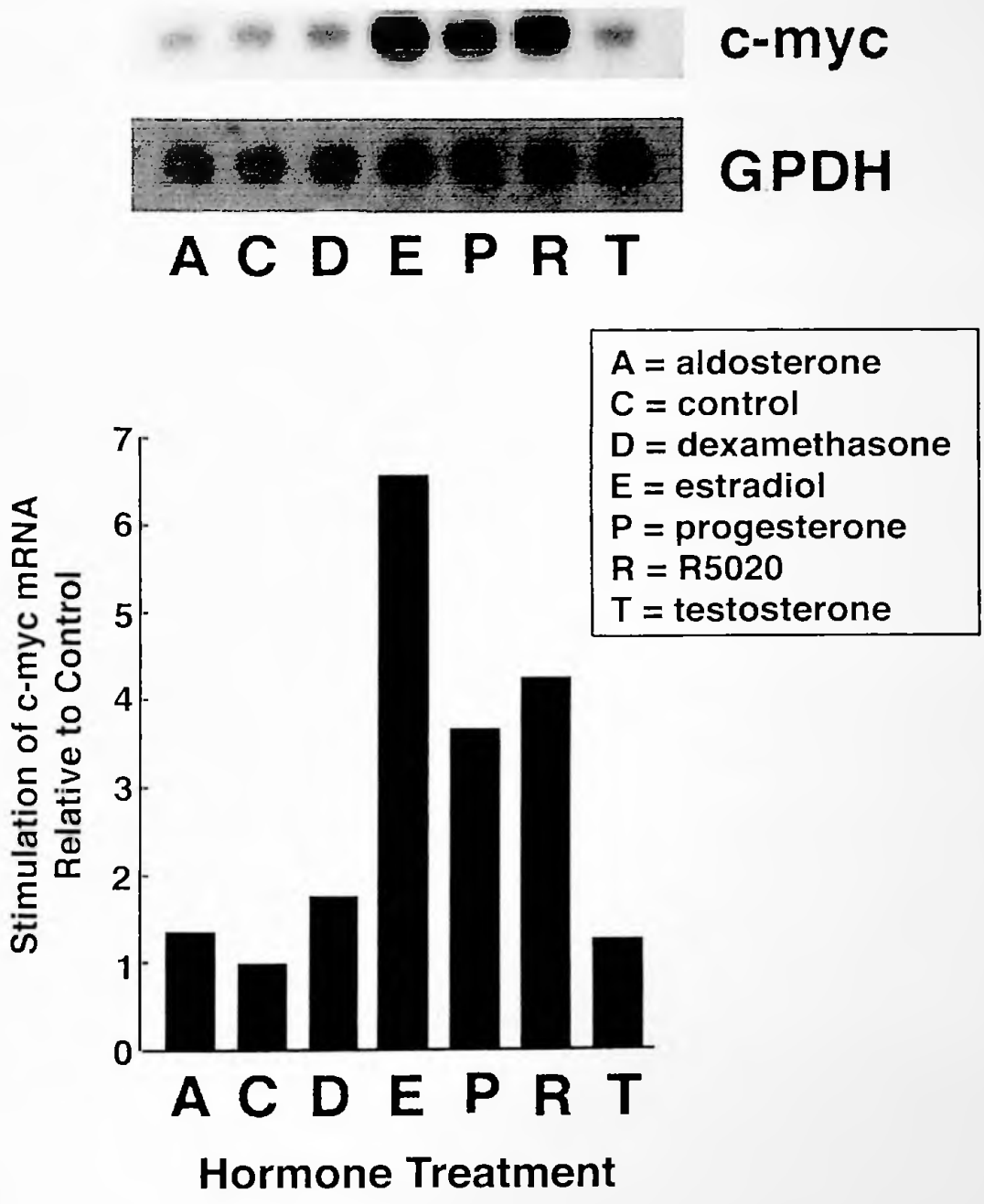

c-myc
GPDH
A C D E P R T
A = aldosterone
C = control
D = dexamethasone
E = estradiol
P = progesterone
R = R5020
T = testosterone
Stimulation of c-myc mRNA
Relative to Control
0
1
2
3
4
5
6
7
A C D E P R T
Hormone Treatment

FIGURE 5: Cycloheximide Inhibition of Protein Synthesis

In plastic 6-well plates approximately 2 X 10^5 cells/well were seeded , giving a starting confluency of 10%. Treatments were done in duplicate. Three different treatment regimens were used.

I. Solid bars

Cells were pretreated for 2 days in medium containing 10% double charcoal-stripped fetal bovine serum (CS^2FBS). Cells between 20 and 30% confluency were treated for 1 hour either with fresh CS^2FBS **[CTRL]** or cycloheximide **[CHX]** dissolved in CS^2FBS at 10 µg/ml or 20 µg/ml concentrations. ^3H-leucine **[Leu]** (50 µCi), specific activity of 33 Ci/mmol, was added to each well. Incubation continued 4 hours.

II. Hatched-crossed bars

Cells were pretreated 5 days in CS^2FBS medium with one change of medium. On the day of treatment, cells at approximately 45% confluency were treated with fresh CS^2FBS medium **[CTRL]** or with 10 or 20 µg/ml cycloheximide **[CHX]** in fresh CS^2FBS, simultaneously with 50 µCi ^3H-leucine per well, for 1 hour.

III. Diagonally striped bars

Cells were pretreated in CS^2FBS medium for 5 days as above. Cells were then treated simultaneously with 50 µCi ^3H-leucine and cycloheximide **[CHX]** or vehicle **[CTRL]** for 4 hours.

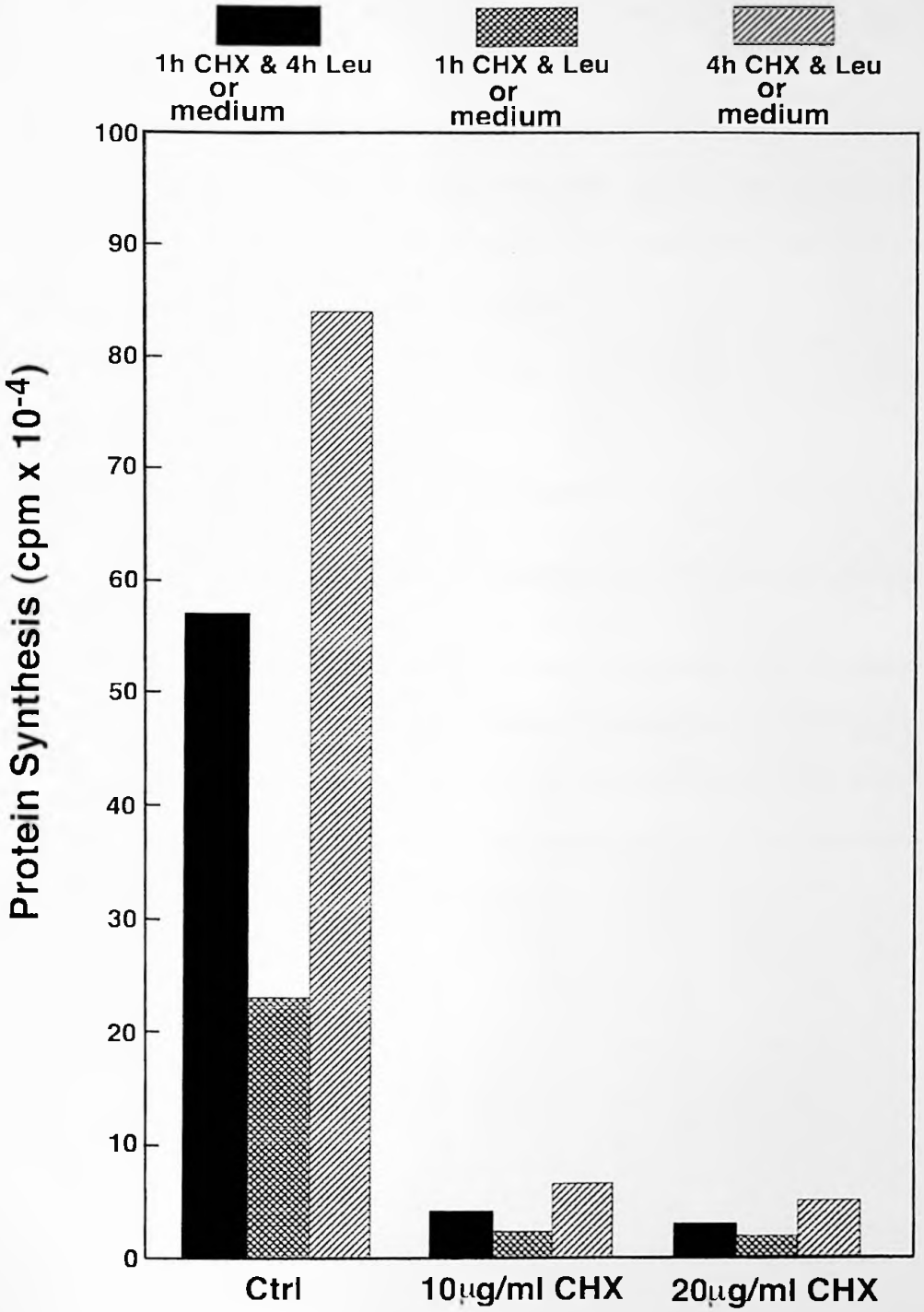

1h CHX & 4h Leu
or
medium
1h CHX & Leu
or
medium
4h CHX & Leu
or
medium
Protein Synthesis (cpm x 10-4)
100
90
80
70
60
50
40
30
20
10
0
Ctrl
10μg/ml CHX
20μg/ml CHX

FIGURE 6: Effect of Cycloheximide and R5020 on c-myc mRNA

Cells were grown for 5 days in regular basal medium Eagle (BME) growth medium with one change of medium. On the day of the experiment, medium was replaced with serum-free, insulin-free, phenol red-free medium for 4 hours . Cells between 20 and 30% confluency were then exposed for 1 hour to one of four treatments:

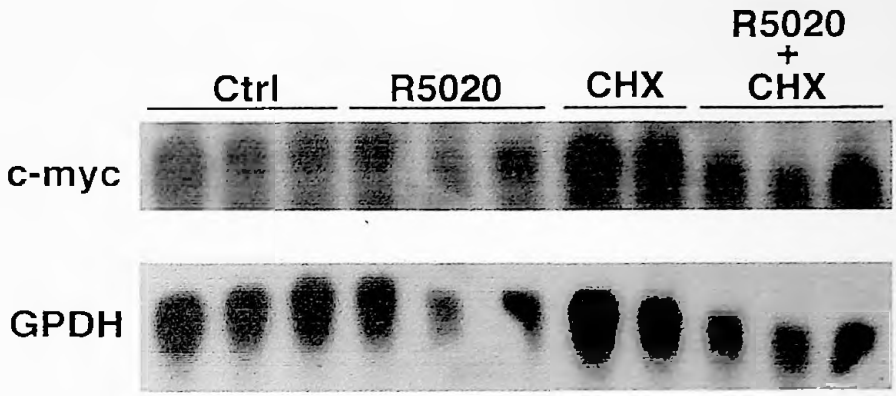
CTRL - 0.1% ethanol

R5020 - 10^{-8} M R5020 in 0.1% ethanol

CHX - 15μg/ml cycloheximide + 0.1% ethanol

CHX + R5020 - 10^{-8} M R5020 + 15 μg/ml cycloheximide in 0.1% ethanol final concentration

Treatments were performed in triplicate. RNA was isolated, analyzed, and quantitated as in Figure 2. Data are from one experiment. The magnitude of stimulation by R5020 and cycloheximide varied among experiments, but in all cases was statistically different from controls ($p < 0.05$). In each experiment, the cycloheximide + R5020 treatment caused a greater increase in the levels of c-myc mRNA than did treatment with R5020 alone.


Ctrl
R5020
CHX
R5020
+
CHX
c-myc
GPDH

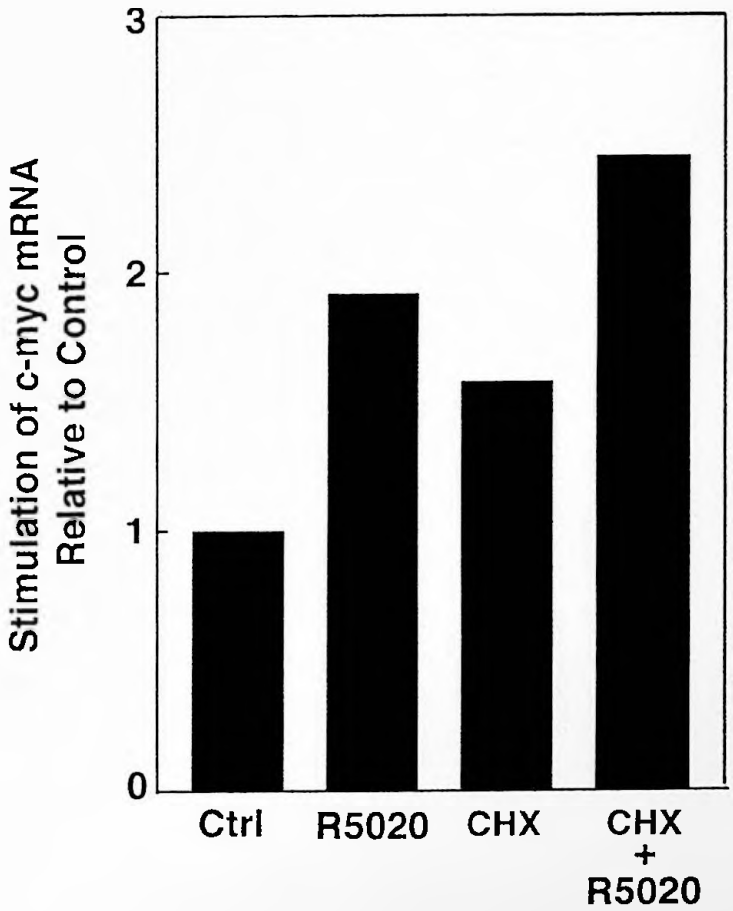

Stimulation of c-myc mRNA
Relative to Control
3
2
1
0
Ctrl
R5020
CHX
CHX
+
R5020

FIGURE 7: CHX/R5020: Mean Stimulation of c-myc mRNA

Treatments were as described in Figure 6. This represents the mean of three such experiments. R5020, cycloheximide, and cycloheximide + R5020 treatments were all significantly different from controls, but were not statistically different from one another, using the Student-Newman-Kuels multiple comparison analysis ($p < 0.05$).

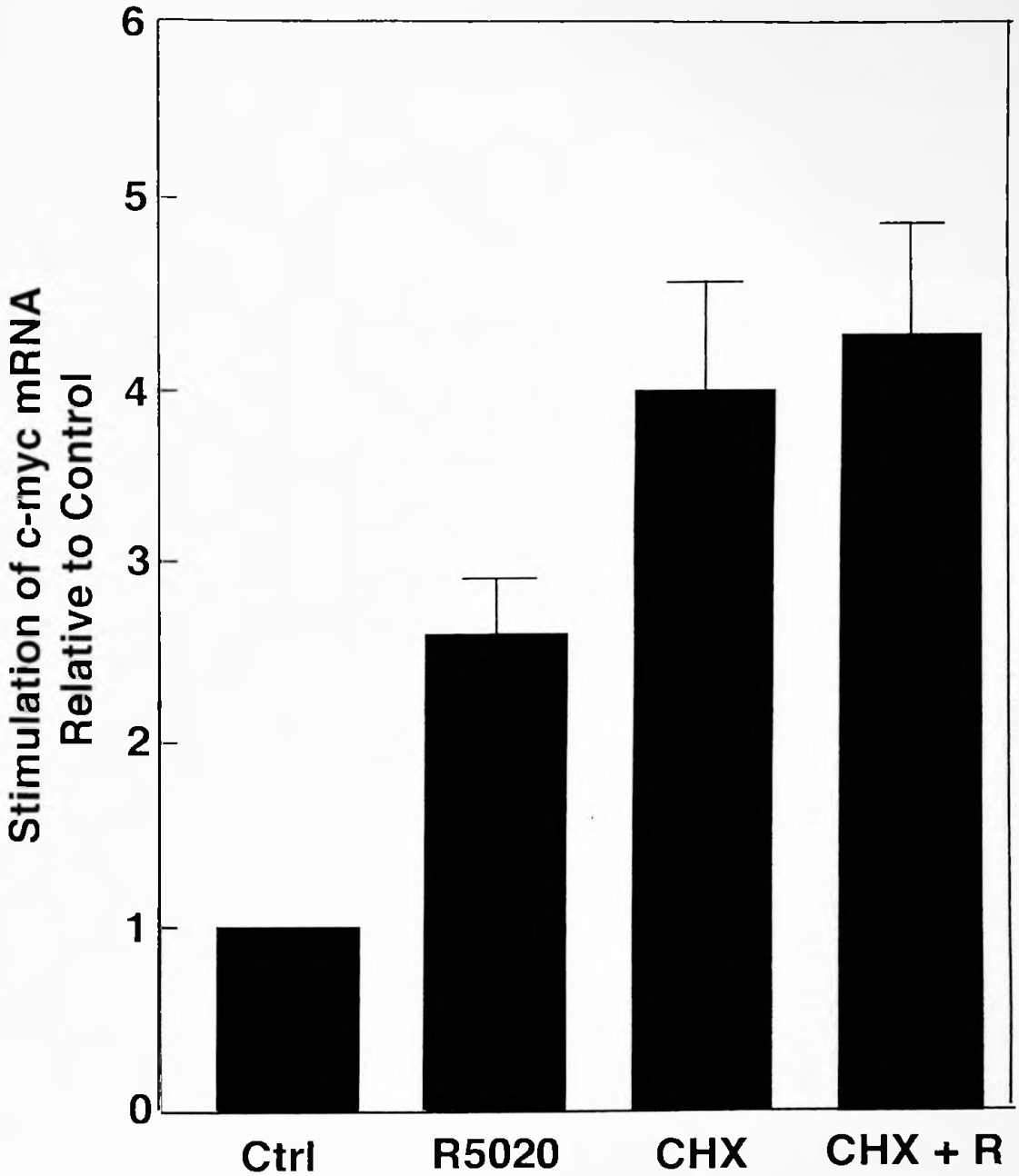
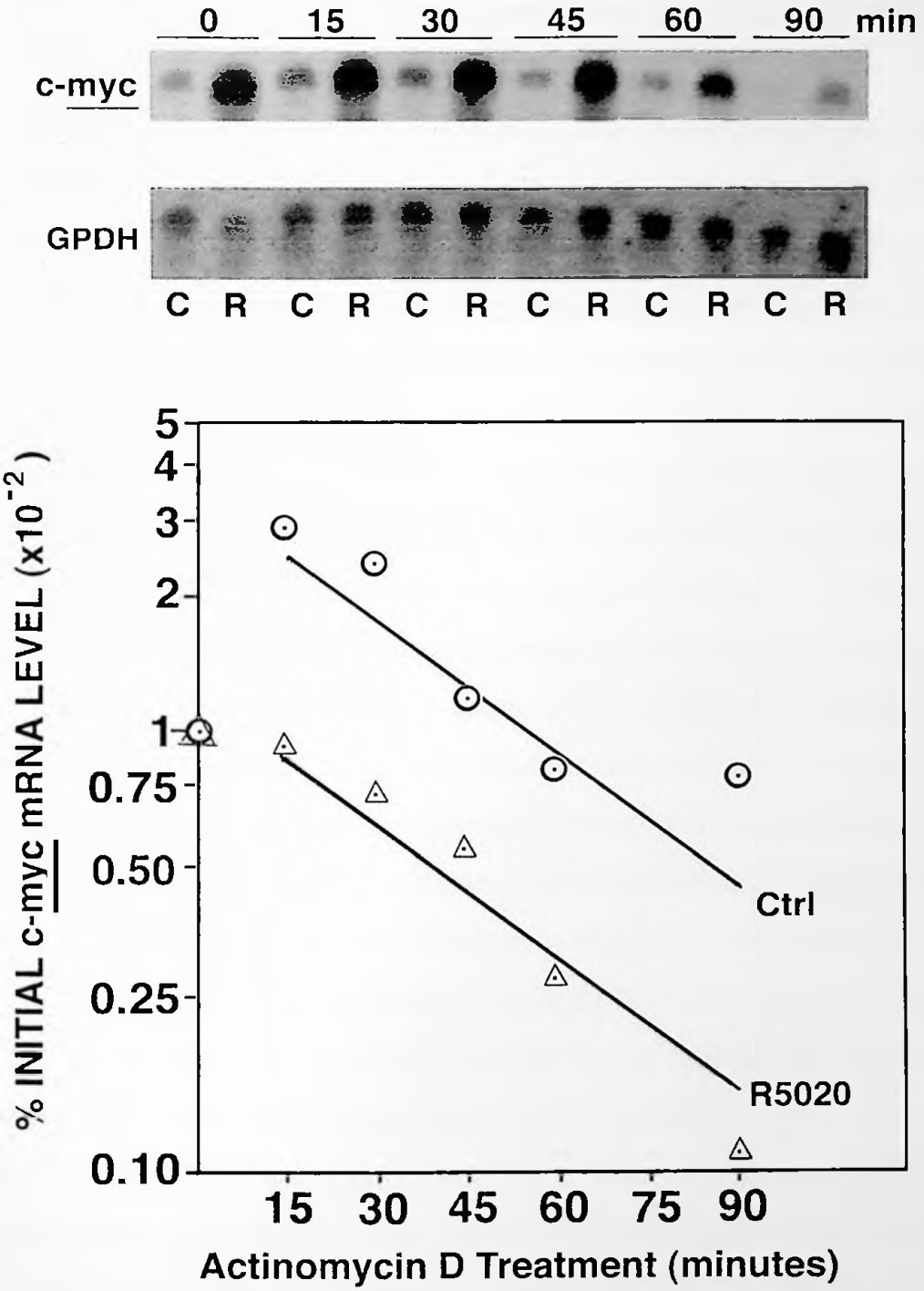

Stimulation of c-myc mRNA
Relative to Control
6
5
4
3
2
1
0
Ctrl
R5020
CHX
CHX + R
Error bars = S.E.M.

FIGURE 8: Effect of Actinomycin D on c-myc mRNA

Cells were grown for 5 days in medium containing 10% double charcoal-stripped fetal bovine serum (CS^2FBS). On the day of the experiment the medium was replaced with serum-free, insulin-free, phenol red-free medium (SF/IF/PRFM) for 4 hours. Cells (20-30% confluent) were treated in SF/IF/PRFM for 1 hour with either 0.1% ethanol **[CTRL]** or 10^{-8} M R5020 in 0.1% ethanol **[R5020]**. 1 μg/ml actinomycin D was then added to both control and R5020-treated flasks and incubation proceeded for 0-90 minutes. Northern blots of RNA from these treatments were analyzed densitometrically. c-myc values were normalized with GPDH values as in previous figures and expressed as a percentage of initial c-myc mRNA levels for each treatment. The experiment was repeated 3 times. The apparent increase in % initial c-myc mRNA levels for control cells over that of R5020-treated cells was not reproducible.


0 15 30 45 60 90 min
c-myc
GPDH
C R C R C R C R C R C R
% INITIAL c-myc mRNA LEVEL (x10-2)
5
4
3
2
1
0.75
0.50
0.25
0.10
Ctrl
R5020
15 30 45 60 75 90
Actinomycin D Treatment (minutes)

DISCUSSION

In these studies I have investigated a possible mechanism by which progestins stimulate growth in T47D human breast cancer cells, namely through transcriptional up-regulation of the protooncogene c-myc. I have characterized the increase in c-myc mRNA in response to the synthetic progestin R5020 in terms of dose-response, the time dependency for stimulation, and inhibition of R5020 stimulation with the antiprogestin RU486. The hormone specificity for c-myc induction was also tested with steroids from each of the major classes of classical steroid hormones. The protein synthesis inhibitor cycloheximide was used to determine if de novo protein synthesis was necessary for induction of c-myc mRNA. Actinomycin D, an inhibitor of transcriptional elongation, was used to determine if progestins affected the stability of c-myc mRNA and to determine the half-life of the c-myc message in T47D cells in our culture conditions.

Expression of c-myc is critical for growth. In the earliest stages of life, c-myc is expressed at high levels. c-myc mRNA has been detected as early as embryonic day 2 in preimplantation mammalian embryos. c-myc expression in initiated during the activation of the zygotice genome after the first cleavage, increases to constitutive levels after the third cleavage (8-cell), and remains high during differentiation of the 8-cell morulae into blastocysts. Interruption of c-myc expression halts development at the 8-cell/morulae stage (Paria, 1992). Whereas family members L- and N-myc's expression is restricted to specific tissue and stages of development, c-myc is more generalized, found in most proliferating cells (Musgrauer, 1988).

The direct involvement of c-myc in cell cycle progression was suggested by the observation that transfection of the c-myc gene or microinjection of the Myc protein into quiescent cells enabled them to enter the cell cycle and replicate (Kaczmarek, 1985; Kingston, 1984). Down-regulation of c-myc in P1798 lymphosarcoma cells resulted in a subsequent 50% decrease in the percentage of cells in S-phase with a corresponding increase in cells in G_o/G_1-phase (Forsthoefel, 1987). Treatment of T-lymphocytes with antisense c-myc oligonucleotides prevented entry into the

replicative S-phase of mitosis (Heikkila, 1987). Progression of cells past the restriction point in late G_1 commits cells to DNA synthesis/replication. This progression is dependent on c-myc expression, but c-myc alone is not sufficient for passage into S-phase. G_1 cyclin dependent kinases, D-type cyclins, the presence of mitogens, and, for many nontransformed cells, an intact cytoskeleton involved in cell adhesion are also required (Bohmer, 1996; Nass, 1997).

Although high levels of c-myc expression have been noted in rapidly proliferating fetal epithelial and mesenchymal cells (Schmid, 1989), in tumor cells (Holt,1988; Marcu, 1992), and in cells stimulated by growth factors (Reuse, 1990; Haugel, 1992), F9 murine teratocarcinoma stem cells were reportedly unaffected by enhanced c-myc expression (Schulz, 1989). Down-regulation of c-myc in F9 cells, however, did lead to growth arrest (Dean, 1986a) and induced differentiation (Dony, 1985). Cell-cycle arrest due to reduction in c-myc expression has also been reported in the HL60 promyelocytic cell line (Bentley, 1986). Inhibition of Myc protein synthesis induced HL60 cells to differentiate into mature myeloid cells (Holt, 1988). Inhibition of proliferation in papovavirus-transformed C129 murine fibroblasts was preceded by reduction in c-myc mRNA levels (O'Banion, 1992). Decreased c-myc expression was accompanied by induction of differentiation in murine erythroleukemic cells (Lachman, 1984), whereas constitutive c-myc expression blocked their differentiation (Coppola, 1986). In medullary thryroid carcinomas a reduction in c-myc gene transcription has also been linked to induced differentiation (de Bustros, 1985).

The emerging picture is that in many cell lines up-regulation of c-myc favors proliferation, whereas attenuation of c-myc expression promotes differentiation. Involvement of c-myc in the enhancement of growth for MCF-7 breast cancer cells under estrogenic stimulation (Dubik, 1988; Thomas, 1995; van der Burg, 1989) raises the possibility that progestins stimulate the growth of breast cancer cells through a similar induction of this protooncogene. In the progesterone receptor (PR)-rich breast cancer cell line T47D, the synthetic progestin ORG 2058 stimulated and then inhibited cell cycling. c-myc was rapidly but transiently induced by this progestin (Musgrove,

1991). The experimental conditions employed during this investigation, however, included phenol red which may have masked a pure progestin response with respect to c-myc induction since commercial phenol red has been shown to act as an estrogen (Berthois, 1986). In addition, the control cells grew very well under the conditions of Musgrove et al. (Musgrove, 1991). Under our growth conditions (Hissom, 1987; Moore, 1997) control cells grow very slowly or not at all, and progestins stimulate growth in a sustained manner. In our hands, if the control cells are growing rapidly, progestins will not further stimulate their growth (data not shown). The biphasic regulation of breast cancer growth reportedly was also affected by progestin-induced expression of growth factors and their receptors (Murphy, 1985; Murphy, 1991; Musgrove, 1991; Papa, 1990), although entry of cells into S-phase was shown to precede progestin-stimulated increases of these growth factors (Musgrove, 1991). Unfortunately, phenol red was included in culture media for these experiments, as it was in those determining the role of cyclin-dependent kinase inhibitors p21 and $p27^{Kip1}$ in the noted biphasic response of T47D cells to progestins (Groshong, 1997). The effect of progestins on c-myc mRNA expression in the absence of phenol red, under conditions in which the control cells grew very slowly or not at all (as in ours), had not been determined in human breast cancer cells expressing adequate levels of PR such as in T47D cells.

Maximal stimulation of c-myc mRNA occurred with a 10 nM concentration of R5020 (Figure 1). In MCF-7 cells, estradiol at the same dose elicited peak production of the Myc protein within 90 minutes of treatment (Watson, 1991). In the same cell line a 5.3-fold increase in c-myc mRNA levels in phenol red-free medium occurred with a 10-fold lower dose of estradiol (van der Burg, 1989). A 10-fold higher concentration, 10^{-7} M, was needed to achieve a 10-fold induction of c-myc transcription in medium containing phenol red and 10^{-6} M tamoxifen, an anti-estrogenic compound (Dubik, 1988). In normal human breast epithelial (HBE) cells, 10 nM estradiol in phenol red-free medium was the optimal concentration for stimulation of c-myc mRNA (Leygue, 1995). The concentration of progestin demonstrated as the optimal dose in our experiments is in agreement with concentrations used in in vitro studies by other researchers and is within the

physiologically relevant range of hormone concentrations in vivo.

During the normal menstrual cycle, levels of progestogens undergo marked fluctuations. The menstrual cycle is divided into two phases, a follicular, or proliferative, phase and a luteal, or secretory, phase. The luteal phase begins at approximately day 14 of the menstrual cycle when the dominant ovarian follicle ovulates. Changes in cellular structure and function of the follicle result in the formation of the corpus luteum. With increased vascularization and stimulation by the luteinizing hormone (LH), the corpus luteum maximally sythesizes and secretes progesterone. Plasma levels typically are reported at an average of 36 nM for the duration of the luteal phase although actual secretion of progesterone is episodic, itself correlating with pulsatile secretion of LH. With the decline of corpus luteal function, approximately 10 days post-ovulation, progesterone levels begin to decrease (Carr, 1992).

Our experimental doses of R5020 eliciting transcriptional responses in the c-myc gene are physiologically relevant and likely reflect actual cellular processes occurring monthly in women. The synthetic progestin is more stable than progesterone and could therefore effect a greater response in the microenvironment of a cell where its "apparent concentration" could be slightly higher due to delays or reductions in its metabolism. Even if this is the case and the concentration of progestin experienced by the cell is 2- or 3-fold higher than the 10^{-8} M treatment we used, the cell is still seeing no greater PR activation than routinely obseved during menstruation.

Peak induction of c-myc mRNA levels occurred with 1 hour of progestin treatment (Figure 2). van der Burg and co-workers also reported maximal c-myc stimulation at 1 hour but with the hormone estrogen. Other researchers noted a biphasic increase in c-myc transcription upon estradiol treatment with the first rise of approximately 10-fold at 20 minutes, followed by maximal stimulation at 1 hour in MCF-7 cells (Dubik, 1988) or in HBE cells an initial increase in mRNA levels of 1.9 +/- 0.3-fold after 30 minutes and a comparable stimulation (1.7 +/- 0.3-fold) again after 60 minutes (Leygue, 1995). c-myc was also maximally stimulated after 1 hour of treatment with thyrotropin in canine epithelial thyrocytes in primary culture (Reuse, 1990). The progestin

ORG 2058 in T47D cells adapted to grow in insulin-containing serum-free medium induced a 3.5-fold increase of c-myc mRNA levels after only 30 minutes with maximal stimulation of 8-fold after 1-2 hours (Musgrove, 1991).

The rapid increase in c-myc mRNA levels that we observed is in agreement with a model of progestin transactivaton of the c-myc gene. One would not expect to observe significant elevation of c-myc message earlier than 15-30 minutes following initiation of transcription. Due to the fact that c-myc mRNA is rapidly degraded or processed in the cell with a reported half-life of only 15-30 minutes (Dean, 1986; Dubik, 1988; Leygue, 1995), accumulation of myc message likely does not occur, and observed increases in total c-myc mRNA reflect ongoing transactivation of the gene. By one hour of hormone exposure to a set of 3 million cells, the majority of c-myc genes being activated has already occurred and the transcripts processed to maturity. After 1 hour, the balance of sythesis and degradation favors degradation. c-myc mRNA levels decline to basal levels following stimulation, unless the mitogen mediates a biphasic increase as has been observed in the case of estrogenic stimulation of c-myc in HBE cells (Leygue, 1995).

The pronounced effect of R5020 on the cells depicted in Figure 2 likely reflects stimulation of a population of cells which were more quiescent than other populations we've tested. Control cells would express much lower c-myc levels, resulting in a higher R5020 stimulation of c-myc mRNA. Even though protocols to reduce or eliminate growth factors and other mitogens are utilized in all experiments, variation is still observed. Differences in commercially-obtained serum is a factor (Forsthoefel, 1987; Leygue, 1995). Preliminary experiments demonstrating progestin's influence on cell growth, not shown here, revealed a variaton in stimulation that was serm lot-dependent.

T47D cells themselves also inherently contain variables. These cells are known to range in growth and responses due to slight genetic instability (Reddell, 1988). Passage number, or the amount of time a particular cell population has been grown in culture, often correlates with such changes. Although I seldom used cells in experiments that had been maintained in cell culture for

more than 400 total passages, I still could have unknowingly used populations with varying basal growth rates. The time-course experiments were repeated three times over a span of 2-3 years and in all cases demonstrated maximal stimulation at 1 hour. The maximum achievable stimulation, however, fluctuated with passage number and/or serum-medium conditions. As c-myc is known to respond to a considerable number of reagents, reagents which may not alsways be constant in cells/serum/medium, it is understandable that stimulation ranged from 2- to 4-fold in the course of these experiments.

As described earlier, progestins can operate via PR- and PRE-independent pathways to elicit cellular responses as well as through conventional PR-PRE interactions. RU486, a type I progesterone antagonist, interferes with progestin activation of genes by binding the progesterone receptor and effecting PR conformational changes that render it less capable of interacting properly with transcriptional machinery. Treatment with RU486 should significantly reduce progestin stimulation if that stimulation proceeds by PR-mediated mechanisms.

The anti-progestin RU486 abrogated R5020-induced stimulation of c-myc mRNA (Figure 3). This is consistent with reports of RU486 inhibition of proliferation in T47D cells (Bardon, 1985; Gill, 1987) and antagonism of transient increases in percentage of cells in the S-phase of the cell cycle upon progestin treatment (Musgrove, 1991). Because RU486 does not behave as a pure antagonist at elevated concentrations, RU486 concentrations of 0.1 nM in our experiments and 0.5-1 nM in the work of Musgrove and co-workers were used. A treatment time of 2 hours for this experiment deviates from the treatment schedule of the other experiments presented here. Preliminary work by Dr. Moore and co-workers had suggested 2 hours were optimal in eliciting cellular progestin-induced responses in T47D cells, and the RU486 experiment was conducted before I had completed the R5020 time course experiments for c-myc induction. A more pronounced R5020 stimulation of c-myc mRNA levels would have likely occurred had RNA been collected after only 1 hour of progestin treatment. Although the effect may have been more dramatic with a 1 hour treatment regimen, the conclusions would have remained the same. RU486

negation of R5020 stimulation of c-myc mRNA suggests that progestin enhancement of c-myc occurs via progesterone receptor-mediated pathways.

These experiments provide support for the hypothesis that progestins stimulate c-myc as a primary response in which the gene is activated by the liganded PR binding a progesterone response element (PRE). Additional work performed by Jian-Lang Zhou, a post-doctoral fellow working in Moore's laboratory, also supports this hypothesis. Using chimeric c-myc reporter gene constructs including a putative PRE [GGAACC-GCC-TGTCCT] identified by Moore in the 5' upstream region of the human c-myc gene and using the c-myc gene's own promoter, Zhou found that R5020 treatment caused a statistically significant increase in CAT activity (acetylation of chloramphenicol) in a hormone- and PR-dependent manner. Fold stimulation was similiar to levels I had observed for c-myc mRNA stimulation in T47D cells. Gel mobility shift assays conducted by Renee Gentry and Moore also demonstrated specific binding of human progesterone receptor isoforms A and B with this putative PRE. This binding was enhanced with progestin treatment (Moore, 1997). Together these data strongly suggest that progestin activation of c-myc is via stimulation of the rate of transcription.

In Figure 4 the hormone specificity of c-myc induction is illustrated. The only two classes of steroid hormones which significantly stimulated c-myc expression were progestins (R5020 and progesterone) and estrogen. None of the other steroids tested here significantly altered c-myc mRNA levels. Dubik and co-workers likewise did not see alterations in c-myc message with dexamethasone or testosterone even at 0.1 μM concentrations. The progestin, medroxyprogesterone acetate (MPA), under their conditions, however, also failed to enhance c-myc expression (Dubik, 1988). This is likely due to the presence of phenol red in their medium, such that progestin stimulation was counter-balanced by progestin's anti-estrogenic effects. In P1798 lymphosarcoma cells, clone C7, the glucocorticoid dexamethasone reversibly inhibited initiation of c-myc transcription which was statistically significant at 3 hours and maximal at 90%

inhibition within 6 hours (Forsthoefel, 1987). In the L929 fibroblast cell line, 0.1 μM dexamethasone did not inhibit the c-myc gene (Frost, 1994), although numerous other investigators have reported glucocorticoid inhibition of c-myc in various cell lines (Eastman-Reks, 1986; Forsthoefel, 1987; Ma, 1992; O'Banion, 1992; Yu, 1989). The effect of other steroid or steroid-like hormones on c-myc transduction has been reported as inhibition by retinoic acid (Dubik, 1992; Schulz, 1989), stimulation by thyrotropin (Reuse, 1990), by 10 nM hydrocortisone (Leygue, 1995), and by 30 nM ACTH (Liu, 1996).

The protein synthesis inhibitor cycloheximide at concentration of 10-20 μg/ml has been reported to superinduce c-myc expression (Dubik, 1988; Kelly, 1983; Leygue, 1995; Lindsten, 1988; Reed, 1986; Reuse, 1990), most likely by de-represson of a labile protein. In order to determine if on-going protein synthesis was required for hormonal stimulation of c-myc expression, investigators used cycloheximide in cells pre-treated with medium of reduced mitogenic potential by eliminating serum (Reuse, 1990), by inclusion of micromolar concentrations of the anti-estrogen tamoxifen (Dubik, 1988), or by removal of serum, phenol red, and any other known mitogens (Leygue, 1995). Only when cells were sufficiently quiescent at onset of treatment was the hormonal stimulation of c-myc evident in the presence of cycoheximide. Mauvis-Jarvis and co-workers demonstrated that after 4 hours of pre-treatment with basal medium, 10 nM estradiol + 14 μg/ml cycloheximide caused a 2.5-fold increase in c-myc mRNA levels which surpassed the 2-fold stimulation by estadiol alone and the 1.5-fold stimulation by cycloheximide (Leygue, 1995). In tamoxifen-treated MCF-7 cells pre-exposed 2 hours to 50 μM (14 μg/ml) cycloheximide, Dubik and co-workers demonstrated a relative 12-fold increase in c-myc mRNA levels after 20 minutes of co-exposure to estradiol and cycloheximide. This was 4X higher than stimulation seen by cycloheximide alone and more than double that seen by estradiol alone (Dubik, 1988).

A modest increase in c-myc mRNA levels was observed in cells co-treated with 10 nM R5020 and 15 μg/ml cycloheximide for 1 hour compared to levels elicited by either reagent alone (Figure 6). Although this result suggested that de novo protein synthesis was not required for progestin stimulation of c-myc, additional work did not unequivocally support this (Figure 7). Because the cells were not equally quiescent at the onset of treatment, fold stimulation by progestin and/or cycloheximide varied among experiments such that statistical significance was not achieved.

In future studies I would test the various pre-treatment serum conditions reported to be used in the laboratories of Sutherland (Musgrove, 1991), Mauvis-Jarvis (Leygue, 1995), and Shiu (Dubik, 1988) for reduction of basal c-myc expression. Combinations of these pre-treatments with maintenance of cells for 5 days in CS²FBS medium would likely arrest cells in the G_0-phase of the cell cycle in which c-myc expression is repressed (Hann, 1985). Subsequent treatment for only 20-30 minutes as was done in the experiments of Dubik and Leygue above might allow detection of hormone induction of c-myc before the effects of cycloheximide reached maximal levels, coincidentally masking the hormonal effect. Progestins typically do not stimulate c-myc as much as estrogens do, so the level of c-myc expression after only 30 minutes may not always be suficiently high to gain statistical significance. Further work is necessary to determine if cycloheximide can be used in our culture conditions to answer definitely whether or not protein synthesis is required for progestin stimulation of c-myc. The use of different protein synthesis inhibitors with less pleiotropic effects may also warrant investigation, although work by Forsthoefel et al. with the inhibitor emetine could not provide conclusive evidence either as the requirement of on-going protein synthesis for stimulation of c-myc expression (Forsthoefel, 1987).

Although data from our cycloheximide experiments did not exclude the possiblility of de novo synthesis of proteins being involved in R5020 stimulation of c-myc, data from our timecourse (Figure 1) and reporter gene experiments (Moore, 1997) suggest that protein synthesis

is not required. In systems where estrogens stimulated c-myc transcription in the presence of cycloheximide, thus demonstrating an absence of the requirement for on-going protein synthesis, estrogens elicited maximal response in the same range of hormonal concentration as did R5020 and in the same time period (Dubik, 1988; Leygue, 1995). In both estrogen and progestin cases, up-regulation was rapid, being evident at 15 minutes (see Figure 2). The rapidity alone at with mRNA elevation was demonstrated strongly suggests no new proteins were necessary for hormonal induction of c-myc.

Studies utilizing the inhibitor of transcriptional elongation, actinomycin D, have demonstrated that estrogens did not increase c-myc mRNA levels via stabilization of the message. In the work by Shiu and co-workers the half-life of c-myc mRNA in both quiescent and estrogen-stimulated MCF-7 cells was determined to be 18 +/- 7 minutes (Dubik, 1988). In HBE cells estrogen likewise did not alter c-myc mRNA half-life, claculated to be 12 +/- 3 minutes in this cell line (Leygue, 1995). R5020 stimulation of c-myc mRNA also did not involve alteration of myc half-life in T47D cells (Figure 8). Initial experiments involving actinomycin D yielded inconclusive results, but showed no consistent difference between prpgestin-treated and control cells. The determination of a half-life of 23 minutes for control and R5020-stimulated cells is the result of only one experiment with duplicate samples. The value of 23 minutes is similar to that reported by other investigators and reflects the short-lived nature of c-myc mRNA. The main point of these experiments was to determine if progestins affected message stability. The fact that R5020 treatment did not enhance message stability as a means of increasing mRNA levels further supports the model that progestins induce increases in c-myc mRNA by action at the transcriptional level. Direct transduction of the c-myc gene by progestins may be part of the mechanism by which progestins stimulate the growth of human breast cancer cells.

The down-stream effects of c-myc up-regulation have just begun to be elucidated. Much information from this on the mechanisms of growth in normal and transformed cells will ensue. That progestins participate in initiating this proliferative cascade is valuable information that may be

useful in designing better therapies for the control and treatment of breast cancer.

In conclusion, evidence has been presented supporting the notion that progestins activate c-myc expression in T47D breast cancer cells, probably by stimulation of the rate of transcription. Additional work to elucidate the complete ramifications of progestin-induction of c-myc in T47D cells will no doubt show that this phenomenon is part of a complex system of controls that balance life, growth, and death. This balance is at the heart of a non-cancerous existence which every individual craves and, hopefully, someday will achieve.

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APPENDIX

SOLUTIONS

In all solutions, "water" was deionized distilled water unless otherwise specified. Manufacturers of reagents and supplies are listed below.

General Solutions

Gel Loading Buffer: 30% Ficoll, 1 mM EDTA pH 8, 0.25% bromophenol blue, 0.25% xylene cyanole FF. 3 g Ficoll-400, 40 µl 0.25 M EDTA pH 8, 25 mg bromophenol blue, and 25 mg xylene cyanole FF per 10ml water.

Prehybridization Solution: 5X SSPE, 5X Denhardt's solution, 0.5% SDS, 50% deionized formamide, warmed to 42°C before addition of 0.2 mg/ml denatured salmon sperm DNA

10% Sarcosyl: 10 g sarcosyl (N-Lauroylsarcosine, sodium salt) per 100 ml water.

20% SDS: 20 g sodium dodecyl sulfate (SDS) per 100 ml water.

20X SSC: In 900 ml water, 175.3 g sodium chloride (NaCl) and 88.2 g Na Citrate were dissolved. The pH was adjusted to 7.0, the volume brought to 1 liter, and the solution autoclaved.

20X SSPE: In 480 ml water, 105.19 g NaCl, 9.45 g monobasic sodium phosphate (NaH_2PO_4 H_2O), 8.44 g dibasic sodium phosphate (Na_2HPO_4 $7H_2O$), and 2.92 g EDTA were dissolved. Twenty pellets of NaOH were added. The pH was adjusted to 7.7 with more solid NaOH or 1N NaOH. The volume was brought to 500 ml with water and autoclaved.

TE Buffer: 10 mM Tris-HCl pH 8, 1 mM EDTA pH 8.0

Low TE: 10 mM Tris, 0.1 mM EDTA pH 8.0

TE-saturated Phenol: TE buffer pH 8.0, 3 ml per 10 ml melted phenol, was mixed in a 50ml disposable centrifuge tube, vortexed for 5 minutes, then centrifuged at 500 x g for 15 minutes to separate phases. Vortexing/centrifugation was repeated until a bilayer was seen. The upper aqueous layer was removed with a Pasteur pipet. Alternately, 0.05 g 8-hydroxyquinoline, 50 ml liquified phenol, and 50 ml 50 mM Tris base were stirred in a 250ml beaker for 10 minutes at low

speed. Phases were allowed to separate at room temperature and the aqueous layer discarded into waste container. 50 ml of 50 mM Tris-HCl pH8 were added, mixed as above, and this was repeated once. The final phenol layer was mixed with 25 ml of 50 mM Tris-HCl pH 8.0 and stored protected from light at 4^{0}C (Aushabul, 1995).

TEPC: 1:1 TE-saturated phenol/chloroform. Equal volumes of TE-saturated phenol and chloroform were mixed at room temperature.

TEPCIA: 25:24:1 TE-saturated phenol: chloroform: isoamyl alcohol. For 50 ml, 25 ml of TE-phenol, 24 ml of chloroform, and 1 ml of isoamyl alcohol were combined in a 50ml polypropylene centrifuge tube. Care was taken not to use polystyrene tubes as these would disintegrate in TEPC.

Cell Culture

Charcoal-stripped Fetal Bovine Serum (CS-FBS): One liter of dextran-coated charcoal (DCC) was divided into 250ml Nalgene centrifuge bottles and pelleted by centrifugation at 2000 x g for 10 minutes (4000 rpm in JA-14). Heat-inactivated FBS was incubated with the pellets from 2 volumes of DCC for 30 minutes at 45^{0}C with constant agitation. To achieve this, the FBS-DCC mixture was divided into several smaller fractions in separate bottles and placed in the temperature-controlled Gyrotory Water Bath Shaker, Model G76, at an agitation setting of "5". Throughout incubation the water level in the bath remained above that of the FBS-DCC fractions. At the end of incubation the separate fractions were pooled and redivided into balanced 250 ml centrifugation bottles, spun at 2000 x g for 10 minutes to remove the DCC, and the FBS supernatant was coarse-filtered using Whatman or Micron Separations, Inc. filters in a Millipore filter system. For double charcoal-stripping, the filtered charcoal-stripped fetal bovine serum (CS-FBS) was incubated again with 2 volumes of fresh DCC, prepared as above, and subjected to the same treatment as above. Following coarse filtration, the charcoal-stripped FBS (CS-FBS or CS^{2}FBS) was filtered under sterile conditions through 0.2 μm filter (Nalgene) into a clean autoclaved medium culture bottle and stored at -20^{0}C.

Dextran-coated Charcoal (DCC): DCC was prepared by mixing 2.5 g Norit A activated charcoal, 25 mg Dextran, and 10 ml 1M Tris-HCl pH 8.0 per final volume of 1 liter of water.

Growth Medium: One bottle of Gibco-BRL Powdered Basal Medium Eagle, Modified, Autoclavable, with phenol red was mixed with 4.5 liters of water, pH adjusted to 4.1-4.2, subdivided into ten clean 500ml tissue culture medium bottles, and autoclaved for 20-30 minutes. After the solution had cooled to room temperature, the following sterile solutions were added: 15 ml of 7.5% $NaHCO_3$, 5 ml of Gibco streptomycin-penicillin (10^4 U/ml penicillin; 10^4 µg/ml streptomycin sulfate in 0.85% saline), 5 ml of 10mM MEM non-essential amino acids, 4 ml of previously prepared stock L-glutamine/insulin solution, and 50 ml of fetal bovine serum (FBS) heat-inactivated at 56°C for 30 minutes. pH was adjusted to 7.2-7.4 with sterile 1N NaOH or 1M HCl. The final concentration of ingredients was 0.2% $NaHCO_3$, 100 µg/ml streptomycin, 100 units/ml penicillin, 100 nM non-essential amino acids, 2 mM L-glutamine, 6 ng/ml insulin, and 10% FBS.

L-glutamine Stock Solution: L-glutamine (1.83 g) was dissolved in 50 ml 0.01N HCl and filter-sterilized into sterile 15ml disposable centrifuge tubes.

L-glutamine/insulin Stock Solution: L-glutamine (3.66g) was mixed in a final volume of 75 ml 0.01N HCl. Insulin (0.15mg) was dissolved in 50 ml 0.01N HCl, and 25 ml of this was added to the glutamine solution. The stock solution was filter-sterilized (Nalgene, 0.2µm filter), dispensed into 15ml polystyrene centrifuge tubes, and stored at -20°C.

Splitting Solution: In a final volume of 995 ml of water, the following were dissolved: 8.0 g NaCl, 1 g Dextrose , 0.4 g KCl, 0.372 g disodium EDTA (Na_2EDTA) or 0.292 g EDTA, 0.09 g dibasic sodium phosphate (Na_2HPO_4-$7H_2O$), and 0.06 g monobasic potassium phosphate (KH_2PO_4). This was divided into two 500ml bottles and autoclaved for 20-30 minutes. Before use, 2.35 ml sterile 7.5% $NaHCO_3$ was added to each bottle.

RNA Isolation

49:1 Chloroform: Iso amyl alcohol: 0.1 ml of isoamyl alcohol per 4.9 ml chloroform.

0.1% DEPC-H_2O: In an open 500ml bottle 0.5 ml of diethylpyrocarbonate (DEPC) was stirred 10 minutes with 495.5 ml water. The solution was autoclaved, then stirred while still hot in the sterile hood for 30 minutes with the lid removed. The sealed solution was stored at room temperature.

Guanidinium Thiocynate Stock Solution: 4M guanidinium thiocynate, 25mM sodium citrate, and 0.5% sarcosyl.

Solution D: 7.2 µl β-mercaptoethanol per ml of guanidinium thiocynate stock solution.

Water-saturated Phenol: Phenol was warmed to 65°C in the Gyrorotory water bath. Warmed phenol (20-50ml) was added to a Kimax 500ml separatory funnel and an equal volume of water added to it. Manufacturer recommendations called for only one-fourth the volume of water to phenol, but experience proved that more water was often necessary to saturate the phenol. The mixture was shaken vigorously, aerated, and then placed in the cold room, protected from light, to allow the phases to separate. In 4-8 hrs the lower phenol layer was recovered into a 50ml polypropylene centrifuge tube. An anti-oxidant, 0.1% (w/v) 8-hydroxyquinoline, was added to the phenol and mixed by inversion. A thin layer of water was applied to the top of the phenol as further protection against oxidation. The tube was wrapped in aluminum foil and stored at 4°C.

RNA Electrophoresis and Dot Blots

Deionized Formamide: BioRad AG501-X8 resin (0.05 g per ml of formamide) was mixed 1 hr on a stir plate. The deionized formamide was then filter-sterilized into a 15ml centrifuge tube using Gelman 0.2µm syringe filters attached to 5ml syringes.

Ethidium Bromide Stock Solution, 10 mg/ml: As ethidium bromide is a putative carcinogen, gloves were worn when weighing out 10 mg of ethidium bromide and dissolving it in 1 ml sterile water. The microcentrifuge tube was wrapped with tape to protect the solution from light and

stored at 4^{0}C, marked as hazardous.

Ethidium Bromide Staining Solution, 5 µg/ml : 150 µl of the 10 mg/ml stock ethidium bromide solution above were added to 300 ml water in a plastic tupperware dish. The lid was marked with a cautionary statement as to the danger of the contents. The solution was stored in the dark.

10X MOPS Buffer pH 7: 0.2M MOPS (3-[N-Morpholino]propanesulfonic acid), 0.5M NaOAc, and 0.01M Na_2EDTA. For a final volume of 500 ml, 20.93 g of MOPS, 34.02 g NaOAc, and 1.86 g Na_2EDTA were dissolved, and the pH was adjusted to 7. The bottle was wrapped in aluminum foil prior to being autoclaved. The wrapped solution was stored at room temperature, but discarded if it became yellow in color.

RNA Denaturing Solution (Dot Blot): 500 µl deionized formamide, 162 µl 37% formaldehyde, and 100 µl 10X MOPS buffer.

RNA Gel (11x14cm^2): One gram of agarose was mixed with 73 ml sterile water and 10 ml sterile 10X MOPS buffer and boiled 2 minutes until the agarose was thoroughly dissolved. The beaker was then taken to the chemical hood in which the horizontal gel apparatus was assembled. In the hood, 17 ml of 13.3M formaldehyde were mixed with the 55-65^{0}C agarose/MOPS solution, and the gel poured into the gel box. The comb was inserted, and all bubbles moved to the periphery with a clean pipet tip. The gel was allowed to polymerize at least 30 minutes before covering with 750 ml of 1X MOPS buffer.

RNA Gel Samples: RNA (20 µg in 6 µl of 0.1% DEPC-H_2O) was mixed with 12.5 µl deionized formamide, 2.5 µl 10X MOPS buffer, and 4 µl formaldehyde. Samples were heated to 65^{0}C for 5 minutes in the Fisher Scientific Isotemp Dry Bath 145, quick-chilled on ice, and 2 µl of RNA loading buffer added. Samples were centrifuged briefly to collect in the microcentrifuge and loaded on the 1% agarose, 2.2M formaldehyde, 1X MOPS gel.

RNA Northern Hybridization

100X Denhardt's Solution: 2% (w/v) BSA (bovine serum albumin), 2% (w/v) Ficoll-400, and 2% (w/v) PVP (polyvinylpyrrolidone). In 10 ml water, 0.2 g of each were dissolved, filter-sterilized, and aliquoted into 6 autoclaved microcentrifuge tubes. This was stored at -20°C.

Salmon Sperm DNA: Salmon sperm DNA (50 mg) was hand-shredded and dissolved in 5 ml TE Buffer pH8.0. TEPCIA (5ml) was added, vortexed 1 minute, and then chilled on ice. The solution was centrifuged 1 minute in an IEC Clinical centrifuge at full speed and the aqueous layer transferred to an autoclaved 30ml Corex tube. DNA was precipitated with 0.1 volume of 3M NaOAc pH 5.2 and 2.2 volumes of 200-proof ethanol, covered with parafilm, and placed at -20°C for 1 hour or more. The precipitant was centrifuged at 10,000 x g for 10 minutes at 4°C. The DNA pellet was washed with 1 ml ice-cold 70% ethanol, dried *in vacuo* in a dessicator, and resuspended in 5 ml 0.1N NaOH. This was divided into 4 aliquots and stored at 4°C.

cDNA Probes Maintenance and Isolation

Equilibration Buffer (Sephadex columns): 10mM Tris-HCl pH 8.0, 50mM NaCl, 0.1mM EDTA pH 8.0, 0.1% SDS.

G-50 Sephadex Slurry: G-50 Sephadex (2g) was mixed with 35 ml of equilibration buffer and stirred for 24 hours.

GET Buffer: 25mM Tris-HCl pH 7.5, 10mM EDTA, 15% sucrose.

Luria-Bertani (LB) Agar Plates: In water, 15g/l Bacto-tryptone + 5g/l Yeast Extract + 10g/l NaCl + 15g/l of agar were dissolved. The solution was autoclaved for 25 minutes. When the mixture had cooled to approximately 50°C, 12.5 µg/ml tetracycline-HCl were added. Into each agar plate, 32-40 ml of this were poured in the sterile hood with the lights off. Lids were removed until after the agar had solidified. Plates were stored in the original plastic sleeve at 4°C in the dark. Plates were identified with "LB/Tet" marked on the lids as well as the date of production.

LB Medium: In water, 10g/l Bacto-tryptone + 5g/l Yeast Extract + 10g/l NaCl were dissolved,

titrated to pH 7.5 with 5N NaOH (approximately 14 drops per .5 liters), and autoclaved. Before use, 12.5 µg/ml tetracycline-HCl was added to the culture.

Lysis Buffer: 5.6 ml of GET buffer per 0.6ml of 40 mg/ml lysozyme.

Lysozyme Stock, 40 mg/ml: In 5 ml of sterile water, 0.2g of lysozyme were dissolved.

13% PEG Solution: 13 g of polyethylene glycol (PEG) per 100 ml water.

Sodium Phosphate Solution: 6.85 ml 0.2M NaH_2PO_4 H_2O + 3.15 ml Na_2HPO_4 $7H_2O$ in 20ml final volume.

RNase Stock, 4 mg/ml: In 10 ml sterile water, 0.04 g of RNase was dissolved, aliquotted into ten autoclaved 1.5ml microcentrifuge tubes, and stored at -85°C.

Solution II: 0.2M NaOH, 1% SDS.

25 mg/ml Tetracycline-HCl Stock Solution: Tetracycline-HCl was shipped in vials of 20 mg and stored at -20°C. Sterile water (0.8 ml) was pipetted into the vial, mixed gently to dissolve the tetracycline, and stored at 4°C with the lid parafilmed. To achieve a final concentration of 12.5 µg/ml tetracycline, 5 µl of this stock solution were added per ml of LB medium.

10% TCA: 1 ml trichloroacetic acid (TCA) per 10 ml water.

Experiments

1M Cycloheximide: 28.13 mg cycloheximide per ml of EtOH.

Denaturing Solution (nuclear run-on exp.): 1.5 M NaCl, 0.5 M NaOH

1M DTT (dithiothreitol): 0.1542 g per ml of water.

Glycerol Storage Buffer: 50 mM Tris, 5 mM $MgCl_2$, 0.1 mM EDTA, 40% (v/v) glycerol, pH 8.3

Neutralizing Solution (nuclear run-on exp.): 1.5 M NaCl, 0.5 M Tris, 1 mM EDTA, pH 7.2

NP-40 Lysis Buffer A: 10 mM Tris, 10 mM NaCl, and 3 mM $MgCl_2$ pH 7.4. The solution was autoclaved and cooled before 0.5% (v/v) NP-40 was added.

2X Reaction Buffer (nuclear run-on exp.): 10 mM Tris, 5 mM $MgCl_2$, 0.3 M KCl, pH 8.0,

autoclaved.

2X Reaction Buffer with Nucleotides: 10 µl each of 100 mM ATP, CTP, GTP, and 5 µl 1M DTT in 1 ml 2X reaction buffer.

TES Solution: 10 mM N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES), 10 mM EDTA, 0.2% (w/v) SDS, pH 7.4 , autoclaved.

REAGENT MANUFACTURERS

Acetic acid, glacial	Fisher
Actinomycin D	Sigma
Activated Charcoal, Norit A	Sigma
AG501-X8 20-50 Mesh Resin	Bio-Rad
Agarose	Gibco-BRL
Alanine	Sigma
Aldosterone	Sigma
Ammonium acetate	Fisher
Bacto-Tryptone	DIFCO
Bacto Yeast Extract	DIFCO
Beta-mercaptoethanol	Sigma
Bromophenol blue	Fisher
Buffer H	Promega
DECAprime II kit	Ambion
DEPC (diethyl pyrocarbonate)	Sigma
Dexamethasone	Sigma
Dextran (clinical grade)	Sigma
Dextrose	Fisher
Dextrose, anhydrous	MCB *
Disodium ethylenediaminetetraacetate	Fisher
DNA, sodium salt from salmon testes	Sigma
DNA Ladder, 1 kb	BRL*
DNA Mass Ladder	Gibco-BRL
EDTA (ethylenediaminetetraacetate)	J.T. Baker or Fisher

Estradiol-17β	Sigma
Ethidium bromide (electrophoresis grade)	Fisher
Ficoll-400	Pharmacia
Formaldehyde, 37%	Fisher
Formamide	Fisher
Glutamine	Sigma
Glycerol	Fisher
Glycine (aminoacetic acid)	Sigma
Guanidine thiocynate	Fisher
Hydroxycortisone	Sigma
8-Hydroxyquinoline	Fisher
Insulin	Sigma
Isopropanol	Fisher
Magnesium chloride	Fisher
Magnesium sulfate	Fisher
MOPS (3-[N-Morpholino]propanesulfonic acid)	Sigma
N-lauroylsarcosine, sodium salt	Sigma
Nonidet P-40	Sigma
Penicillin-Streptomycin	Gibco-BRL
Phenol	BRL Life Technologies
Phosphoric acid, concentrated	J.T. Baker
Potassium chloride	Fisher
Potassium phosphate dibasic, anhydrous	Fisher
Potassium phosphate monobasic	Fisher
Progesterone	Sigma

Pst1 restriction endonuclease	Promega
PVP-40 (polyvinylpyrrolidone)	Sigma
Random Primers DNA labeling system	Gibco-BRL
R5020 (17,21-dimethyl-19-nor-4,9-pregnadiene-3,20-dione)	Roussel-UCLAF
RNA Ladder, 0.24-9.5 kb, 1μg/μl	Gibco-BRL
RU486 (17β-hydroxy-11β-(4-dimethyl-aminophenyl-1)-17α-(prop-1-ynil)-estra-4,9-dien-3-one)	Roussel-UCLAF
SDS (sodium dodecyl sulfate)	Bio-Rad or J.T. Baker
Sephadex G-50	Sigma
Sodium acetate	Fisher
Sodium bicarbonate	Fisher
Sodium chloride, U.S.P. granular	Fisher
Sodium citrate	Fisher
Sodium hydroxide	Fisher
Sodium molybdate	Fisher
Sodium phosphate dibasic heptahydrate	Fisher
Sodium phosphate monobasic	Fisher
TES (N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid)	Fisher
Testosterone	Sigma
Tetracycline-HCl	Sigma
Trizma Base (Tris[hydroxymethyl]aminomethane)	Sigma
Xylene Cyanole FF	Sigma

* MCB - Matheson, Coleman, & Bell

* BRL - Bethesda Research Laboratories

ABBREVIATIONS & ACRONYMS

A	adenine
ACTH	adrenocorticotropic hormone
AR	androgen receptor
ATP	adenosine triphosphate
AUG	codon for methionine
BBC1	breast basic conserved-1 gene
bp	basepair
BRCA1	Breast cancer gene-1
BRCA2	Breast cancer gene-2
8-Br-cAMP	8-bromo-cyclic 3', 5'-adenosine monophosphate
BRL	Bethesda Research Laboratories
BUS	B-upstream segment of hPR-B
C	cytosine
cAMP	cyclic 3',5'-adenosine monophosphate
CAT	chloramphenicol acetyltransferase
CBG	corticosteroid-binding globulin
CBP	CREB-binding protein
cc	cubic centimeter
C/EBPα	CCAAT/enhancer-binding protein α-gene
CHX	cycloheximide
cM	centiMorgan
CS²FBS	double charcoal-stripped fetal bovine serum
DBD	DNA-binding domain

DEPC	diethyl pyrocarbonate
DHFR	dihydrofolate reductase gene
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
DPEP1	dipeptidase-1 gene
dsRNA	double-stranded ribonucleic acid
dTTPs	deoxythymidine triphosphates
EDTA	ethylenediamine tetraacetate
e.g.	exempli gratia (Latin): for example
EGF	epidermal growth factor
ER	estrogen receptor
ERE	estrogen response element
et al.	et alia (Latin): and others
EtOH	ethanol
FBS	fetal bovine serum
FGF	fibroblast growth factor
FGFR	fibroblast growth factor receptor
FMO5	flavin-containing monoxygenase 5
G	guanine
GR	glucocorticoid receptor
GRIP	glucocorticoid receptor interacting protein
H_2O	water
H8	cyclic nucleotide-dependent protein kinase inhibitor
HAT	histone acetyltransferase
HBE	human breast epithelia (cells)
HD1	histone deacetylase-1

hER	human estrogen receptor
^{3}H-leu	leucine radioactively labeled with tritium
H-L-H	helix-loop-helix
HMG-1	high mobility group-1 chromatin protein
hPR-x	human progesterone receptor-x where x = A, B, or C isoforms
hr	hour
HRE	hormone response element
hsp	heat shock protein
IGF-1	insulin-like growth factor-1
IL-2	interleukin-2
k	first-order rate constant
k_{-1}	dissociation rate constant
K_d	equilibrium constant for dissociation
kb	kilobase
kDa	kilodaltons
Leu	leucine
LDH	lactate dehydrogenase
LH	luteinizing hormone
LOH	loss of heterogosity
m	symbol for the slope of a line
M	molar
Max	myc Associated X
MCB	Matheson, Coleman, & Bell
MEN-1	multiple endocrine neoplasia type 1
min.	minute
ml	milliliter, 10^{-3} l

mM	millimolar, 10^{-3} M
MOPS	3-(N-Morpholino)propanesulfonic acid
MPA	medroxyprogesterone acetate
MR	mineralocorticoid receptor
mRNA	messenger ribonucleic acid
NCoA	nuclear co-activator
NLS	nuclear localization signal
nM	nanomolar, 10^{-9} M
NME-1	encodes metastasis inhibitor protein nm23
p	short arm of the chromosome, from the French petit
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
p/CIP	p300/CBP/co-integrator-associated protein
PDGF	platelet-derived growth factor
PDI	protein disulfide isomerase
PEG	polyethylene glycol
PHA	phytohemagglutinin
PKC	protein kinase C
PKI	protein kinase A inhibitor
PMSG	pregnant mare serum gonadotropin
PVP	polyvinylpyrrolidone
q	long arm of the chromosome
R5020	17,21-dimethyl-19-nor-4,9-pregnadiene-3,20-dione
RA	retinoic acid
RAR	retinoic acid receptor
RBF	receptor binding factor

rpm	revolutions per minute
rRNA	ribosomal ribonucleic acid
RU486	17β-hydroxy-11β-(4-dimethyl-aminophenyl-1)-17α-(prop-1-ynil)-estra-4,9-diene-3-one
SCLC	small cell lung cancer
SDS	sodium dodecyl sulfate
Ser	serine
Sf9	Spodoptera frugiperda insect cells
SF/IF/PRFM	serum-free, insulin-free, phenol red-free medium
SR	steroid receptor
SRC	steroid receptor coactivator
SRE	steroid response element
T	thymine
T3B ($T_3\beta$)	thyroid hormone (3, 5, 3'-triiodothyronine)
TAF-n	transcription activation (or transactivation) function-n where n=1,2, or 3
TBP	TATA-binding protein
TCA	trichloroacetic acid
TeBG	testosterone-binding globulin
TES	N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid
TGF	transforming growth factor
TIF-2	transcriptional intermediate factor-2
TNF-α	tumor necrosis factor-α
TPA	12-O-tetradecanoyl phorbol-13-acetate
TSG	tumor suppressor gene
μg	microgram, 10^{-6} gram

µl	microliter, 10^{-6} liter
UTP	uridine triphosphate
VDR	vitamin D receptor
vitERE	vitellogenin estrogen response element
YY1	Yin-Yang-1 transcription protein

ABSTRACT

Moore and co-workers have previously demonstrated that progestins stimulate the growth of T47D human breast cancer cells. We now investigate the possibility that progestins might transactivate the protooncogene c-myc as part of the mechanism for growth stimulation. Treatment of T47D cells with the synthetic progestin R5020 results in a rapid, dose-dependent increase in c-myc mRNA. This stimulation is evident as early as 5 minutes, increases up to 4-fold at 1 hour, and then returns toward basal levels. The optimal concentration of R5020 for induction of c-myc gene expression is 10 nM, which is within the physiologically relevant range of hormone exposure. Co-treatment of T47D cells with R5020 and the anti-progesterone RU486 results in abrogation of R5020-induced increases in c-myc mRNA levels. These results suggest that R5020 is operating through the progesterone receptor in transactivating the c-myc gene. Stimulation of c-myc expression is specific for progestins and estrogen. Treatment with 10 nM androgens, glucocorticoids, and mineralocorticoids has little or no affect c-myc mRNA levels. An increase in c-myc mRNA levels upon exposure to progestins occurs even in the presence of the protein synthesis inhibitor cycloheximide. Treatment with cycloheximide itself, however, also results in significant elevation of this mRNA. Experiments utilizing actinomycin D demonstrate that R5020 does not alter c-myc message half-life. The half-life of c-myc mRNA in T47D cells grown under our culture conditions is 23 minutes, which is consistent with values reported by other investigators. Together the data suggest that progestins stimulate c-myc expression as a primary response, that is, by direct enhancement of transcription of the c-myc gene via classical steroid hormone receptor pathways. This conclusion will have to be confirmed by nuclear run-on assays. Increased expression of the c-myc gene may be part of the mechanism by which progestins stimulate growth of T47D human breast cancer cells and impact on the progression of cancerous lesions of the breast.