Oestrogen and Antioestrogen induced Gene Expression

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Summary

The aim of this project was to study the effect of oestrogen and the antioestrogen tamoxifen on the expression of specific genes in the immature rat uterus and MCF-7 human breast cancer cells in order to try and further understand the mechanism of action of these two compounds. In order to do this a cDNA library was constructed using mRNA from 4 hour oestrogen-stimulated rat uteri. This was then screened with cDNA to mRNA from oestrogen stimulated and unstimulated rat uteri in order to isolate clones of oestrogen-regulated mRNAs.

Twelve such clones were isolated and the expression of three of these, F4, B11 and E10, together with clones to a number of oncogenes, v-myc, v-Ha-ras, v-Ki-ras and c-sis, the oestrogen-regulated pS2 clone from MCF-7 cells, and an actin clone, p749, were studied in the immature rat uterus and MCF-7 cells in response to oestrogen and tamoxifen.

In the immature rat uterus oestrogen caused a biphasic stimulation of expression of total mRNA with peaks at 4 and 16-20 hours after administration and, although the extent of induction was variable, it had a similar effect on all the clones studied, except actin which showed a continual increase from 0-20 hours after administration.

Taking into account the slower uptake of tamoxifen, when compared to oestrogen, by uterine cells, and its metabolism to a more active derivative, this compound was found to be agonistic with respect to the induction of all the genes studied except the oestrogen-regulated clone from the rat uterine cDNA library, F4, which showed only one early peak of induction in response to tamoxifen.

In MCF-7 human breast cancer cells oestrogen caused a 2-2.5 fold increase in mRNA levels over control cells between 3 and 36 hours after administration, whereas tamoxifen treatment resulted in no increase in mRNA levels over the first 8 hours, and a decrease to half control level by 36 hours. Oestrogen also stimulated the expression of all the clones studied in this system, except the uterine library clone E10 which did not cross-react with MCF-7 cell RNA, though not to the same extent as in the immature rat uterus. However, the effect of tamoxifen on the amount of total mRNA available from MCF-7 cells meant that only two of the clones, pS2 and p-myc-2, could be studied in full. Of these the level of myc specific RNA was not increased at all but decreased steadily over the 36 hours studied, and although the level of pS2 specific RNA was increased within 1 hour of tamoxifen administration, this increase was only a fraction of that caused by oestrogen at 24 hours, and had fallen to control levels by 36 hours.

List of Abbreviations

DNA	- deoxyribonucleic acid
cDNA	- complementary DNA
RNA	- ribonucleic acid
mRNA	- messenger RNA
rRNA	- ribosomal RNA
tRNA	- transfer RNA
hnRNA	- heterogeneous nuclear RNA
polyA+RNA	- polyadenylated RNA
dATP	- doexyribosyladenine - 5' - triphosphate
dCTP	- deoxyribosylcytosine - 5' - triphosphate
dGTP	- deoxyribosylguanine - 5' - triphosphate
dTTP	- deoxyribosylthymidine - 5' - triphosphate
DNase	- deoxyribonuclease
RNase	- ribonuclease
Oestradiol-17β	- 1, 3, 5, (10) - estratriene - 3, 17β-diol
Tamoxifen	- [1-(4-β-dimethyl-aminoethoxyphenyl) 1, 2-di phenylbut-1-ene]
X-gal	- 5-bromo-4-chloro-3-indolyl - βD galactose
EDTA	Ethylene diamine tetra acetic acid

INTRODUCTION

1. Introduction

The aim of this project was to study the effect of oestrogen on the expression of individual genes and, in order to try and elucidate the mechanism of action of the non-steroidal antioestrogens, to study the effect of the antioestrogen tamoxifen on these oestrogen-regulated genes. This introduction therefore describes the nature of both oestrogens and non-steroidal antioestrogens, what is known of their mechanism of action, and points out the necessity of studying their effects on the expression of individual genes. The specific genes studied are also described.

2. Steroid Hormones

Steroid hormones in higher organisms regulate a variety of functions within the cells of their target tissues which result in the development of these tissues and maintenance of their role in the organism itself.

Early studies on the mechanism of action of steroid hormones showed that the activity of certain cellular enzymes was increased by administration of steroid hormones (Knox and Auerbach, 1955; Lin and Auerbach, 1957), and that this increase was due to increased de novo synthesis of these enzymes (Kenney; 1962).

The mechanism by which steroid hormones stimulate de novo enzyme synthesis however remained unclear until new techniques involving the use of radioactively labelled hormones were developed. Administration of these radioactively labelled hormones to rats showed that they accumulate in the nuclei of the cells of their specific target tissues, (Jensen and Jacobson, 1960; Edelman et al., 1963) suggesting that this may be their site of action. Also, injection of the insect steroid hormone, ecdysone,

which causes moulting from the caterpillar to the pupal stage, into the insect Chironomous Tentans, resulted in the formation of 'puffs', that is regions of unfolded DNA, on the giant salivary gland chromosomes of this insect. Simultaneous administration of actinomycin D, an inhibitor of RNA synthesis, prevented formation of these 'puffs' implying that this steroid hormone also acted in the nucleus and that its effect was to alter gene expression (Clever and Karlson, 1960).

Since then, steroid hormones have been shown to increase the synthesis of specific mRNAs and proteins in a number of systems for example, chick oviduct, rat ventral prostate, rat uterus, and MCF-7 human breast cancer cells implying that they act by altering gene expression (Palmiter et al., 1976; Higgins et al., 1979; Westley and Rochefort, 1979).

3. Oestrogens

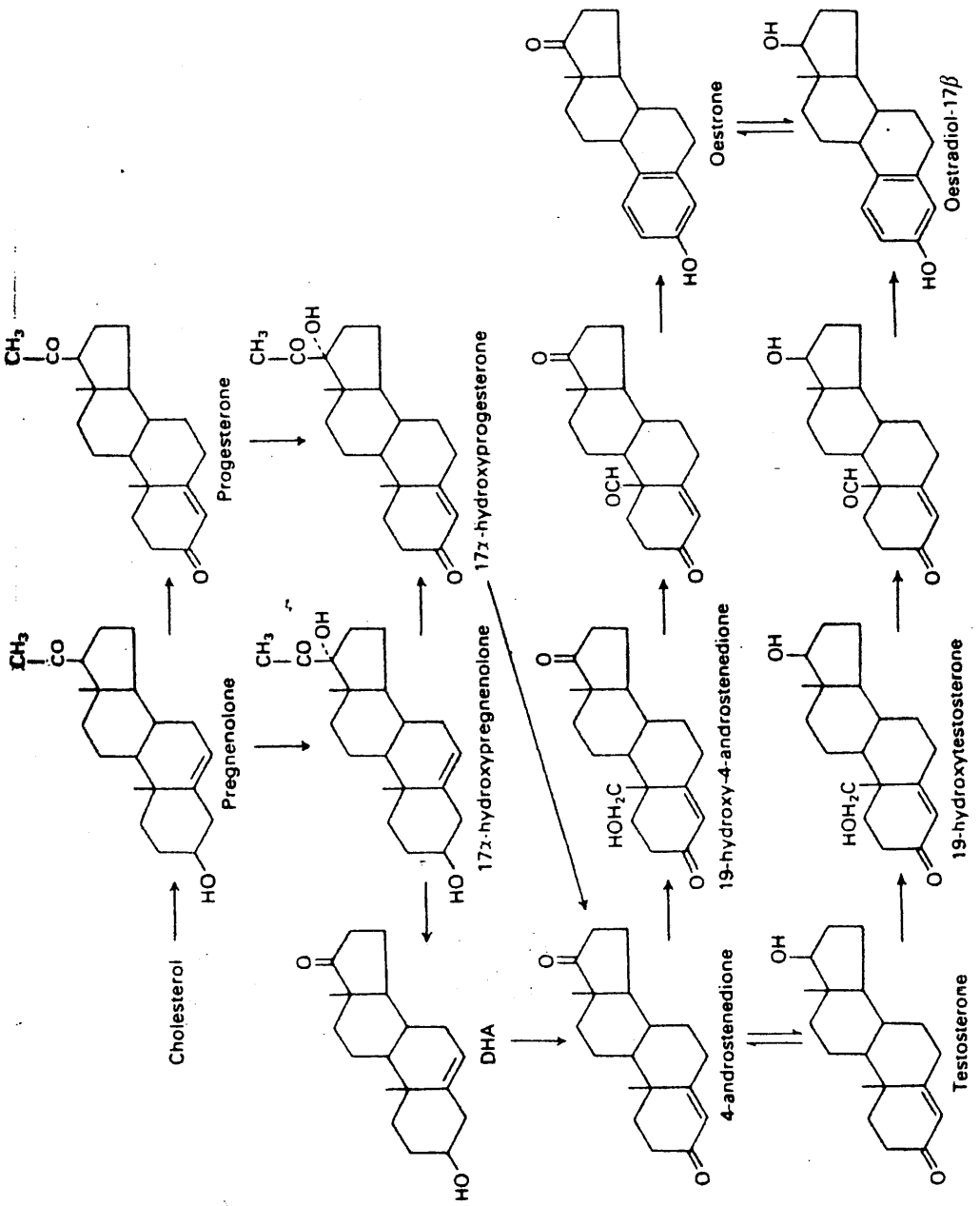
Oestrogens are a class of steroid hormone and, like all steroid hormones, they are derived from cholesterol, a 27-carbon compound, synthesised mainly in the liver from isoprene and transported to the ovaries, the site of oestrogen synthesis, by low density lipoproteins. Cleavage of a side chain from cholesterol results in the formation of a 21-carbon molecule, pregnenolone. This then undergoes a series of dehydration reactions which lead to the formation of testosterone, a 19-carbon molecule, which, after further dehydration and aromatisation, gives the 18-carbon oestrogens (Fig.1).

There are three major oestrogens found in mammals; oestrone, oestradiol and oestriol, of which oestradiol is the most active. The

Figure 1

Biosynthesis of the principal oestrogens.

From Fotherby, 1984.


Cholesterol
Pregnenolone
Progesterone
17α-hydroxypregnenolone
17α-hydroxyprogesterone
DHA
4-androstenedione
19-hydroxy-4-androstenedione
Oestrone
Testosterone
19-hydroxytestosterone
Oestradiol-17β

target tissues for oestrogen include the uterus, vagina and mammary glands, and the effect of oestrogen on a number of these will be discussed later.

Oestradiol is metabolised to oestrone and then to oestrone sulphate in the target tissue cells which, as it has no affinity for the oestrogen receptor, is rapidly excreted from the cells and, passes via the blood, to the liver (Fotherby, 1984).

3.1 Oestrogen Uptake

Oestradiol, a hydrophobic molecule, is thought to enter cells by passive rather than active diffusion, as studies with radioactively labelled hormone showed that:

(1) it was initially taken up by all tissues of the immature rat (Jensen and DeSombre, 1973)

(2) uptake was linearly related to the concentration of oestradiol in the surrounding medium (Clark et al., 1974)

(3) the rate of uptake by target and non-target tissues was not affected by dinitrophenol, an uncoupler of oxidative phosphorylation (Clark et al., 1974)

The involvement of a specific protein has however also been proposed by a number of groups (Vriel et al., 1976; Siiteri et al., 1982).

3.2 The Oestrogen receptor

Jensen and DeSombre (1960), demonstrated that, although tritiated - oestradiol was taken up by all the tissues of the immature rat, it was selectively retained in only a few; the uterus and the vagina. They therefore proposed that these target tissues contained a receptor molecule which bound oestradiol. The fact that treatment with proteases but not nucleases caused release of oestradiol, and that oestradiol binding was competitively inhibited by steroidal and non-steroidal oestrogens but not by non-oestrogenic steroids, led to the conclusion that the receptor was an oestrogen-specific binding protein (Noteboom and Gorski, 1965; Toft and Gorski, 1966).

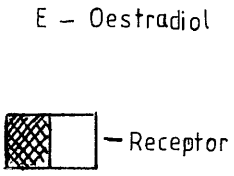
The demonstration of the existence of an oestrogen receptor which, using tritiated oestradiol and cell fractionation, was found in both nuclei and cytoplasm (Noteboom and Gorski, 1965), and that the level in the nuclei increased as that in the cytoplasm decreased (Jensen et al., 1968), led to the proposal of a two-step model for steroid hormone action, based on the action of oestradiol in the immature rat uterus (Jensen et al., 1968) {Figure 2}.

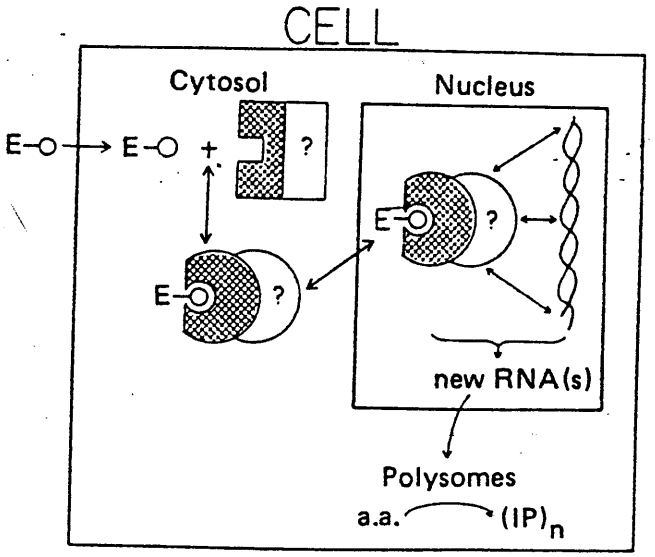
This proposed that oestrogen entered the target tissue cell and bound to a cytoplasmic receptor. The resulting complex was then translocated to the nucleus where it acted to alter the transcription of specific genes and therefore the levels of specific proteins.

Although a lot of work has been carried out which confirms and adds detail to this model, recent data has put in doubt the existence of a cytoplasmic receptor, suggesting that receptor exists only in the nucleus. This new data and its effect on the above model will be discussed later.

Figure 2

The two-step model of steroid hormone action




CELL
Cytosol
Nucleus
E
E
+
?
E
?
E
?
new RNA(s)
Polysomes
a.a.
(IP)n

The cytoplasmic oestrogen receptor was first characterised in the rat uterus (Toft and Gorski, 1966) where it is estimated to be present at a concentration of 15,000-20,000 molecules per cell (Clark and Gorski, 1970; Barbanol and Assenmacher, 1980), and binds oestradiol with high affinity (Kd-InM) (Rochefort and Capony, 1975; Weichman and Notides, 1977). A range of sedimentation values have been observed for the cytoplasmic oestrogen receptor depending on the ionic conditions employed in fractionation; at high salt concentrations it consists of a single polypeptide with a sedimentation value of 4S and one oestrogen binding site.

Two classes of oestrogen binding sites have been identified in rat uterine cytosol. Clark et al. (1970) have classified these as Type I sites, which are equivalent to the classical oestrogen receptor in that they bind oestrogen with a high affinity and translocate to the nucleus, and Type II sites, which are present at four times the concentration of Type I sites, bind oestrogen with a lower affinity (Kd - 30nM) and do not translocate to the nucleus but are present in both nuclei and cytoplasm. (Clark and Markaveritch, 1981). It has been suggested that the large number of Type II sites may serve to concentrate oestrogen in its target tissue cells (Clark et al, 1978).

Oestradiol also binds, with low affinity (Kd - 10-30nM), to a nuclear receptor not derived from Type I or Type II cytoplasmic sites, termed nuclear Type II sites. Oestradiol stimulated accumulation of these sites has been correlated with the late oestrogen responses of the uterus, cell growth and division, and is also dependent on long term nuclear occupancy by nuclear Type I sites (Markaveritch and Clark, 1979; Clark and Markaveritch, 1981).

3.3 Activation and Transformation of the Oestrogen Receptor

On binding oestrogen the oestrogen receptor exhibits two changes:-

(1) it shows an increased affinity for isolated nuclei, chromatin and DNA-cellulose (Yamamoto, 1974; Thampan and Clark, 1981; Gschwendt and Kittstein, 1980) in a process termed activation which follows first-order reaction kinetics (Bailley et al., 1980).

(2) its sedimentation value increases from 4S to 5S in a process called transformation (Notides et al., 1981) which shows second-order reaction kinetics (Bailley et al., 1980).

These processes can occur independently and both are temperature dependent, occuring maximally at 37°C but only slightly at 4°C (Jensen and DeSombre, 1972).

3.3.1 Mechanism of Activation

A number of theories on the mechanism of activation of the oestrogen receptor have been proposed: Dissociation of an inhibitory molecule has been suggested (MacDonald and Leavitt, 1981) and is supported by binding studies (Chamness and McGuire, 1972) and the fact that dialysis leads to activation of the oestrogen receptor (Sato et al., 1980). Association of an activating factor has also been proposed by a number of groups (Thampan and Clark, 1981; Yamamoto, 1974). These propose that the function of this factor is to increase the affinity of the oestrogen receptor complex for DNA leading to its migration to the nucleus. The 5S activated receptor also displays an increased affinity for oestradiol, therefore, the association or dissociation of an additional molecule would be driven by oestradiol binding (Weichman and Notides, 1977).

Notides and Neilson (1974) have shown that both the 4S and 5S forms of the oestrogen receptor complex are capable of undergoing marked conformational changes. However conformational change as a mechanism of activation was proposed by Bailley et al., (1980) and Muller et al. (1983) who suggested that the increased DNA affinity and transformation were due to exposure of new domains on the receptor, on binding of oestrogen.

A further proposal suggests that activation is the result of phosphorylation of the receptor by a cytoplasmic ATP-dependent kinase. This, and a related phosphatase found in nuclei, have been characterised in the mouse uterus by Aurrichio et al., (1981) who further propose that a phosphorylation/dephosphorylation cycle controls the amount of oestrogen receptor available for hormone binding.

The above proposed mechanisms of activation are not however mutually exclusive.

3.3.2 Transformation

The 4S to 5S transformation was suggested by Muller et al., (1983) and Notides and Neilson (1974), to be a result of dimerisation of the 4S cytoplasmic receptor. However, the fact that transformation was shown to follow second-order reaction kinetics, implied that it involved association of a second subunit (Bailley et al., 1980). In fact a protein which caused activation and transformation of the oestrogen receptor was isolated by Thampan and Clark (1981) from rat uterine cytosol and by Murayama et al., (1980) from bovine and porcine uterus.

3.4 Translocation

Translocation of the oestrogen receptor from the cytoplasm to the nucleus was shown by Jensen et al, (1968) to be a temperature dependent process. Again association of a second subunit to the receptor was proposed, in this case a DNA binding protein which increased the affinity of the oestrogen-receptor complex for chromatin and resulted in net movement of the complex to the nucleus (Yamamoto, 1974). However, Horowitz (1972), proposed that activation of the receptor decreases its interaction with one or more molecules in the cytoplasm and allows it to equilibrate with the larger water-space of the nucleus.

3.5 Cytoplasmic Oestrogen Receptor Replenishment

The number of oestrogen receptor complexes in the cytoplasm of uterine cells decreases to a minimum within one hour of administration of oestradiol to the immature rat due to receptor translocation (Clark et al., 1973). However, after 24 hours, although oestrogen receptor complexes were still present in the nucleus, the number of receptors in the cytoplasm was double the control-value implying that oestradiol induced the synthesis of its own receptor (Cidlowski and Muldoon, 1974; McGuire, 1977; Gardner et al., 1978). Simultaneous administration of actinomycin D inhibited this increase suggesting that the receptor was being synthesised de novo and that, if receptor recycling occurred, it was a process dependent on protein synthesis (Cidlowski and Muldoon, 1974; Dix and Jordan, 1980).

The theory of receptor recycling was proposed by Kassis and Gorski (1981) and was based on studies using the short-acting oestrogens, such as 16α-oestradiol, which only induces the early oestrogenic events (Section 5.1.3). Administration of this oestrogen to immature rats led to total replenishment of cytoplasmic receptor levels after only four hours. This correlated well with the disappearance of receptor from the nucleus and was not subject to inhibition by cycloheximide. Studies with the long-acting oestrogens, 17β-oestradiol and diethyl-stilbestrol, however did not show such good correlation of cytoplasmic receptor increase with nuclear receptor decrease, and the extent of replenishment of cytoplasmic receptor levels by recycling observed with 16α-oestradiol may be a property of short-acting oestrogens.

Use of the long acting oestrogens led to a decrease in the total number of receptors by a mechanism known as receptor 'processing' (Koseki et al., 1977), which is thought to involve the irreversible loss of the ligand binding site possibly as a result of a conformational change (McGuire, 1982). This 'processing' of the oestrogen receptor complex is inhibited by actinomycin D, but not by other inhibitors of protein synthesis.

3.6 Nuclear Binding of the Oestrogen - Receptor Complex

Transformation of the oestrogen-receptor complex is associated with an increase in its affinity for chromatin (Yamamoto, 1974; Thampan and Clark, 1981). In vitro studies of the binding of transformed oestrogen-receptor complex to DNA (Andre and Rochefort, 1975) and isolated nuclei (Chamness et al, 1974) have shown that this process is non-saturable, that

is, uptake by nuclei and binding to DNA was proportional to the concentration of oestrogen-receptor complex in the cytosol or surrounding medium. This relationship implied that binding was due to a large number of low affinity oestrogen-receptor complex binding sites on DNA.

The presence of specific high affinity binding sites could not however be ruled out as, if they occurred in relatively small numbers, they would be masked by the large number of low affinity sites (Yamamoto and Alberts, 1974). The existence of two types of binding sites on chromatin was demonstrated using the sensitivity of oestrogen-receptor complex binding to low salt concentrations (Clark and Peck, 1976; Ruh et al., 1979). They found that 80-90% of chromatin bound oestrogen-receptor complexes were found to be salt-extractable and therefore bound to lower affinity sites than the remaining 10-20% which were resistant to salt extraction.

The minimum number of nuclear-bound oestrogen-receptor complexes necessary for a maximum uterine growth response was estimated by Clark and Peck (1976) to be 1000-3000/cell. This number correlated with the number of salt-resistant oestrogen-receptor complex binding sites in the nucleus and suggested that the interaction of the oestrogen-receptor complex with this small number of high-affinity sites regulated the genes responsible for uterine growth.

Studies using the weak oestrogen, oestriol, show that its inability to stimulate true uterine growth was due to a failure to maintain its receptor complex in the nucleus, whereas those of the long-acting oestrogen, oestradiol, were maintained in the nucleus for up to six hours

(Anderson et al., 1975; Clark et al., 1977). However, if the level of oestriol-receptor complexes in the nucleus was maintained by chronic administration a full uterotrophic response was elicited implying that long term nuclear retention of the oestrogen-receptor complex, probably on the high affinity chromatin binding sites, is necessary for a full oestrogenic response. The function of the large number of low affinity binding-sites for the oestrogen-receptor complex in the nucleus is not known.

3.7 The Interaction of the Oestrogen-Receptor Complex with Chromatin

The components of chromatin involved in its association with the oestrogen-receptor complex and the nature of this interaction have been studied by a number of groups. The fact that nuclear retention of the oestrogen-receptor complex is dependent on ionic strength (Clark and Peck, 1976; Ruh et al., 1979) implied that at least one component of the interaction was electrostatic in nature. Also, a change in conformation of the receptor itself on binding to chromatin has been proposed, based on the different dissociation rates from free and chromatin-bound receptor complexes. Clark and Peck (1976) and MacDonald and Leavitt (1981) have suggested that the receptor functions as a helix destabilising protein, thereby opening up DNA for transcription.

Many components of chromatin have been proposed to function as acceptor sites for the oestrogen-receptor complex. The increased affinity of the activated receptor complex for DNA-cellulose (Yamamoto, 1974) suggested a role for DNA in its nuclear binding. Studies using the 5S oestrogen receptor showed that it could bind with low affinity to a

variety of DNAs from different species and indeed, studies with the steroid receptor for progesterone have shown that it too binds non-specifically to DNA and that this is a function of one of its two domains (O'Malley et al., 1972). This non-specific binding to DNA cannot account for the specific control of gene expression demonstrated by these steroid hormones and therefore it was once again proposed that, in addition, there existed a small number of specific sequences responsible for binding the receptor complex. Studies have been directed mainly at finding a consencus sequence responsible for binding steroid receptor complexes in genes activated by a particular hormone. These have now been proposed for glucocorticoids (Hynes et al., 1983) and progesterone (Mulvihill et al., 1982; Schrader et al., 1977; Dean et al., 1983) but not so far for the oestrogens. The binding of steroid receptor complexes to these sequences however is only 10-40 times that to heterologous DNAs and therefore the specificity of receptor complex binding may be due to, or involve, other components of chromatin, for example, the proteins.

In particular, removal of the acidic non-histone chromosomal proteins from chromatin results in a loss of specific binding of the receptor (O'Malley et al., 1972) leading to a 'specifier function' being proposed for these proteins.

Other components of chromatin, for example, the nuclear matrix (Puca et al., 1974), nuclear envelope (Jackson and Chalkley, 1974), and histones (Kallos et al., 1981) have also been proposed to be involved, to various extents, in the specific binding of steroid hormone-receptor complexes to chromatin.

Binding of the oestrogen-receptor complex in the nuclei of its target-tissue cells leads to changes in RNA, DNA and protein synthesis, which varies with the target tissue and is discussed in later sections dealing with oestrogen-responsive tissues.

3.8 Nuclear Versus Cytoplasmic Oestrogen Receptor

Recent data has brought into doubt both the subcellular location and physical state of the unfilled oestrogen receptor.

Firstly, King and Greene (1984), using a batch of monoclonal antibodies raised against purified oestrogen receptor, showed that, in all the target tissues examined, and under all the conditions used, staining was confined to the nucleus.

This data was supported by the GH3 rat pituitary tumour cell fractionation experiments of Welshons et al., (1984). In these cytoplasts (cytoplasm plus intact plasma membrane) and nucleoplasts (nuclei plus small amount of cytoplasm plus intact plasma membrane) were isolated and unoccupied receptor was found predominantly in the nucleoplasts, and very little in the cytoplasts. Conventionally homogenised GH3 cells however showed a high level of unoccupied receptor in the cytoplasm.

Secondly, binding of oestrogen to either intact cells or immobilised oestrogen receptor has been shown to be non-co-operative, whereas binding at concentrations of greater than 1nM of soluble receptor is cooperative (Sakai and Gorski, 1984; Muller et al., 1984; Notides et al.,

1981). As the concentration of steroid receptor per cell has been estimated at 10nM, this implied that the oestrogen-receptor complex may be immobilised in the target tissue cells, possibly by attachment to some component of chromatin or the nuclear matrix (Gorski et al., 1984).

These experiments led to the proposal of a new model for the oestrogen receptor (Gorski et al., 1984) which suggest that the cytoplasmic oestrogen receptor was an artefact and that all the oestrogen receptors are found in the nucleus where they are bound to some nuclear component. The immobilised oestrogen receptor can however be both activated and transformed (Gorski et al., 1984), therefore the only step in the original model of oestrogen action, (Fig. 2), affected is that of translocation. Replenishment of cytoplasmic receptor would, by the new model, be better thought of as replenishment of unoccupied receptor.

The methods used to obtain the above data on which this new model of the oestrogen receptor was based has however been extensively critizised by Szego and Pietras (1985), who suggest that they may create just as many artefacts as the older methods. Much more work is necessary in order to resolve this problem.

4. Antioestrogens

4.1 Introduction

Antagonism of oestrogen action can occur in a number of different ways, for example, by inhibition of FSH/LH secretion, inhibition of oestrogen synthesis, or by inhibition at any stage of oestrogen-induced gene activation. Antioestrogens are however generally defined as the compounds which bind to the oestrogen receptor and antagonise the action of oestrogen within its target tissue cells.

The main reasons for studying antioestrogens were that:

(1) By studying the molecular basis of oestrogen antagonism the mechanism of oestrogen action might become clearer.

(2) These compounds might be of use in the control of fertility and possibly in the treatment of hormone-dependent cancers. The non-steroidal antioestrogens, derivatives of triphenyl-ethylene or diphenylnapthalene, have proved to be of some use in the later case, and have therefore been most extensively studied. This is particularly true of the antioestrogen tamoxifen which is widely used in the treatment of metastatic mammary carcinomas.

The extent of antagonism by these compounds is dependent on their chemical structure and geometric conformation and shows a wide species variation, for example, tamoxifen has been found to be a complete antagonist in chick oviduct (Sutherland et al., 1977), a complete agonist in mouse uterus (Terenius, 1971), and a partial agonist/partial antagonist

in rat uterus (Harper and Walpole, 1967). The extent of antagonism can also vary between different target tissues and different cell populations within a tissue (Martin, 1981) and is dependent on the conditions used and the parameters measured.

Antioestrogens have also been shown to modulate almost every step of the response of a target tissue cell to oestrogen.

4.2 Structure of the Non-Steroidal Antioestrogens

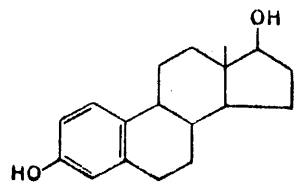
The structure of oestradiol and a number of antioestrogens are shown in Figure 3. The trans-isomers of these compounds are more potent oestrogen antagonists than the cis-isomers (Harper and Walpole, 1966; Robertson, 1982).

The main structural feature of the compounds responsible for their antioestrogenic activity is the ether side chain. Removal of this results in potent oestrogenic, but no antioestrogenic activity (Lednicer et al., 1966; Jordan et al., 1981). Also, modification of the triphenylethylene structure has been shown to affect antioestrogenicity (Sutherland and Murphy, 1980), in particular, hydroxylation of the antioestrogens at a position analogous to position 3 of oestradiol leads to an increased affinity for the oestrogen receptor and a greater antioestrogenic potency in vitro (Jordan et al., 1977; Binart et al., 1979; Borgna and Rochefort, 1980).

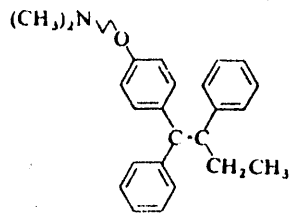
Figure 3

The structure of oestrogen and non-steroidal antioestrogens

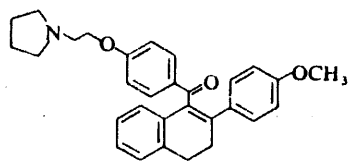
The structure of oestradiol and of the antioestrogens tamoxifen, trioxifene, 6-hydroxy-2-(*p*-hydroxyphenyl)-benzo(*b*)thien-3-yl *p*-<2-(1-pyrrolidinyl) ethoxyphenyl ketone (LY 117018) and 6-hydroxy-2-(*p*-hydroxyphenyl)-benzo(*b*)thien-3-yl *p*-<2-(1-piperidinyl) ethoxyphenyl ketone (LY 139481)



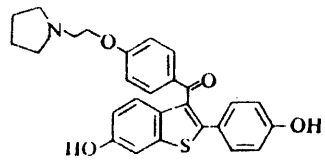
Oestradiol-17β



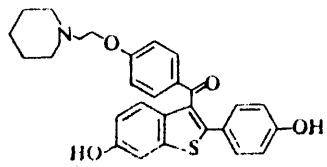
Tamoxifen



Trioxifene



LY 117018



LY 139481

4.3 Metabolism of the Antioestrogens

The metabolism of the antioestrogens has been found, like the extent of its antagonism of oestrogen action, to vary between different species and different tissues. In the chick and the rat the antioestrogens are metabolised, mainly in the liver, to the more potent mono-hydroxylated forms. This conversion can also take place in the chick oviduct but not in the rat uterus (Borgna, 1981; Hayes et al., 1981). In humans the major metabolite of the antioestrogen tamoxifen is desmethyl tamoxifen, which has equal potency as its parent compound (Sutherland and Whybourne, 1981).

The fact that the metabolites of the antioestrogens bind to the oestrogen receptor with greater than, or equal affinity to, and have more or equal antioestrogenic potency than their parent compounds, led to the suggestion that the parent compounds were actually pro-drugs which required metabolic activition. Studies with the MCF-7 human breast cancer cell line which does not metabolise antioestrogens have shown that the parent compounds have inherent antioestrogenic activity and that, although metabolism may enhance this, it is not a prerequisite for the expression of this activity (Coezy et al., 1981; Jordan et al., 1977; Horowitz et al., 1978).

4.4 Antioestrogen Action in Oestrogen Target Tissue Cells

4.4.1 Interaction with the oestrogen receptor

Antioestrogens competitively inhibit the binding of tritiated oestradiol to the oestrogen receptor (Korenman, 1970; Skidmore et al.,

1972; Katzenellenbogen and Katzenellenbogen, 1973; Rochefort and Capony, 1972) and studies under equilibrium and non-equilibrium conditions suggest that oestrogen and antioestrogens bind to the same site on the receptor (Nicholson et al, 1978; Rochefort and Capony, 1972). However the rates of dissociation of antioestrogens from the receptor are faster than that of oestradiol (Nicholson et al, 1978; Korenman, 1970) and the antioestrogen receptor complex is also more susceptible to tryptic digestion and thermal inactivation than the oestrogen-receptor complex. It has been suggested that this is due to an incomplete conformational change induced in the receptor on binding antioestrogen. However, although the stability of the antioestrogen-receptor complex was closely related to the affinity of the compound for the receptor, it was not related to their antagonistic potency (Pavlik and Katzenellenbogen, 1980). This implied that a low binding affinity and high dissociation rate of antioestrogen from the receptor were not sufficient criteria to discriminate agonists and antagonists (Borgna and Rochefort, 1980; Rochefort et al, 1977).

These findings do not negate the theory that antioestrogens cause a partial conformation change in the receptor compared to that induced by oestradiol. They do however indicate that this conformational change, measured as affinity, thermal stability or resistance to proteolysis cannot be related to antioestrogenic potency (Pavlik and Katzenellenbogen, 1980).

The antioestrogen-receptor complex and oestrogen-receptor complex are in fact very similar, differing only in a quantitative rather than a qualitative manner in, for example, their affinity for DNA-cellulose and their sedimentation properties (Katzenellenbogen et al, 1981). This

suggests that the antagonism of oestrogen action by antioestrogens occurs at a stage beyond their binding to the oestrogen receptor.

4.4.2 Antioestrogen Specific Cytoplasmic Binding Sites

Competition studies using tritiated oestrogens and antioestrogens in the chick oviduct showed that, although the antioestrogens could completely inhibit binding of oestradiol to the cytoplasmic receptor, oestradiol could only partially inhibit antioestrogen binding, implying that an additional antioestrogen-specific binding site existed in the chick oviduct cytosol (Sutherland and Foo, 1979).

Antioestrogen-specific cytosol binding has also been demonstrated in the rat uterus where it occurs with a high affinity (Kd 2-10nM) and varies with the oestrus cycle. This suggested a possible physiological role for these sites. But neither this, nor the existence of a natural antioestrogen have been demonstrated (Faye et al., 1980). Nevertheless, high affinity (Kd 60nM) antioestrogen - specific binding sites have also been demonstrated in the cytosol of human mammary carcinoma cells, and in the microsomal fractions of both oestrogen receptor positive and oestrogen receptor negative human breast cancer cell lines (Sutherland and Murphy, 1980; Katzenellenbogen et al., 1983).

Studies using the oestrogen receptor positive MCF-7 cell line demonstrated that the structural feature important in binding of triphenylethylene antioestrogens to their specific site, was the amino ether side chain (Murphy and Sutherland, 1981), which is the group found to be essential for antioestrogenic activity (Lednicer et al., 1966).

Although the role of these sites is unknown, it has been suggested that they may act in a 'passive' manner by altering the apparent distribution volume of the antioestrogens and therefore their pharmacokinetics (Katzenellenbogen et al., 1983).

4.4.3 Activation, Transformation and Translocation of the Oestrogen Receptor by Antioestrogens

Binding of oestradiol to the receptor causes its activation, a process which results in the oestrogen-receptor complex demonstrating an increased affinity for both DNA and oestradiol (Jensen and DeSombre, 1972; Weichman and Notides, 1977). Binding of the antioestrogen to the receptor results in a complex which shows a smaller increased affinity for DNA than the oestrogen-receptor complex (Ruh et al., 1979) and no increased affinity for the antioestrogen (Borgna and Rochefort, 1980). This led to the suggestion that the receptor is only partially activated on binding the antioestrogen.

Antioestrogens have been shown however to cause the 4S-5S transformation of the receptor (Borgna et al., 1979) and to result in translocation of this complex to the nucleus in both immature rat uterus and chick oviduct (Sutherland et al., 1977; Dix and Jordan, 1980; Davies et al., 1979; Koseki et al., 1977). It occurs however at a slower rate than observed with oestradiol; translocation occuring within one hour of oestradiol administration (Makku et al., 1981; Clark et al., 1973).

4.4.4 Interaction of the Antioestrogen-Receptor Complex with Nuclear Acceptor Sites

Long term (4-6 hours) nuclear retention of oestrogen-receptor complexes was shown to be necessary for a full oestrogenic response (Clark et al, 1973; 1977). Antioestrogen-receptor complexes however demonstrate very prolonged nuclear retentions of up to 24 hours (Sutherland et al, 1977; Katzenellenbogen et al, 1979; Clark et al, 1973), but only elicit a partial response in some tissues and no response in others. Part of this apparent long retention might be due to the prolonged serum half-lives of the antioestrogens (Dix and Jordan, 1980). Furthermore, it was shown that, in the immature rat uterus, whereas the oestrogen-receptor complex bound to both salt-extractable and salt-resistant chromatin sites, the antioestrogen-receptor complexes only bound to the low affinity salt-extractable sites (Ruh et al, 1979; Ruh and Baudendistal, 1976). This may in turn be due to the partial activation of the receptor induced on binding antioestrogen (Section 4.4.3). Nevertheless this did demonstrate the importance of the low affinity chromatin binding sites in that binding to them alone was sufficient to induce the early oestrogen responses in the immature rat uterus (Katzenellenbogen et al, 1979).

4.4.5. Effect of Antioestrogens on Receptor Replenishment and Processing

It has been proposed that antioestrogens antagonise oestrogen action by inhibiting the replenishment of cytoplasmic receptors (Clark et al., 1973; Ferguson and Katzenellenbogen, 1977). By virtue of their long half-lives, they could immediately bind newly synthesised or recycled receptor and translocate it to the nucleus, thus prolonging nuclear

retention. This cannot however be the primary antagonistic event as, in chick oviduct, some triphenylethylene derivatives have been shown to be antagonistic after only one injection, when the number of cytoplasmic receptors was not a limiting factor on the ability to respond to oestrogen (Sutherland, 1981). Also, tamoxifen has been shown to antagonise oestrogen action in the chick oviduct when administered up to 6 hours after oestrogen, when the cytoplasmic receptor level is low (Catelli et al., 1980; Mester and Geynet, 1977). Cytoplasmic receptor replenishment has been demonstrated after antioestrogen, but occurs much later than with oestrogen (Katzenellenbogen and Ferguson, 1975; Capony and Rochefort, 1975; Koseki, 1977).

It is probable therefore that cytoplasmic receptor replenishment is not inhibited by antioestrogen, but is impaired, possibly as a result of an altered interaction of the antioestrogen-receptor complex with chromatin. Thus, it is a secondary event in oestrogen antagonism, rather than the primary one.

4.4.6. Conclusion

From the above discussion it would seem that the favoured primary action of the antioestrogens is the partial activation of the receptor by the antioestrogen, compared to oestrogen, which results in altered binding of the receptor complex to chromatin. This in turn would lead to induction of an altered mRNA population and therefore protein population by the antioestrogens when compared with those induced by oestradiol.

Comparison of the mRNA and protein populations induced by antioestrogen and oestrogen should therefore further elucidate the molecular mechanism of antioestrogen action.

5. The Effect of Oestrogens and Antioestrogens on Different Oestrogen Target Tissues

5.1 The Immature Rat Uterus

5.1.1 Introduction

The immature rat uterus has been used extensively as a model in which to study the mechanism of action of oestrogen, as well as that of oestrogen antagonism. The triphenylethylene antioestrogens have been studied in the immature rat uterus as they have been shown to be partial agonists/partial antagonists in this system (Harper and Walpole, 1967)and determination of the stage at which they cease to be agonistic would lead to a better understanding of the mechanism of action of the antioestrogens, but possibly also of the mechanism of action of oestrogen.

5.1.2 The Uterus

The uterus consists of three tissues; the endometrium, which consists of luminal and glandular epithelial cells and stromal cells, the myometrium, which consists of smooth muscle cells, and the perimetrium or outer sheath.

The endometrium, which undergoes cyclic changes in growth in response to the hormones secreted by the ovaries, consists of two layers; the lamina functionalis and the lamina basilis. The endometrium also contains a number of tubular glands which are often branched and almost reach the myometrium. The epithelial cells lining these glands appear very similar to the luminal epithelial cells.

The myometrium surrounds the endometrium and consists of densely packed muscle cells and a few fibroblasts.

Oestrogen has been shown not to be equally mitogenic in the different cell populations of the uterus, and therefore the response of this tissue to oestrogen is a result of the response of the individual cell populations. Oestrogen causes stromal and luminal epithelial cell proliferation, hypertrophy of glandular epithelium, but virtually no myometrial cell division (Martin, 1981).

5.13 The Effect of Oestrogens and Antioestrogens on the Immature Rat Uterus

The response of the immature rat uterus to oestrogen is complex. In this tissue, oestradiol evokes a series of biochemical and physiological responses which result in cell growth and division and initiate preparation of the uterus for implantation by a fertilised egg. These changes occur over a number of weeks in the developing rat, but can be induced to take place in a few days by administration of exogenous oestrogen to immature rats (Katzenellenbogen et al., 1979). The sequence of these events is shown in Fig.4.

Figure 4

Effect of oestrogen on the immature rat uterus

Temporal sequence of events initiated in the immature rat uterus following an injection of oestradiol-17β at zero time. The time (hours) is on a logarithmic scale (0.01 hours = 0.6 minutes)

From Katzenellenbogen and Gorski, 1975.

Hours after oestrogen.	
30	Cell division.
20	Histone synthesis, DNA synthesis. Net protein synthesis.
10	Net RNA synthesis.
5	Water imbibition. Protein synthesis. Nucleolar RNA polymerase activated. Phospholipid synthesis, glucose metabolism.
1	IP synthesis.
0.5	Nucleoplasmic RNA polymerase II and hnRNA synthesis. IP RNA synthesis (and other RNA's).
0.1	
0.05	
0.01	Oestrogen binding in nucleus. Oestrogen binding in cytoplasm.
0	Administration of oestrogen.

The non-steroidal antioestrogens are agonistic in that they bind to the oestrogen receptor and cause its translocation to the nucleus where they stimulate the early uterine events, that is, increased uterine weight, protein synthesis, RNA polymerase activity and, in some cases, DNA synthesis. They do not, however, bring about cell division or oestrogen-like tissue growth (Davies et al., 1979; Katzenellenbogen and Ferguson, 1975; Markaveritch et al., 1978; Cowan and Leake, 1979; Jordan and Dix, 1979).

The degree of antagonism of oestrogen action also varies with the antioestrogen under study, and with the cell types. Martin (1981) studied the effect of oestradiol and the antioestrogen tamoxifen, which induces uterine growth to a certain extent, on the different cell populations within the uterus, using mitotic index as a marker. He found that tamoxifen caused more DNA synthesis in stromal cells than oestradiol, but that, although it induced hypertrophy of both luminal and glandular epithelial cells, it resulted in almost no epithelial cell division and in fact led to death of glandular epithelial cells.

The contribution of the different uterine cell populations to the events induced by oestrogen and antioestrogen are very difficult to estimate as separation of the component cells, especially in the numbers necessary, is very difficult. The effects of oestrogens are therefore usually studied in the intact uterus.

5.1.4 The Effect of Oestrogens and Antioestrogens on Protein and RNA Synthesis in the Immature Rat Uterus

The uterus, unlike most oestrogen target tissues, has been shown to respond to the hormone by altering the synthesis of a large number of proteins to a small extent (Beaumont and Knowler, 1983).

(a) 'Induced-Protein'

The first detectable response to oestrogen was the increased synthesis of the mRNA for a specific protein, termed the oestrogen-induced protein (IP), within 15 minutes after oestrogen administration.

Increased synthesis of IP mRNA was not blocked by inhibitors of protein synthesis which suggested that it was a result of primary transcription and not dependent on translation of existing or earlier induced, mRNAs (DeAngelo and Gorski, 1970). Accumulation of the protein was detectable by 30 minutes (Notides and Gorski, 1966) and it was identified as creatine kinase (Walker and Kaye, 1980), an enzyme which regulates the intracellular concentration of ATP (Jacobus and Lehninger, 1973).

This protein has been used as a marker for oestrogen activity as its synthesis is rapidly increased to several fold the constitutive level, and it has been found in most oestrogen target tissues, as well as in breast tumours (Kaye, 1983).

In vitro studies suggested that antioestrogens did not, or only slightly, increase the levels of IP (Katzenellenbogen and Katzenellenbogen, 1973).

However, *in vivo*, they promoted a full IP response in the rat uterus and this was maintained as long as the receptor complexes were in the nucleus (Mairesse and Galand, 1979).

(b) DNA Polymerase and Thymidine Kinase Activity

The activity of DNA polymerase-1 has been shown to increase in a dose-dependent, oestrogen-specific, manner in the cells of the immature rat uterus. Maximal activity occurred 24-30 hours after oestrogen administration, and correlated with the times of increased DNA synthesis in these cells (Harris and Gorski, 1978).

Antioestrogens, even when given more than once, only stimulate one cycle of DNA synthesis in the immature rat uterus suggesting that they, either directly or indirectly, affect DNA polymerase activity (Holinka *et al.*, 1980). In MCF-7 cells, antioestrogens cause a decrease in the activity of DNA polymerase (Edwards *et al.*, 1980), but the mechanism is not known.

The enzyme thymidine kinase, which is involved in providing precursors for DNA synthesis, is also affected by oestrogen. In this case oestrogen has been found to increase thymidine kinase activity, by stimulating the *de novo* synthesis of the enzyme, to a maximum 24 hours after administration, that is, just before the oestrogen-induced increase in DNA polymerase activity.

Administration of one injection of the antioestrogen tamoxifen also increased thymidine kinase activity, however, further injections led to a complete inhibition of the activity of this enzyme (Baurtourault *et al.*, 1984).

(c) RNA Polymerases

Both oestrogens and antioestrogens have been shown to affect the activities of the DNA-dependent RNA polymerases (I and II).

Oestrogen stimulated an increase in RNA polymerase II activity, the enzyme responsible for hnRNA synthesis, which peaked 1 hour after administration and returned to control levels within 2 hours. A second increase was observed between 2-12 hours after oestrogen administration (Glasser et al., 1972; Markaveritch et al., 1978). Antioestrogens were found to stimulate RNA polymerase II activity in a similar manner (Kurl and Borthwick, 1980; Hardin et al., 1976).

The activity of RNA polymerase I, the enzyme responsible for the synthesis of RNA, was however differentially affected by oestrogens and antioestrogens; oestrogen administration led to an increase in RNA polymerase I activity within 1 hour which was still detectable after 12-24 hours (Teng and Hamilton, 1968), whereas the antioestrogens, clomiphene and tamoxifen, increased the activity of RNA polymerase I, but did not sustain the increase (Kurl and Borthwick, 1980).

The rapid increase in RNA polymerase II activity in response to oestrogen was shown to be the result of increased transcription and not of direct hormonal activation of the enzyme and, using inhibitors of RNA and protein synthesis, to be necessary for the subsequent increase in RNA polymerase I activity (Borthwick and Smellie, 1975). This was supported by the demonstration of a lag between hnRNA and rRNA synthesis in the oestrogen-treated immature rat uterus (Merryweather and Knowler, 1980).

(d) Enzymes Involved in Metabolism

Oestrogens stimulate the activity of a number of enzymes involved in meeting the increased metabolic requirements of the stimulated uterus (Table 1). Among the most studied of these are glucose-6-phosphate dehydrogenase (G6PDH) and ornithine decarboxylase (ODC).

Ornithine Decarboxylase

This enzyme is the initial and rate-limiting enzyme in the synthesis of polyamines, and its activity has been found to relate to tissue growth in a number of systems (Kaye, 1983). In the uterus oestrogen elicits a biphasic increase in ODC activity, that is, it stimulates two peaks of activity, one at 6 hours and another at 16 hours after oestrogen administration. This increase is via a stimulation of mRNA synthesis, that is, de novo enzyme synthesis (Kaye et al., 1971).

The non-steroidal antioestrogens also stimulate an increase in ODC activity, but over a different time-scale, that is, it starts increasing at 6 hours and reaches a peak, of the same magnitude as that due to oestrogen, after 24 hours. However, on multiple injections of antioestrogen, an inhibition of ODC activity is observed (Roke et al., 1984).

Glucose-6-Phosphate Dehydrogenase

Oestrogen also increases the activity of G6PDH, the first enzyme in the pentose phosphate pathway which catabolises glucose and produces NADPH and ribose. The increase in activity, which reached a peak 36 hours after

Table 1

Examples of uterine enzyme activities stimulated by a single dose of oestradiol-17β

From A.M. Kaye, 1983

Examples of Uterine Enzyme Activities Stimulated by a Single Dose of Estradiol-17β[1]

Enzyme	Test system	Dosage	Earliest response (hr)	Earlier(est) time tested (hr)	Peak response (hr)
t-boc-Ala-Ala-Pro-Ala AMC hydrolase	Weanling mice	0.15 μg/mouse	0.5	0.5	6
Ornithine decarboxylase	20-day-old rats	0.5 μg/rat	2	1	4
	ovex or hypox[a]	5 μg/rat	4	4	—
SAM decarboxylase	20-day-old rats	0.5 μg/rat	3	1	5
Mg^{2+}-dependent ATPase	Ovex	Not given	3–6	1–2	—
Aldolase	Ovex	10 μg/100 g rat	4	2	16
Hexokinase	Ovex	10 μg/100 g rat	4	2	16
Poly(adenosine diphosphoribose synthetase)	Immature mice	2 μg/mouse	6	3	18
Pyruvate kinase	Ovex	15 μg/100 g rat	8	4	16
Aspartate transcarbamylase	20–22-day-old rats	10 μg/rat	12	12	—
Leucine aminopeptidase	Ovex	1 μg/rat	12	6	24
Isocitrate dehydrogenase	Ovex	1 μg/rat	12	6	48
Glucose-6-PO_4dehydrogenase	Ovex	1 μg/rat	12	6	24
DNA polymerase α	20-day-old rats	5 μg/rat	16	12	20–36
	21-day-old rats	1 μg/rat	18	12	24
Lysyl oxidase	Mouse (cervix)	1 μg/mouse	12–18	12	18
Succinic dehydrogenase	Ovex	30 μg/rat	24	24	48

[a] Ovex, ovariectomized adult rats; hypox, hypophysectomized adult rats.

oestrogen administration, has been shown to be a result of both de novo enzyme synthesis (Barker et al., 1981) and an increased half-life of the enzyme (Smith and Barker, 1974; 1977).

The effect of antioestrogens on G6PDH activity is similar to that demonstrated by the other metabolic and DNA synthetic enzymes, that is, it showed an initial increase followed by a sustained decrease (Lerner, 1981).

The activities of these enzymes therefore correlates well with the oestrogen/antioestrogen induced growth response in the uterus.

(e) Plasminogen Activator

Plasminogen activators are proteases which, by their action, specifically activate plasminogen, a serine protease with a broad substrate specificity. Their levels are increased by oestrogens in human breast cancers, human breast cancer derived cell lines, and in the epithelial, stromal and myometrial cells of the uterus. The uterine plasminogen activator levels increase in response to oestrogen in line with the general protein synthesis induced by the hormone, that is, approximately 12 hours after hormone administration. This increase is suppressed by the administration of the non-steroidal antioestrogens (Kneifel et al., 1982).

(f) Oestrogen and Progesterone Receptors

Administration of oestrogen has been shown to increase both the level of its own receptor and that of the progesterone receptor in the immature rat uterus (Jordan and Prestwich, 1978; Cidlowski and Muldoon,

1974; Milgrom et al., 1973; Davies et al., 1979). However, although progesterone acts to increase the level of oestrogen receptors in the endometrium, it antagonises the oestrogen induced increase in the myometrium and in this respect is a partial antioestrogen in the uterus. The non-steroidal antioestrogens also, after translocating the existing cytoplasmic oestrogen receptors to the nucleus, cause a replenishment of these receptors, either by recycling or by de novo synthesis, but much more slowly than oestrogen. This may be due to the long half-lives and long-term retention of the receptors complexes of these compounds in the nucleus.

The induction of progesterone receptor also occurs in response to antioestrogens. Monohydroxytamoxifen, an antioestrogen with a short half-life but high affinity for the oestrogen receptor induces progesterone receptor synthesis with similar kinetics as oestradiol, but tamoxifen itself causes a much slower increase.

5.1.4.2 RNA Synthesis

An increase in the synthesis of all classes of RNA was the first detectable synthetic response of the immature rat uterus to oestrogen and a prerequisite for subsequent protein synthesis, ribosome formation and DNA synthesis, that is for synthesis of the macro-molecules essential for cell growth and division (Aziz and Knowler, 1978; Nicollete and Babler, 1974).

(a) Overall Effects on RNA Synthesis

The first effect of oestrogen on RNA synthesis was found to be a two-fold stimulation of hnRNA synthesis which was detectable within 30 minutes of oestrogen administration and which increased to a maximum of ten-fold control levels by 2 hours (Knowler and Smellie, 1973; Aziz and Knowler, 1977). This was followed 2-4 hours after administration by an aggregation of pre-existing ribosomes on the newly made mRNA and this in turn is followed by an increase in rRNA and tRNA synthesis (Knowler and Smellie, 1971; 1973; Merryweather and Knowler, 1980). This increase in rRNA and tRNA synthesis was shown to be dependent on: (a) the initial increase in hnRNA synthesis and (b) previous protein synthesis, thus implying that processing of the hnRNA to mRNA and its subsequent translation to protein was essential for the formation of new ribosomes (Knowler and Smellie, 1971; Borthwick and Smellie, 1975).

A second increase in hnRNA synthesis occurred between 4-12 hours after oestrogen, coincidental with the increase in protein synthesis and the formation of new ribosomes (Borthwick and Smellie, 1975; Merryweather and Knowler, 1980).

Tamoxifen was found to cause similar changes in RNA levels but over a completely different time-scale, that is, it stimulated a broad peak of RNA synthesis between 12-32 hours after administration, involving changes in hnRNA, tRNA and rRNA synthesis, with a maximum seven-fold control levels at 24 hours (Waters and Knowler, 1981; Waters et al, 1983).

The different kinetics of the response to oestrogen and tamoxifen was thought to be caused by a combination of the different serum

half-lives of the two compounds; oestradiol having a half-life of approximately 30 minutes, compared to tamoxifen with a half-life of 4 days, the slower uptake of tamoxifen compared to oestradiol by uterine nuclei, and the fact that tamoxifen is slowly metabolised in the liver of the rat to a more active monohydroxylated derivative (Adam et al., 1980; Borgna, 1981, Hayes et al., 1981).

Administration of this monohydroxylated derivative to immature rats resulted in an oestradiol-like increase in RNA synthesis after 2-4 hours (Waters et al., 1983).

Tamoxifen therefore did not demonstrate antagonistic effects on general RNA synthesis in the immature rat uterus in that it stimulated synthesis of all classes of RNA.

(b) The Effect on PolyA+ RNA

The effects of oestrogen and the antioestrogen tamoxifen on poly A+RNA synthesis in the immature rat uterus were analysed by Rot curve analysis. These studies showed that the 4 hour oestrogen stimulated rat uterus contained approximately 12,000 diverse polyA+RNA sequences, compared to about 56,000 in the mature rat uterus (measured at pro-oestrus when the level of circulating oestrogen is maximal). They also demonstrated that there were a number of sequences present after 4 hours of oestrogen treatment that were not present after only 2 hours, and that this difference was mainly in the sequences present in intermediate abundance (Aziz and Knowler, 1979). Thus the complexity of the mRNA population seemed to increase from the unstimulated immature rat uterus, to that exposed to oestrogen, to the mature rat uterus (Table 2).

Table 2

Rot curve analysis of uterine mRNA populations

From Waters *et al.*, (1983)

Aziz *et al.*, (1979 *a*, *b*)

	Abundant	Mid-Abundant	Rare
4hr E_2 homologous hybridisation	9	150	7800
4hr E_2 minus 2hr E_2	1-2	151	2533
12hr tamoxifen homologous hybridisation	15	790	8000
Mature rat (at proestrus)	18	2100	34000

The kinetics of the hybridisation of mRNA from 12 hour tamoxifen treated rat uteri with its own cDNA was found to be very similar to those of 4 hour oestrogen stimulated uterine mRNA with its own cDNA.

Heterologous hybridisations using 4 hour oestrogen induced mRNA and 12 hour tamoxifen induced mRNA, and their respective cDNAs, showed that the 12 hour tamoxifen stimulated mRNA population contained all the sequences present in the 4 hour oestrogen stimulated population, but with quantitative differences in the sequences of intermediate abundance. That is, some sequences were more abundant in response to oestrogen than to tamoxifen, and vice versa.

Furthermore, some sequences in the 12 hour tamoxifen mRNA population were not found in the 4 hour oestrogen-induced mRNA population (Waters et al, 1983).

Rot curve analysis therefore demonstrated that, although tamoxifen stimulated transcription of the same mRNAs as oestrogen in the immature rat uterus, it stimulated some to a different extent.

5.2 The Chick Oviduct

Administration of oestrogen to chickens leads to the differentiation of the cells of the oviduct, and results in the synthesis and secretion of the egg white proteins, ovalbumin, conalbumin, ovomucoid and lysozyme, from the tubular gland cells of the differentiated oviduct. Oestrogen induces the synthesis of these few specific proteins by a mechanism similar to the induction of the more general protein synthesis

observed in the immature rat uterus, that is, binding to a specific receptor, binding of the resulting complex to chromatin acceptor sites which results in increased transcription and protein synthesis.

The induction of ovalbumin and conalbumin synthesis by oestrogen is both rapid and significant, conalbumin mRNA synthesis increasing after only 30 minutes, and ovalbumin mRNA increasing after 3 hours. It has been suggested that the 'lag' in ovalbumin mRNA synthesis is related to the nuclear oestrogen receptor levels; that is, whereas conalbumin gene transcription is directly related to the level of oestrogen receptor in the nucleus, ovalbumin mRNA synthesis could require co-operative binding of the receptor (Palmiter et al., 1977; Thomas and Teller, 1981; Mulvihill and Palmiter, 1977).

The extent of the induction of the mRNAs for the egg white proteins was demonstrated by hybridisation studies. These showed that the mRNAs for ovalbumin, ovomucoid and lysozyme represented 0.01%, 0.0039% and 0.0046% respectively of the polyadenylated RNA in the hormone withdrawn oviduct, whereas in the mature oviduct, they represented 50%, 6.6% and 3.4% (Hynes et al., 1977; Tsai, 1978; Harris, 1975; Cox, 1977).

In addition to stimulating the synthesis of the egg white proteins, oestrogen also caused an increase in the total oestrogen receptor concentration, progesterone receptor concentration, ODC activity, DNA content and wet weight of the chick oviduct.

The non-steroidal antioestrogens were found to be pure antagonists of oestrogen action in the chick oviduct. Even though they bound to the

cytoplasmic oestrogen receptor causing its translocation to, and long-term retention in, the nucleus, they did not increase ovalbumin or conalbumin synthesis, oestrogen and progesterone receptor concentration, ornithine decarboxylase activity or DNA content (Catelli et al., 1980; Sutherland et al, 1977).

A comparison of the nuclear binding of the oestrogen-receptor and antioestrogen-receptor complexes however demonstrated that the antioestrogen-receptor complexes did not bind, or bound very weakly, to the same chromatin acceptor sites as the oestrogen-receptor complex (Lebeau et al, 1981). The existence of a specific binding site, distinct from the oestrogen receptor, for the non-steroidal antioestrogens has been demonstrated in the chick oviduct (Sutherland and Foo, 1979).

5.3 Avian Liver

Oestrogen administration does not result in proliferation or differentiation of the cells of the avian liver, but does induce the synthesis of a few specific proteins, for example, vitellogenin, very low density lipoproteins (VLDLs) and the oestrogen receptor (Bergink et al, 1974).

Vitellogenin, a large phospholipoglycoprotein, is a precursor for the egg yolk proteins phosvitin and lipovitlellin (Bergink et al, 1974). Administration of oestrogen leads to an increase in chromatin-bound oestrogen-receptor complexes and results in an increased transcription of the vitellogenin genes and an increased synthesis of the oestrogen

receptor. This in turn results in a further increase in vitellogenin mRNA synthesis, so that oestrogen induces a biphasic stimulation of the synthesis of this protein (Shapiro et al., 1980).

The VLDL apo-proteins, the plasma carriers of large amounts of triglycerides, are also synthesised in the liver in response to oestrogen, the smaller apo-VLDL-II has kinetics of induction very similar to that of vitellogenin whereas those of VLDL-B are much slower (Williams et al., 1979; Chan et al., 1978).

The non-steroidal antioestrogens are pure antagonists in the avian liver in that they do not induce the synthesis of vitellogenin or the VLDL's (Gschwendt et al., 1982; Chan et al., 1977), and in fact inhibit oestrogen-induced synthesis. However, unlike other oestrogen target tissues, the antioestrogen-receptor complexes accumulate in the nucleus very slowly and to a much smaller extent than the oestrogen-receptor complexes. Antioestrogens with a much lower affinity for the receptor than oestradiol can still significantly inhibit the oestrogen-induced increase in nuclear receptor concentration. This later effect may be a result of the antioestrogens being metabolised to higher affinity forms in the liver (Lazier et al., 1981).

This slower nuclear accumulation of the antioestrogen-receptor complexes implies a defect, either in their ability to enter, or to be retained by, the nucleus. Again, however it must be noted that specific antioestrogen binding sites have been found in liver and could possibly, at least in part, be responsible for the above effect.

5.4 Human Breast Cancers

5.4.1 Introduction

The dependence of certain breast cancers on hormones was first demonstrated by Beatson (1896) when he observed mammary tumour regression in a number of patients following oophorectomy. About one-third of breast cancer patients were then found to respond to endocrine manipulation.

After the discovery of specific steroid hormone receptors in hormone target tissues (Toft and Gorski, 1966), and the proposal of the two-step mechanism for steroid hormone action (Jensen and DeSombre, 1968), it seemed logical to suppose that the presence of functional receptors would be necessary to evoke a response to endocrine therapy.

Approximately 60-70% of postmenopausal breast tumours were found to contain oestrogen receptors and about half of these were shown to respond to endocrine therapy (Edwards et al., 1979). Initially endocrine therapy involved either ablative surgery or administration of high doses of steroids which resulted in a number of unpleasant side-effects. However, a group of non-steroidal compounds which antagonised the actions of oestrogens in a number of its target tissues were found to cause regression of hormone dependent tumours and to inhibit the growth of oestrogen receptor positive human breast cancer cells in culture (Horowitz and McGuire, 1978; Lippman et al., 1976). One of these, tamoxifen, a derivative of triphenylethylene, which exhibits very low toxicity (Manni et al., 1979), is now the agent of choice for treatment of hormone dependent metastatic mammary carcinoma.

5.4.2 The Effect of Oestrogen and Antioestrogens on Human Breast Cancers

1. General Effects

The effect of oestrogens on breast tissue is even less understood than that in the other extensively studied oestrogen responsive tissues. This is, at least in part, because a number of hormones are involved in the regulation of development and function of mammary tissue. Oestrogen may either act directly, or indirectly, with other hormones, or oestrogen-induced growth factors, on breast tissue and on tumours.

In order to study the mechanism of action of oestrogens, and antioestrogens in the development of breast tumours, and to try and identify possible clinical markers for breast cancer, a number of human breast cancer cell lines have been utilised. In these, the problems of metabolism, transport, and accessibility of both oestrogens and antioestrogens to the tumour cells are much reduced when compared to *in vivo* models. The MCF-7, T47D, and ZR-75.1 oestrogen receptor positive human breast cancer cell lines have been most studied (Soule *et al.*, 1973; Brooks *et al.*, 1973; Keydar *et al.*, 1979; Engel *et al.*, 1978).

In these cell lines, as in other target tissues, oestrogen acts via its specific receptor protein, that is, oestrogen enters the cell and is bound to the receptor, the resulting complex then binds to specific chromatin acceptor sites and stimulates cell growth and division (Lippman *et al.*, 1976; Allegra and Lippman, 1978; Lippman *et al.*, 1981). Oestrogen administration to the cells leads to an increase in the activity of a

number of proteins involved in their increased metabolic requirements. It also causes the synthesis of a number of proteins of unknown function (Adams et al., 1983).

Antioestrogens are partial agonists/partial antagonists of oestrogen in human breast cancer cell lines. They bind to the oestrogen receptor and cause its translocation to the nucleus, but they do not stimulate cell proliferation (Lippman et al., 1976). They are also both agonistic and antagonistic at the level of oestrogen-induced protein synthesis, depending on the conditions used, and protein studied.

2. The Effect of Oestrogens and Antioestrogens on the Synthesis of Specific Proteins in Human Breast Cancer Cells

(a) Progesterone Receptor

As in a number of other oestrogen target tissues, oestrogen induces the synthesis of the progesterone receptor in human breast cancer cells (Horowitz and McGuire, 1978). The antioestrogen tamoxifen also induces progesterone receptor synthesis in these cells, though at a slower rate than oestrogen, whereas nafoxidine does not induce any progesterone receptor synthesis. This observation parallels the effect of these three compounds on oestrogen receptor processing in this system: complete processing of oestrogen receptors occurs with oestrogen, is impaired with tamoxifen, and is absent altogether with nafoxidine (McGuire and Horowitz, 1984; Horowitz et al., 1978).

The above correlation has led to the presence of progesterone receptors in breast tumours being taken to indicate the existence of a functional oestrogen receptor pathway. Indeed patients with both oestrogen and progesterone receptor positive tumours have a higher response rate to endocrine therapy (Osborne et al., 1980). However the progesterone receptor is present in such small amounts that its clinical usefulness is limited.

(b) Metabolic and DNA Synthetic Enzymes

Again the activity of enzymes involved in the metabolic functions necessary for growth, including DNA synthesis, are stimulated by oestrogen, and inhibited by antioestrogens, in the human breast cancer cell lines. For example, the activity of DNA polymerase and thymidine kinase were shown to increase in a dose-specific manner in MCF-7 cells responding to oestrogen (Edwards et al., 1980; Bronzert et al., 1981), but were inhibited by a single treatment of antioestrogen.

The activity of ornithine decarboxylase and lactate dehydrogenase were also increased, whether due to a direct increase in enzyme activity or de novo enzyme synthesis, in oestrogen treated human breast cancer cells in culture (Lima and Shio, 1985). The activity of ornithine decarboxylase does not increase in response to antioestrogens.

(c) Plasminogen Activator

Plasminogen activator activity has been found to be high in oestrogen receptor positive human breast cancers (Sherman et al., 1980; Sutherland et al., 1980). In MCF-7 cells the level was stimulated by low concentrations of oestrogen (Butler et al., 1979), whereas in response to

the antioestrogens tamoxifen and monohydroxytamoxifen, it was decreased to a level below that found in control cells. The antioestrogens also largely inhibited the oestrogen induced increase in plasminogen activator activity (Katzenellenbogen et al., 1984).

(d) Oestrogen-induced Proteins of Unknown Function

A protein, with a molecular weight originally estimated to be 46Kd, secreted by MCF-7 cells in culture, was discovered by Westley and Rochefort (1979) using two-dimensional gel electrophoresis. Further analysis however showed the protein to be a 52Kd glycoprotein, and to be distinct from the 52Kd plasminogen activator, which was also secreted by these cells (Mossot et al., 1984). The protein is synthesised in response to oestrogen in oestrogen receptor positive, but not oestrogen receptor negative, breast cancer cell lines (Westley and Rochefort, 1980). It does not appear to be present in the uterus and may therefore be a better marker for oestrogen induced growth of breast tumours than more generally responsive proteins such as the progesterone receptor. The 52Kd protein was further related to the growth response in that in two tamoxifen resistant sublines of MCF-7 it was secreted in the presence of tamoxifen (Vignon et al., 1984; Westley et al., 1984). Whether, in responsive cells, the antioestrogens inhibit the synthesis, or the secretion, of this protein is unknown.

A monoclonal antibody, raised to the protein was found not to react with MCF-7 plasma membrane, implying that the protein was secreted, rather than shed from the membrane (Garcia et al., 1981). A different monoclonal antibody however, produced against the cell surface of MCF-7 cells, associated with the secreted 52Kd protein, and also with membrane associated 54Kd and 56Kd proteins which may therefore be precursors (Brabon et al., 1984).

A second protein of 160Kd was found to be secreted into the culture medium of MCF-7 cells in response to oestrogen (Westley and Rochefort, 1980) and, like the 52Kd protein, was not secreted in response to the antioestrogen tamoxifen. However, unlike the 52Kd protein the 160Kd protein was not found in response to tamoxifen, in the tamoxifen resistant cell lines (Rochefort et al., 1983; Westley et al., 1984).

The basis for the induction of the 52Kd, but not the 160Kd protein, in the tamoxifen resistant cell is unknown, but could possibly involve an altered oestrogen receptor, and/or an alteration in the gene for the 52Kd protein. It has been suggested that the 52Kd protein is a growth factor, and that this would account for the tamoxifen resistance of the cells (Vignon et al., 1983; Jakesz et al., 1984).

5.4.3 pS2, An Oestrogen-regulated mRNA in Human Breast Cancer

In order to study the regulation of specific genes in human breast cancer, Chambon and co-workers (Masiakowski et al., 1982) constructed a cDNA library to mRNA from MCF-7 cells grown in the presence of oestrogen. By differential screening, using homologous cDNA and cDNA to mRNA from cells grown in medium containing charcoal-stripped serum and the antioestrogen nafoxidine, they isolated clones of mRNAs whose abundance was regulated by oestrogen. One such clone, pS2, has been extensively studied. This clone specifically hybridises to an mRNA of about 600 bases, and the fact that the level of pS2 mRNA in MCF-7 cells was increased rapidly, within 15 minutes, in response to oestrogen, even in the presence of cycloheximide implied that transcription of the pS2 gene was a primary effect of oestrogen on these cells (Brown et al., 1984).

Recent work however, using growth arrested MCF-7 cells, showed no change in pS2 mRNA levels occurred in the 24 hours following reversal of growth arrest, whereas levels of thymidine kinase mRNA did increase. This suggests that a change in pS2 mRNA is not a primary event in the growth response to oestrogen (Aitken and Lippman, 1985).

pS2 RNA was not synthesised in response to tamoxifen in either MCF-7 cells or the tamoxifen resistant sublines of MCF-7 (Westley et al., 1984), and, in fact, its expression seems to be limited to MCF-7 cells and a number of other breast cancer cells. It is not expressed in normal breast tissue (Chambon et al., 1984).

5.4.4 The Effects of Oestrogens and Antioestrogens on Cell Growth and Cell Cycle Kinetics of Human Breast Cancer Cells in Culture

Direct stimulation of both MCF-7 and ZR-75.1 cell proliferation by oestrogen has been shown to occur in vitro (Lippman et al., 1976; Weichselbaum et al., 1978; Allegra and Lippman, 1978). High doses of oestrogen however inhibit cell growth, but this inhibition does not involve the oestrogen receptor, and is possibly due to the effect of the steroid on the cell membrane (Riley et al., 1978).

The effect of the non-steroidal antioestrogens on these two cell lines is also well documented. That is, administration of antioestrogen at concentrations of 4μM inhibits growth and DNA synthesis (Sutherland et al., 1983). The effect can be reversed by the addition of a 100-fold lower concentration of oestrogen, and the fact that the inhibitory effect of the antioestrogen is not seen when administered with oestrogen, suggests that

the antioestrogen acts by competing with oestrogen for the oestrogen receptors (Lippman et al., 1976). Antioestrogens at the same concentration have no effect on the growth of the oestrogen receptor negative cell line BT20 (Green et al., 1981).

At higher concentrations, about 6μM tamoxifen, the growth inhibitory effect on the cells cannot be reversed by oestrogen. Thus they exert a cytostatic effect which is followed by cell death. A concentration of 10μM tamoxifen is immediately cytotoxic (Sutherland et al., 1983; Patterson et al., 1982). These responses of mammary cell lines to high concentrations of tamoxifen appear not to be oestrogen receptor mediated. This is supported by the observation that concentrations of greater than 4μM tamoxifen also inhibit the growth of the oestrogen receptor negative breast cancer cell line BT20. Oestrogen has been shown to have an effect on the cell cycle kinetics of its target tissue cells by increasing the number of cycling cells. Cells are recruited from the quiescent Go state, into the cycle, and the length of the cycle is decreased (Weichselbaum et al., 1978; Lippman et al., 1976; Jozan et al., 1979).

The growth inhibitory effect of tamoxifen has been shown to result from an accumulation of cells in Go/G1 phases of the cycle (Sutherland et al., 1983), due to an arrest of cells in G1 (Green et al., 1981). The inhibition of growth of the oestrogen receptor negative BT20 cells was also due to an accumulation of cells in the Go/G1 phases, again implying that the oestrogen irreversible effects of tamoxifen are not receptor mediated. The cytotoxic effect of tamoxifen at high concentrations was not associated with a notable change in the cell cycle kinetics of the cells, and therefore appeared to be a non-specific event.

6. Oncogenes

6.1 Introductions

The ability of retroviruses to cause cancer was first demonstrated using Rous sarcoma virus (RSV). The ability of this virus to convert a normal cell to a cancer cell was found to reside in a single gene, the v-src oncogene (Stehelin et al., 1976). A number of other retroviruses were then found to contain genes responsible for tumorigenesis in a variety of animals and to transform cells in culture (Coffin et al., 1981).

The discovery of cellular homologues of these viral oncogenes (v-oncs) in all vertebrate species examined, (Bishop 1983; Weiss et al., 1982) and in Drosophila and yeast (Shilo and Weinberg, 1981; Gallwitz et al., 1983; Ze'ev et al., 1984) led to the suggestion that the v-oncs had arisen by transduction of cellular genes. These were obviously not tumorigenic in normal cells, and were termed proto-oncogenes (Bishop, 1983). The extent of conservation of oncogene sequences among different species implied a fundamental role for the proteins encoded by them in normal cells (Stehelin et al., 1976; Bishop, 1981).

Transfection studies, using pieces of DNA isolated from human tumours and studying their ability to transform mouse NIH 3T3 cells in culture to anchorage independent growth, also demonstrated the presence of active oncogenes in human tumours, many of which were found to be homologous to those found in retroviruses, and all of which had normal cellular counterparts (Bishop, 1983; Weiss et al., 1982).

Approximately 30 oncogenes have so far been identified. Comparison of the normal cellular gene with those isolated from tumours and viruses demonstrated a number of differences which could be responsible for 'activating' these genes to, either directly or indirectly, cause the alteration in proliferation and differentiation observed in both tumours and cells in culture. These include over-expression, gene amplification, gene translocation, insertional and point mutations (Schwab et al., 1983; Kozbur and Croce, 1984; Klien, 1983; Hayward et al., 1981), and possibly loss of cell cycle dependent regulation of expression (Campisi et al., 1984; Kelly et al., 1983; Robertson, 1984).

The types of oncogene, their mode of action, and possible functions, will be described below.

6.2 Classes of Oncogenes

Tumour cells in culture differ from normal cells in a number of ways, for example, they show anchorage independent growth, decreased dependence on growth factors, and an ability to be passaged without limit, that is they are said to be 'immortalised'. In vivo tumour cells have the ability to metastisize. The development of a spontaneously occurring tumour is viewed as a multistep process and it would appear that activation of individual oncogenes can occur at any stage, that is, it is not a stage-specific process (Mougneau et al., 1984; Keath et al., 1984; Little et al., 1983). It was therefore proposed that the activation of a single oncogene is insufficient to cause all the changes that result in a normal cell becoming tumorigenic, and this led to the idea of co-operating, or 'complementation' groups of oncogenes.

It was shown that in normal rat embryo fibroblast cells, transfection with an activated ras gene only resulted in the cells acquiring anchorage independence, however if the cells were transfected with both the ras and myc oncogenes, they became fully transformed. That is, different groups of oncogenes are responsible for conferring different phenotypic changes, but only when acting together can they result in full cellular transformation (Land et al., 1983; Riley, 1983). The multistep process of tumorigenesis was therefore attributed to a cascade activation of a number of oncogenes.

Recent work, however, has demonstrated the ability of a single oncogene to transform cells to a completely cancerous phenotype (Spandidos and Wilkie, 1984). These workers have proposed that the environment of a cell affects its ability to be transformed, and that the effects of different classes of oncogene are complementary and not necessarily additive. That is, that the cytoplasmic oncogenes found to be potent inducers of anchorage independent growth, are also weak inducers of 'immortalisation', whereas the nuclear oncogenes which confer 'immortalisation' also weakly induce anchorage - independence (Weinberg, 1985; Land et al., 1983; Mougneau et al., 1984). The large T antigen of SV40, which can transform cells on its own has been shown to be present in both the nucleus and cytoplasm of cells (Soule and Butel, 1979; Landford et al., 1985; Fischer-Fantuzzi and Vesco, 1985). Furthermore, certain nuclear oncogenes have been found to act in a complementary fashion (Jenvwien et al., 1985), and to transform previously immortalised cells (Keath et al., 1984). These facts demonstrate that the classification of oncogenes into complementation groups, or into nuclear and cytoplasmic groups, is not a strict one.

Oncogenes can also be classified on the basis of the function of the proteins encoded by them. For example, a number of oncogene proteins have been found to share a common domain responsible for their tyrosine kinase activity (Brugge and Erikson, 1977; Hunter and Cooper, 1985; Decker, 1985; Gilmore, 1985) (Table 3). The similarity of the sequences encoding these domains also suggested a common evolutionary origin for these genes (Hunter and Cooper, 1985).

Tyrosine protein kinases in normal cells have been found to be either membrane associated proteins, or transmembrane receptors for growth factors, and the oncogene proteins with this activity also fit these categories (Bishop, 1985). More striking however is the fact that most protein kinases in normal cells phosphorylate serine or threonine residues, and the phosphorylation of tyrosine is uncommon, and perhaps confined to specific classes of regulatory pathways (Sefton et al., 1980). That the tyrosine kinase activity of oncogene - encoded proteins was a function of their transforming ability and not simply a coincidence, was shown by the fact that the amount of phospho-tyrosine was much higher in transformed, compared to normal cells, and that mutations leading to an inactivation of this activity resulted in a loss of transforming ability (Stoker et al., 1984; Synder et al., 1985). A small number of proteins have been shown to be phosphorylated on tyrosine in transformed, as opposed to normal cells, however only the effect of this on the adhesion plaque protein, viculin, could be associated with, but not wholly responsible for, the phenotypic changes associated with transformation (Hunter, 1984; Hunter and Cooper, 1985).

Table 3

Classification of oncogenes

From Hunter, T. (1982).

NAME OF ONCOGENE	RETROVIRUS	TUMOR	ONCOGENIC PROTEIN		
			CELLULAR LOCATION	FUNCTION	CLASS
src	CHICKEN SARCOMA	—	PLASMA MEMBRANE	TYROSINE SPECIFIC PROTEIN KINASE	CYTOPLASMIC TYROSINE PROTEIN KINASES
yes	CHICKEN SARCOMA	—	PLASMA MEMBRANE (?)		
fgr	CAT SARCOMA	—	(?)		
abl	MOUSE LEUKEMIA	HUMAN LEUKEMIA	PLASMA MEMBRANE		
fps	CHICKEN SARCOMA	—	CYTOPLASM (PLASMA MEMBRANE ?)		
fes	CAT SARCOMA	—	CYTOPLASM (CYTOSKELETON?)		
ros	CHICKEN SARCOMA	—	?		
erb-B	CHICKEN LEUKEMIA	—	PLASMA AND CYTOPLASMIC MEMBRANES	EGF RECEPTOR'S CYTOPLASMIC TYROSINE SPECIFIC PROTEIN KINASE DOMAIN	POTENTIAL PROTEIN KINASES
fms	CAT SARCOMA	—	PLASMA AND CYTOPLASMIC MEMBRANES	CYTOPLASMIC DOMAIN OF A GROWTH FACTOR RECEPTOR (?)	
mil	CHICKEN CARCINOMA	—	CYTOPLASM	(?)	
raf	MOUSE SARCOMA	—	CYTOPLASM	(?)	
mos	MOUSE SARCOMA	MOUSE LEUKEMIA	CYTOPLASM	(?)	
sis	MONKEY SARCOMA	—	SECRETED	PDGF LIKE GROWTH FACTOR	GROWTH FACTOR
Ha-ras	RAT SARCOMA	HUMAN CARCINOMA RAT CARCINOMA	PLASMA MEMBRANE	GTP-BINDING	CYTOPLASMIC GTP-BINDING
Ki-ras	RAT SARCOMA	HUMAN CARCINOMA LEUKEMIA AND SARCOMA	PLASMA MEMBRANE		
N-ras	—	HUMAN LEUKEMIA AND CARCINOMA	PLASMA MEMBRANE		
fos	MOUSE SARCOMA	—	NUCLEUS	(?)	NUCLEAR
myc	CHICKEN LEUKEMIA	HUMAN LYMPHOMA	NUCLEUS	DNA BINDING	
myb	CHICKEN LEUKEMIA	HUMAN LEUKEMIA	NUCLEUS	(?)	
B-lym	—	CHICKEN LYMPHOMA HUMAN LYMPHOMA	NUCLEUS (?)	(?)	
ski	CHICKEN SARCOMA	—	NUCLEUS (?)	(?)	
rel	TURKEY LEUKEMIA	—	(?)	(?)	UNCLASSIFIED
erb-A	CHICKEN LEUKEMIA	—	(?)	(?)	
ets	CHICKEN LEUKEMIA	—	(?)	(?)	

Several workers have recently shown that tyrosine kinases interact with the lipid kinases which act on the membrane lipid, phosphatidyl inositol (Sugimoto et al., 1984; Macara et al., 1984). They have proposed a scheme in which tyrosine kinases activate the lipid kinases. These in turn catalyse the metabolism of the inositol phosphates eventually to the compounds diacylglycerol and inositol triphosphate, which are responsible for the activation of protein kinase, and the mobilisation of intracellular calcium (Berridge and Irvine, 1984). That intracellular calcium is affected by transformation has been demonstrated but there is no direct evidence to implicate the rest of these processes(Bishop, 1985).

Another functional group of oncogenes are those of the ras family; Ha-ras, Ki-ras and N-ras, all of which demonstrate a guanine nucleotide binding, and GTPase activity (McGrath et al., 1984; Sweet et al., 1984). In yeast, these proteins have been shown to be functionally homologous to the G proteins which are assoiated with the cell membrane, and are thought to be involved in the transduction of signals from cell surface receptors by activating adenylate cyclase and increasing the levels of cAMP, that is again acting via a second messenger (Gilman, 1984; De Feo-Jones et al., 1985; Katoaka et al., 1985; Spandidos, 1985). It is possible that G proteins are also involved in the inositol lipid second messenger pathway (Berridge and Irvine, 1984).

Yet another set of oncogenes, v-mil/raf and v-mos demonstrate serine/threonine protein kinase activity (Hunter and Cooper, 1985). And very recently the oestrogen receptor protein (Green et al., 1986), and glucocorticoid receptor protein, (Weinberger et al., 1985) have been shown to exhibit some homology with the protein product of the oncogene v-erb.

Thus there is growing evidence that oncogene-encoded proteins may be involved in a number of the regulatory pathways by which cells respond to their environment, including the inositol lipid, and cAMP secondary messenger pathways, steroid hormone action and responses to growth factors.

DNA binding proteins also form a functional group of oncogene proteins and are, as their function suggests, found in the nucleus. These are thought to act as direct regulators of transcription (Bishop, 1985; Kingston et al., 1985; Gaynor et al., 1984). These, and oncogenes which affect growth factor function, are discussed in the following sections on the relationship of oncogene products to cell transformation.

6.3 Mechanism of Activation of Oncogenes: Relationship to Cell Transformation

The ability of oncogenes to cause cellular transformation has been associated with changes in the control or coding sequences of the genes. This in turn leads to a loss of regulation of expression of the gene, or of the function of the gene product. Such transforming oncogenes are said to be 'activated' and, by comparing the genes from retroviruses and tumours with those in normal cells, a number of mechanisms for this activation of proto-oncogenes have been discovered.

The normal c-myc oncogene product, a nuclear DNA binding protein, is expressed in response to growth factors (Kelly et al., 1983; Armelin et al., 1984) in differentiating, but not in terminally differentiated cells (Gonda and Metcalf, 1984; Grosso and Pitot, 1985), and is thought to

function in stimulating growth competence in cells (Campisi et al., 1984; Kelly et al., 1983). Altered expression of these genes therefore, not only confers growth factor autonomy on cells, but also causes increased expression of these growth related genes.

In both human Burkitts lymphoma and mouse plasmacytomas, the myc gene is constitutively expressed (Robertson, 1984). This deregulation of control of myc gene transcription is associated with a translocation of the gene to a constitutively expressed immunoglobulin locus (Klein, 1981; Keath et al., 198). This could result in overexpression of this gene in a number of ways:

(a) by positioning the myc gene such that it was under the control of the promoter/enchancer sequences responsible for transcription of the immunoglobulin locus (Klein, 1981; Taub et al., 1984) or

(b) by resulting in the loss of all, or part, of the highly conserved, non-coding first exon of the gene which has been proposed to contain the myc regulatory sequences (Taub et al., 1984; Rabbits et al., 1983).

Activation of the myc gene, in the above examples, is due to chromosome translocation, and results in constant production of a protein which is responsible for promoting entry of cells into the cell cycle, and decreasing their dependence on exogenous growth factors. In human neuroblastomas however the over expression of the N-myc gene is a result of amplification of the gene (Kohl et al., 1984).

In general, the activation of the nuclear DNA binding oncogenes has been shown to result in overexpression, or constitutive expression, of these genes which are normally only activated transiently in response to growth factors (Cochran et al., 1984; Kruijer et al., 1984).

Another mechanism of oncogene activation is by mutations which affect the structure of the protein products. This mechanism has been described for a number of proteins, mostly those associated with the cell membrane (Newbold, 1984; Parker et al, 1984). A well studied example are the ras gene proteins.

Ras genes are commonly activated by point mutations in either codon 12, which results in substitution of a glycine, or in codon 61, which results in substitution of a glutamine (Spandidos, 1985). They can also be activated by attaching strong promoters, but, in this oncogene, overexpression is not as efficient at transformation as mutation. Normal ras proteins bind GTP and hydrolyse it, whereas those mutated in codon 12 have a much reduced GTPase activity (McGrath et al, 1984; Sweet et al, 1984). As the postulated role for the normal ras proteins is in transducing signals from the cell surface, for example from growth factors, mutation could lead to growth factor independence, an attribute of transformed cells.

Oncogenes are therefore activated by chromosome translocation, amplification, or point mutation.

6.4. Oncogenes and Growth Factors

Several oncogene proteins have been shown to be related, structurally and functionally, to both growth factors and their receptors, and to be involved in the mitogenic response of cells to growth factors.

The transforming oncogene product of the sis gene has been found to be structurally related to platelet-derived growth factor (PDGF) (Waterfield et al., 1983). The first N-terminal 109 amino-acids of the B chain of PDGF are virtually identical to the c-sis protein, and the B chain is about 60% homologous with the A chain. Thus the three polypeptides probably share a common evolutionary origin for these genes (Waterfield et al., 1983; Doolittle et al., 1983; Johnsson et al., 1984). PDGF itself is thought to be involved in tissue repair. It is released from the α-granules of blood platelets and binds to specific saturable cell surface receptors stimulating a receptor associated tyrosine kinase activity (Heldin et al., 1981; Ek et al., 1982a; Ek et al., 1982b).

Cells transfected with an activated sis gene were found to contain high levels of this PDGF-like protein compared to normal cells (Doolittle et al., 1983; Huang et al., 1984). Secretion of this protein into the extracellular medium will result in its binding to cells displaying the PDGF receptors, and result in the stimulation of cell growth, independent of exogenous growth factors.

The product of the v-erb-B oncogene is a truncated form of the epidermal growth factor receptor (EGF-R) (Downward et al., 1984; Ullrich et al., 1984), and that of the c-fms oncogene is related to the mononuclear phagocyte growth factor; CSF-1 (Sherr et al., 1983). Both of these growth factors, and their related oncogene proteins, are tyrosine kinases (Hunter, 1984; Decker, 1985; Gilmore et al., 1985).

The ability of the oncogene proteins to cause transformation when the normal gene products do not may reside in the fact that both the

oncogene proteins are smaller than their normal cellular counterparts. That is, they lack all or part of the extracellular domain of the normal protein and cannot therefore bind their respective ligands (Heldin and Westermark, 1984). Ligand binding is necessary for activation of the tyrosine kinase activity of the normal receptor, and also leads to its own autophosphorylation (Bishop, 1985; Heldin and Westermark, 1984). The tyrosine kinase activity of the oncogene products has been found to be constitutive (Hayman and Beug, 1984; Roussel et al., Schmidt et al., 1985) and does not therefore require ligand binding for activation. This presumably accounts for their transforming ability, since their phosphorylation of intracellular proteins is no longer under ligand control.

The third link between oncogenes and growth factors was shown by the fact that admnistration of PDGF to cells in culture resulted in activation of a number of proto-oncogenes, c-myc and c-fos (Kruiger et al., 1984; Cochran et al., 198 ; Kelly et al., 1983). The proteins encoded in these genes are both DNA binding and activate transcription. This involvement in the mitogenic effect of PDGF supports previous data implying a role for these proteins in the regulation of expression of the genes controlling cell proliferation.

Recent work has also demonstrated that mammalian cells, which are transfected with an activated ras or myc oncogene, release transforming growth factor-α (TGF-α) into the extracellular medium. This is a growth factor which induces anchorage independence and binds to the epidermal growth factor receptor. Release of the α-TGFs from transfected cells may be responsible for an autocrine stimulation of cell growth (Spandidos, 1985).

7. Control of Gene Expression

Steroid hormones, growth factors, and oncogenes, are similar in that they all bring about a change in gene expression in their host or target tissue cells. Alteration of gene expression can be the result of:

(a) a change in structure of the chromatin, causing specific genes to adopt an active, transcribable, conformation.

(b) an induction of transcription of active genes, possibly involving promoter/repressor molecules.

7.1. Changes in Chromatin Structure

A number of changes in structure have been demonstrated between active and inactive chromatin using the sensitivity of chromatin to digestion by non-specific nucleases. These studies showed that, although both active and nonactive genes were packaged in nucleosomes (Weintraud and Groudine, 1976) actively transcribed or potentially transcribable genes were differentially sensitive to DNase I digestion (Flint and Weintraub, 1977; Stalder et al., 1978; Groudine et al., 1978; Garel et al., 1977) and were also often hypomethylated (Tsanev, 1983; Mandel and Chambon, 1979). The structure of the nucleosomes in active chromatin has also been found to be slightly different.

Nucleosomes, which consist of a stretch of DNA wound round a core of proteins called histones, appeared in inactive chromatin, to be attached or cross-linked by another histone, HI. This protein appeared to be associated with the 'tail' or 'linker' DNA between the nucleosomes and

is thought to be involved in the condensation of the chromatin into a solenoid-like structure (Carpenter et al., 1974; Renz, 1977). Active chromatin appears to contain a decreased amount of HI (Djundurov et al., 1977) which could therefore result in a relaxation of its structure. The histones in the nucleosomes of active chromatin are also modified, usually by acetylation (Dobson and Ingram, 1980) but also by methylation and ADP-ribosylation (Allfrey et al., 1977) and histone I appears to be modified by phosphorylation (Glover et al., 1981).

It has been suggested that these modifications to the histones may cause an alteration of chromatin conformation and exposure of promoter or repressor sequences.

Some non-histone chromosomal proteins have also been shown to be associated preferentially with active chromatin, in particular the highly conserved, low molecular weight, high mobility group (HMG) proteins (Levy-Wilson et al., 1979). The rapid association of two of these proteins, HMG 14 and 17, with active chromatin appears to confer DNase I sensitivity on these genes (Weisbrod et al., 1980; Weintraub, 1979).

HMG 14 and 17 have been shown to associate with the ends of the nucleosome core DNA, and histone free linker DNA (Sandeen et al., 1980; Bakeyev et al., 1977) and it has been proposed that they may replace HI on active chromatin (Weisbrod, 1982), a suggestion supported by the fact that these proteins are, like HI, modified by phosphorylation at different stages in the cell cycle (Bhorgee, 1981).

Other non-histone chromosomal proteins have been shown to bind to specific sequences in the DNA and either positively (Engelka et al., 1980; Payvar et al., 1981) or negatively (Alwine et al., 1977; Nevins and Winkler, 1980) control transcription. They may prove in time to be synonymous with trans-acting factors that modulate gene expression by their interaction with promoters, enhancers, and other DNA elements associated with gene expression. Steroid hormone-receptor complexes, and the nuclear proteins encoded by some oncogenes may prove to be example of trans-acting factors.

Mild digestion with DNase 1 showed the presence of hypersensitive DNase 1 sites in active chromatin which were located upstream of the 5'-end of the coding regions of the transcribed genes (Groudine et al., 1981; Wu and Gilbert, 1981). The finding of these sites in both tissues that expressed and did not express the specific gene (Stadler et al., 1980; Groudine et al., 1981) suggested that they may be necessary, but not sufficient for, gene activation (Tsanev, 1983). The absence of nucleosome protection from DNase 1 digestion of these sites led to them being proposed as RNA polymerase binding sites (Grosschedt and Birnsteil, 1982).

7.2 DNA Modification

The major modification found in DNA is in the methylation of the 5'-position of cytosine residues (Stein et al., 1982). A change in the methylation pattern of a number of genes, depending on whether they are active or not, has been demonstrated, for example, hypomethylation of the globin gene (Van der Ploeg and Flavell, 1980; Shen and Manniatis, 1980), ovomucoid and ovalbumin genes (Mandel and Chambon, 1979) and certain

oncogenes (Feinberg and Vogelstein, 1983), has been shown to be related to their ability to be transcribed. This hypomethylation also correlates with DNase 1 sensitivity of these genes, and with their ability to be transcribed *in vitro* and *in vivo* by RNA polymerase II (Weintraub *et al.*, 1981; Kuo *et al.*, 1979).

Inhibition of methylation by incorporation of the cytosine analogue, 5-azacytosine, into DNA led to the transcription of potentially active genes, but not of inactive genes (Groudine *et al.*, 1981; Weisbrod, 1982), again implying a role for demethylation in control of gene expression. However, examples have also been found of genes which are under-methylated but not transcribed, or which are activated without a detectable change in methylation (Weintraub *et al.*, 1982), suggesting that hypomethylation of a gene may be necessary for its expression, but is not sufficient for its activation (Tsanev, 1983).

Methylation of cytosine residues is further implicated in the control of gene expression in that it results in the methyl group occupying the major groove of the B-helical form of DNA (Stein *et al.*, 1982) which then might interfere with the actual mechanics of transcription (Razin and Riggs, 1980).

The functional significance of the ability of methylation to induce the transformation of B-DNA to Z-DNA is unclear (Tsanev, 1983).

It therefore seems that gene activation cannot be attributed to a single mechanism, but is more likely the result of the co-ordination of a number of mechanisms.

As the aim of this project was to study the mechanism of action of antioestrogens, and given the information discussed above, it was decided that this could be best achieved by studying the effects of the antioestrogen on the expression of some oestrogen-regulated genes. The methods used to obtain these oestrogen-regulated genes and to study their regulation by oestrogen and antioestrogen, and the results obtained, are described and discussed in the remaining sections.

MATERIALS AND METHODS

List of Suppliers

All chemicals used were of analytical grade and were obtained from BDH Chemicals Limited unless otherwise stated. All solvents used were obtained from May and Baker Limited except for absolute alcohol which was obtained from James Burrough Limited.

Names and Addresses of Suppliers

Amersham International P.L.C., Lincoln Place, Green End, Alyesbury, Bucks. HP20 2TP.

BDH Chemicals Limited, Poole, England.

Bio-Rad Laboratories Limited, Caxton Way, Watford Business Park, Watford, Hertfordshire.

Boehringer-Mannheim; BCL, Boehringer Mannheim House, Bell Lane, Lewes, East Sussex BN7 1LG.

Difco Laboratories, Detroit, Michigan, U.S.A.

James Burrough Limited, 70 Eastways Industrial Park, Witham, Essex.

GIBCO-BRL Limited, P.O. Box 35, Paisley, Scotland.

May and Baker Limited, Dagenham, England.

New England Biochemicals, (PLabs, P.O. Box 22, Bishops Stortford, Herts. LM23 3DH.

Pall Filtration Processes Limited, Europe House, Havant Street, Portsmouth, England.

Pharmacia Fine Chemicals, Pharmacia House, Midsummer Boulevard, Milton Keynes, England.

Sigma Chemical Company, Fancy Road, Dorset, England BH17 7NH.

Rohm and Haas (U.K. Ltd.), Croyden, Surrey.

Travenol Labs SA, D-7860 Lessines, Belgium.

1.2 Sterilisation Procedures

Ribonuclease is a major component of human sweat, and is therefore a major contaminant of glassware and solutions. In order to decrease the extent of degradation of RNA during isolation and manipulation, the following precautions were taken:

(a) disposable protective gloves (Travenol) were worn during all procedures involving RNA.

(b) all solutions were autoclaved at 15 p.s.i. for approximately 20 minutes.

(c) all glassware used in the preparation and manipulation of RNA and cDNA was siliconised by treatment with 2% Dimethyldichlorosilane in 1.1.1 trichloroethane (BDH) before being autoclaved, again at 15 p.s.i., for approximately 20 minutes.

(d) all eppendorfs and microcaps were autoclaved at 25 p.s.i. for approximately 20 minutes.

1.3 Preparation of Organic Reagents

(a) Phenol

Crystalline phenol (Sigma) was redistilled at 160°C and stored in aliquots at -20°C. When needed, aliquots were melted and made 0.1% (w/v) with respect to 8-hydroxyquinoline, an antioxidant. The melted phenol was then extracted with an equal volume of 1M Tris-HCl, pH8 until the pH of the aqueous phase was >7.6. This Tris-saturated phenol was then stored in a dark bottle at 4°C.

(b) Formamide

Formamide (Sigma) was deionised by mixing 50ml of formamide and 5g of analytical grade mixed bed resin A G501-X8/D (Biorad) for 1 hour, at room temperature, and then filtering this twice through Whatman 1MM paper. Deionised formamide was stored at -20°C until required.

2. Biological Methods

2.1 Experimental Animals

All rats used were of the Wistar strain and were bred at either Glasgow University or Alderley Park, Macclesfield. Rats were fed ad libitum on diet PRD. Immature female rats, 18-21 days old and weighing 25-45g, were used. The rats were anaesthetised using ether and killed by cervical dislocation. Uteri were then removed, dissected free of connective tissue, and quickly frozen in liquid nitrogen before treatment.

2.2 Administration of Hormone and Anti-Hormone to Animals

(a) Hormone

Oestradiol-17β (Sigma) was used at a concentration of 10μg/ml in 0.3M NaCl/05% (v/v) ethanol. 1μg of oestradiol-17β was administered to immature female rats by the intraperitoneal injection of 0.1ml of the above solution. Control rats received 0.1ml of 0.3M NaCl/0.5% (v/v) ethanol alone.

(b) Anti-Hormone

Tamoxifen citrate (ICI pharmaceuticals) was dissolved in ethanol, mixed with vegetable oil, and the ethanol evaporated off to give a final concentration of 300µg/ml. Immature female rats each received 30µg tamoxifen in 0.1ml vegetable oil by subcutaneous injection.

The levels of oestradiol-17β, and of tamoxifen citrate used, were equal to, or greater than, those shown to elicit maximal stimulation of transcription in the immature rat uterus (Knowler and Smellie, 1973; Waters, 1982).

2.3 Cell Culture

2.3.1 Growth and Maintenance of MCF-7 Cells

MCF-7 human mammary carcinoma cells were used. These were derived from a malignant pleural effusion in a female patient with metastatic breast cancer (Lippman et al., 1976), and have been shown to be oestrogen receptor positive (Brooks et al., 1973).

MCF-7 cells were obtained from ICI pharmaceuticals, Alderley Park, Macclesfield, where they were grown in Eagles MEM plus Hank salts supplemented with ImM sodium pyruvate, Penicillin (G) 100 units/ml, streptomycin 100mg/ml, 2mM L-glutamine, insulin 10mg/ml, (all from Gibco), 0.115% sodium bicarbonate, 1% non-essential amino-acids (ICI stock) and 5% charcoal stripped foetal calf serum (Gibco). Cells were grown in 20oz flasks to approximately 70% confluence before administration of hormone and anti-hormone.

2.3.2 Administration of Hormone and Anti-Hormone to MCF-7 Cells

Stock solutions of oestradiol-17β and tamoxifen citrate in ethanol were added to flasks of MCF-7 cells, which were about 70% confluent, such that the final concentration of oestradiol-17β was 10^{-8}M and that of tamoxifen was 10^{-6}M, control flasks recieved ethanol only to 0.1%.

2.3.3 Harvesting of Cells

Cells were harvested by treatment with EDTA (10ml of 0.1M EDTA/ flask). Cells were resuspended in phosphate buffered saline, 20ml /flask, and transferred to 50ml Falcon tubes. Cells were then pelleted by centrifugation at 2000rpm for 5 minutes in a Benchtop centrifuge before RNA extraction.

3. Preparation of Uterine RNA

The isolation of intact, viable RNA from the immature rat uterus was hampered by the high endogenous level of ribonuclease (Greenman and Kenney, 1964) and secondly, because the smooth muscle was difficult to homogenise by conventional methods.

A number of isolation procedures, described below, were tried in order to optimise the yield of intact biologically active mRNA from the immature rat uterus.

3.1 Isolation of Polysomal RNA

3.1.1 Preparation of Polysomes

Polysomes were prepared from the uteri of immature rats by a modification (Merryweather and Knowler, 1980) of the method of Berridge et al., (1976).

Groups of 20-40 uteri were frozen in liquid nitrogen and ground to a powder using a mortar and pestle. The powder was quickly transferred to a 10ml glass homogeniser and, after the addition of 9mls of homogenising buffer (200mM Tris-HCl, pH8.5, 50mM KCl, 15mM $MgCl_2$ containing 5mg/ml cycloheximide (Sigma), 7mM β-mercaptoethanol (Sigma), homogenised with a motor driven teflon pestle.

The homogenate was then adjusted to 2% Triton X-100 (Rohm and Haas) and centrifuged at 9000rpm for 10 minutes in the HB4 rotor of the Sorvall RC5 centrifuge (13,200xgav). The resultant supernatant was layered over a 0.5ml cushion of IM sucrose containing 50mM Tris-HCl, pH8.5, 50mM KCl, 15mM $MgCl_2$, 5mg/ml cycloheximide, 7mM β-mercaptoethanol in SW50.1 ultracentrifuge tubes (Beckman) and centrifuged in the SW50.1 rotor for 90 minutes at 47,000rpm (206,000xgav) in a Beckman LC-5 ultracentrifuge at $4^{o}C$ to pellet the polysomes.

3.1.2 Extraction of Polysomal RNA

The supernatant was discarded while the polysomal pellet was washed once with NETS buffer (100mM NaCl, 1mM EDTA, 10mM Tris-HCl, pH7.5, 0.2%

(w/v) sodium dodecyl sulphate, and resuspended in 2ml of the same buffer using a hand-held glass-teflon homogeniser. The polysome suspension was then extracted with an equal volume of phenol/chloroform/isoamylalcohol (25:24:1) by shaking vigourously for 10 minutes at room temperature on a wrist-action shaker in a 30ml corex tube (DuPont). The phases were separated by centrifugation at 6000rpm for 5 minutes in the HB4 rotor of the Sorvall RC5 centrifuge at 4°C (5900xgav). The aqueous phase was collected and the organic phase re-extracted, twice, with an equal volume of 100mM Tris-HCl, pH9, 1% (w/v) sodium dodecyl sulphate as above. The aqueous phases were collected and pooled and the RNA precipitated by the addition of 0.1 volumes 3M sodium acetate, pH5.8, and 2.5 volumes of absolute alcohol and storage overnight at -20°C.

3.2 Preparation of Total RNA

3.2.1 Guanidinium Thiocyanate and Lithium Chloride Method

RNA was prepared from the uteri of immature female rats by a modification of the method of Auffrey and Rougeon (1980).

Uteri were excised from immature rats, frozen in liquid nitrogen, and ground to a fine powder in a mortar and pestle. The powder was then transferred to a 30ml corex tube and 8 volumes of lysis buffer (5M Guanidinium thiocyanate (BRL), 50mM Tris-HCl, pH 7.5, 10mM EDTA) and 1 volume of β-mercaptoethanol added.

The uteri were then further homogenised and the DNA sheared by passing the mixture several times through, firstly, a 19g hypodermic needle, then a 23g needle.

50 volumes of 4M lithium chloride were added and the solutions mixed well and left at 4°C for approximately 20 hours. The RNA was then pelleted by centrifugation for 90 minutes at 6,500rpm in the Sorvall HB4 rotor at 4°C (8,900xgav). The supernatant was discarded and the pellet washed by resuspending it in 30 volumes (with respect to the pellet) of 3M lithium chloride. The RNA was again pelleted, by centrifugation at 6,500 rpm in the Sorvall HB4 rotar for 30 minutes at 4°C, the supernatant discarded and the pellet resuspended in 1-5mls of 10mM Tris-HCl, pH8, 1mM EDTA, 1% (w/v) sodium dodecyl sulphate.

An equal volume of phenol/chloroform/isoamylalcohol (25:24:11) was added and the mixture shaken for 5 minutes on a wrist-action shaker. The phases were separated by centrifugation at 6,500rpm for 10 minutes in Sorvall HB4 rotor at 4°C and the aqueous phase collected. The organic phase was extracted twice as above, and the aqueous phases collected. The RNA was precipitated from the pooled aqueous phases by the addition of 0.1 volumes 3M sodium acetate pH5.8, and 2.5 volumes of absolute alcohol at -20°C overnight.

3.2.2 Guanidinium Thiocyanate and Caesium Chloride Method

RNA was prepared from the uteri of immature rats by a modification of the method of Chirgwin et al, (1979).

Uteri were excised from immature rats, frozen in liquid nitrogen and crushed to a powder in a mortar and pestle as in Section 3.2.1. The powder was transferred to a glass homogeniser and, after the addition of homogenisation buffer (5M guanidinium thiocyanate, 5% (v/v)

β-mercaptoethanol, 50mM Tris-HCl, pH7, 2% (w/v) sarkosyl) at 16ml/gm tissue, it was homogenised using a motor driven teflon pestle.

The homogenate was then passed several times through a 23g needle, in order to shear genomic DNA, and caesuim chloride was added at 1g/2.5ml homogenate. After the caesuim chloride was dissolved, the homogenate was layered over a 1.2ml cushion of 5.7M caesium chloride, 50mM EDTA, pH7, which had been treated with Dowex chelating resin, in SW50.1 ultracentrifuge tubes, and centrifuged in the SW50.1 rotor at 35,000rpm (146,000xgav) for 12 hours at 20°C in a Beckman LC-5 ultracentrifuge. The supernatant was carefully removed, the pellet rinsed with water, and the tube inverted and allowed to drain. The RNA was then dissolved in 1ml water and precipitated with 0.1 volumes of 3M sodium acetate, pH 5.8, and 2.5 volumes of absolute alcohol at -20°C overnight.

3.2.3 Phenol/Chloroform and Caesium Chloride Method

RNA was phenol-extracted by a modification of the method of Kirby (1956), and then further purified by sedimentation through caesium chloride. Uteri were excised from immature rats, frozen in liquid nitrogen and ground to a fine powder in a mortar and pestle. The powder was then added to cold extraction buffer (50mM NaCl, 5mM sodium acetate, pH5, 1mMEDTA, 1% (w/v) sodium dodecyl sulphate), 16mls/gm tissue, and an equal volume of phenol/chloroform (1:1) in a 250ml conical flask.

The mixture was then shaken for 3 minutes at 60°C. The phases were separated by centrifugation at 5,000rpm for 20 minutes in the Sorvall HB4 rotor at 4°C (4,100xgav) and the aqueous phase collected. The organic

phase was then re-extracted with an equal volume of extraction buffer and the phases separated as above. The aqueous phase was again collected and the pooled aqueous phases were re-extracted with an equal volume of phenol/chloroform. The phases were again separated and the aqueous phase collected. Nucleic acids were precipitated from the aqueous phase by addition of 0.1 volumes of 3M sodium acetate, pH5.8, and 2.5 volumes of absolute alcohol at -20°C overnight.

The precipitated nucleic acids were pelleted by centrifugation at 10,000rpm for 20 minutes in the Sorvall HB4 rotor at 4°C (16,300 xgav). The pellet was dried in a stream of nitrogen gas and redissolved in 4ml 1% (w/v) sarkosyl, 10mM EDTA, pH7, and the DNA sheared by passing this through a 23g hypodermic needle. 4g caesium chloride was then dissolved in the above solution which was layered over high density caesium chloride cushions, made by dissolving 5.6g caesium chloride in 4ml 10mM EDTA, pH7, in two SW40 ultracentrifuge tubes. These were then filled with light paraffin oil and centrifuged at 17,000rpm for 17 hours in the SW40 rotor in a Beckman LC-5 ultracentrifuge at 20°C (36,300 xgav).

The paraffin oil and supernatant were carefully removed, the tubes wiped, and the RNA pellets dissolved in 2mls of water. The RNA was then precipitated with 0.1 volumes of 3M sodium acetate, pH5.8, 2.5 volumes of absolute alcohol at -20°C overnight.

3.3 Fractionation of RNA by Oligo (dT) Cellulose Chromatography

3.3.1 Preparing Oligo (dT) Cellulose Columns

Oligo (dT) cellulose columns were made by suspending 0.1gm oligo (dT) cellulose (BRL) in 10ml sterile water and packing this into a 2ml

sterile plastic syringe plugged with sterile, siliconised glass wool. An 18g needle, tubing, and adjustable clamp were then attached. The column was washed successively with 10ml of sterile water, 15ml of 2% (w/v) sodium dodecyl sulphate, and 15ml of binding buffer (1mM EDTA, 10mM Tris-HCl, pH7.5, 0.5M HCl, 0.1% (w/v) sodium dodecyl sulphate).

3.3.2 Running Oligo (dT) Cellulose Column

RNA, precipitated as described in Section 3.2, was pelleted at 10,000rpm for 20 minutes in the Sorvall HB4 rotor (16,300xgav). The pellet was dried under a gentle stream of nitrogen and resuspended in 1ml water. A 5µl aliquot was diluted to 1ml with water and its OD_{260}nm measured. The RNA concentration was then estimated from 1 OD unit 260nm = 40ug RNA (Glison et al., 1974). Sufficient water and 2x binding buffer were added to bring the RNA concentration to approximately 0.5mg/ml in 1X binding buffer.

The RNA was denatured by heating to 65°C for 3 minutes. It was then rapidly cooled, applied to the column, and run through with a flow rate of approximately 0.2ml/min. The eluate was collected, again heated to 65°C, cooled and run through the column. The run through constituted poly A-RNA. The column, which contained bound poly A+RNA, was washed with 1ml aliquots of binding buffer minus sodium dodecyl sulphate until the OD_{260} of the eluate was <0.05 OD units. The poly A+RNA was then eluted using 1ml aliquots of elution buffer (10mM Tris-HCl, pH7.5, 1mM EDTA). The OD_{260} of these fractions was monitored in order to; (a) follow the elution and (b) estimate the amount of poly A+RNA recovered.

Fractions containing polyA+RNA were pooled and the RNA precipitated by addition of 0.1 volumes 3M sodium acetate, pH5.8 and 2.5 volumes absolute alcohol at -20°C overnight. The precipitated polyA+RNA was then pelleted by centrifugation at 10,000rpm (16,300xgav) in the Sorvall HB4 rotor, dried under vacuum and resuspended in 100µl of sterile water. This was then transferred to a sterile, siliconised 1.5ml eppendorf tube, reprecipitated and stored at -20°C until required.

3.3.3 Regenerating Oligo (dT) Cellulose Columns

Oligo (dT) cellulose columns were regenerated by washing successively with 10ml 0.1M NaOH, 10ml water, 15ml 2% (w/v) sodium dodecyl sulphate and 15ml of binding buffer. When not in use the columns were stored at 4°C in binding buffer plus 0.2% (w/v) sodium azide.

3.4 Fractionation of RNA on Sucrose Density Gradients

RNA was fractionated on sucrose density gradients in order to obtain 28S rRNA for use as a standard (see Results section 4.2.1), by a modification of the method of Girard et al, (1965).

0.5 - 1mg of RNA in LETS buffer (10mM Tris-HCl, pH7.4, 0.1M LiCl, 1mM EDTA, 2% (w/v) sodium dodecyl sulphate) was layered on top of a linear 10-25% (w/v) sucrose density gradient in LETS buffer in cellulose nitrate tubes for the Beckman SW27 rotor. These were then centrifuged for 16hours at 23,000rpm (69,000xgav) at 20°C in the Beckman LC-5 ultracentrifuge.

The gradients were then fractionated by pumping them through a flow cell of the Gilford 240 spectrophotometer and monitoring the OD_{260} using an attached chart recorder.

The fraction corresponding to the 28S rRNA peak was collected and, after estimating the amount by measuring the OD_{260} of a 1ml aliquot, was stored frozen at -20°C until required. The integrity and purity of the 28S rRNA was checked by running a sample on a 2% agarose gel as described in Section 3.5.

3.5 Fractionation of RNA on Non-Denaturing Agarose Gels

This method was used routinely to test the purity of 28S rRNA fractions and to check the efficiency of Oligo (dT) cellulose purification of mRNA.

RNA samples, about 2µg/track, in loading buffer (0.04% (w/v) bromophenol blue, 2.5% Ficoll type 400 (w/v), 1X Loening phosphate buffer (36mM Tris-HCl, pH7.7, 30mM NaH_2PO_4, 1mM EDTA) were fractionated in a 2% (w/v) agarose gel in 1X Loening phosphate buffer containing 0.5µg/ml ethidium bromide. The RNA was electrophoresed at 40V, in Loening phosphate buffer made 0.5µg/ml with respect to ethidium bromide, for 2.5 hours. The gel was then transferred to a UV transilluminator (254nm) in order to view the RNA. The gel was then photographed using Polaroid Type 667 film.

4. Plasmid DNA

4.1 Procedures for the Isolation and Purification of Plasmid DNA

These procedures make use of the differences in size and shape of plasmid and host cell DNA. Plasmids being small, usually covalently

closed circles, are resistant to shearing and denaturation. Conversely, host cell DNA can be easily sheared to give long linear molecules. Separation of these two forms of DNA involves differential precipitation and density gradient centrifugation in the presence of an intercalating dye, usually ethidium bromide.

4.1.1 Plasmid Mini-Prep

Plasmid DNA was isolated according to the method of Holmes and Quigley, (1981). This method, which yields between 1-3µg plasmid DNA/1.5ml unamplified culture, involves the selective denaturation of chromosomal DNA by brief boiling and removal of the resulting chromosomal DNA/protein gel by centrifugation.

5ml of L-Broth (1% w/v bactotryptone (Difco), 0.5% (w/v) yeast extract (Difco), 1% (w/v) NaCl) made 100µg/ml with respect to ampicillin, was inoculated with 2µl of a selected colony from a micro-titre plate well, and grown overnight at 37°C in an orbital incubator at 200rpm.

1.5ml of the culture was transferred to an eppendorf and the cells pelleted by centrifugation for 1 min. in an ependorf centrifuge. The medium was then removed and the bacterial pellet resuspended in 0.35ml of 8% (w/v) sucrose, 0.5% (v/v) Triton X-100, 50mM EDTA, 10mM Tris-HCl, pH8. 25µl of a freshly prepared lysozyme solution (10mg/ml in 10mM Tris-HCl, pH8) was then added and mixed by vortexing for 3 seconds. The tube was then placed in a boiling water bath for 40 seconds, and immediately centrifuged for 10 minutes at room temperature in an eppendorf centrifuge. The pellet was removed with a sterile needle and 40µl of 2.5M sodium

acetate and 240µl of isopropanol were added to the supernatant. This was mixed by vortexing and stored for 15 minutes in a dry ice/methanol bath. The mixture was then centrifuged for 15 minutes at 4°C in an eppendorf centrifuge, the supernatant discarded, the pellet dried and resuspended in 50µl 10mM Tris-HCl, pH8, 1mM EDTA containing DNase free RNase (50µg/ml) and incubated for 1 hour at 37°C.

After RNA digestion the solution was extracted with an equal volume phenol/chloroform/isoamyl alcohol (25:24:1), and the aqueous phase precipitated by the addition of 0.1 volumes 3M sodium acetate, pH5.8, and 2.5 volumes of absolute alcohol overnight at -20°C.

4.1.2. Plasmid Midi-Prep

Plasmid DNA was isolated by a modification of the method of Birnboim and Doly (1979). This method, which yields between 200µg - 500µg plasmid DNA, involves selective denaturation of genomic DNA with alkali, and removal of the resulting protein/DNA 'gel' by centrifugation.

100ml of L-Broth, made 100µg/ml with respect to ampicillin, was inoculated with 5µl from either an overnight culture, or a colony in a microtitre plate, and grown overnight in an orbital incubator at 37°C.

The bacteria were then pelleted by centrifugation at 2,500rpm for 15 minutes in a Beckman Benchtop centrifuge in 50ml Falcon tubes. The medium was discarded and the bacterial pellet resuspended in a total volume of 1ml of alkaline lysis I (50mM glucose, 10mM EDTA, 25mM Tris-HCl, pH8). 10µl of stock frozen lysozyme, 50mg/ml, were added and the mixture

left on ice for 15 minutes. 3ml of alkaline lysis II (0.2M NaOH, 1% (w/v) sodium dodecyl sulphate) were added, the solutions mixed, and again left on ice for 5 minutes. After the addition of 2.3ml alkaline lysis III (3M sodium acetate, pH4.8) the solutions were mixed well and left on ice for 30 minutes, after which the mixture was transferred to 15ml corex tubes and centrifuged for 15 minutes at 10,000rpm in the Sorvall HB4 rotor (16,300xgav). The supernatant was collected, and after addition of 0.6 volumes of isopropanol, mixed and left on ice for 5 minutes before being centrifuged at 2,500rpm for 15 minutes at 4°C in the Benchtop centrifuge. The supernatant was then discarded and the pellet dried under a stream of nitrogen. The dried pellet was resuspended in 0.9 volumes of 2M ammonium acetate and left on ice for 5 minutes before being centrifuged for 15 minutes at 2,500rpm at 4°C in the Benchtop centrifuge. The resulting supernatant was collected into a 1.5ml eppendorf tube and 540µl of isopropanol added. This was then centrifuged for 15 minutes in an eppendorf centrifuge at 4°C. The supernatant was discarded, the pellet dried, redissolved in 100µl of 1mM EDTA, 10mM Tris-HCl, pH8, containing pancreatic ribonuclease (20µg/ml) and incubated for 2 hours at 37°C. The solution was then extracted with an equal volume of phenol/chloroform/ isoamylalcohol (25:24:1), and the aqueous phase separated by centrifugation for 3 minutes in an eppendorf centrifuge.

The aqueous phase was collected, and 2µl taken to measure OD_{260} to give an estimate of the amount of plasmid. After making the solution 1mg/ml with respect to plasmid, a 1µl aliquot was taken to run on a 1% agarose gel (Section 4.2) to check the purity and integrity of the DNA. The remainder was frozen in a dry-ice/methanol bath and stored at -20°C until required.

4.1.3. Full-Scale Plasmid Prep

This method yields 500-2000µg of mostly covalently closed circular DNA. It is again a modification of the method of Birnboim and Doly (1979), employing alkali to denature genomic DNA as in the midi-prep. 25ml of L-Broth containing 100µg/ml ampicillin were inoculated with 5µl of a selected colony from a micro-titre plate well and grown overnight at 37°C in an orbital incubator at 200rpm.

Two 5ml aliquots of this overnight culture were then used to inoculate 2x800ml L-Broth in 2x2l conical flasks, and the bacteria grown, in an orbital incubator, until they reached late log phase, that is, OD_{600nm} = 0.8. Chloramphenicol was then added to a final concentration of 165µg/ml and the bacteria left in the incubator overnight. Choramphenicol by inhibiting protein synthesis, stops genomic DNA replication, but not plasmid DNA synthesis, and therefore increases the number of copies of plasmid/cell.

The bacteria were pelleted by centrifugation at 5,000rpm for 10 minutes in a Sorvall GS3 rotor at 4°C, (4,200xgav) the medium discarded, and the bacteria resuspended in a total volume of 9.5mls of alkaline lysis I (50mM glucose, 10mM EDTA, 25mM Tris-HCl, pH8) and transferred to a Ti60 centrifuge tube. 0.5ml of 40mg/ml solution of lysozyme, in alkaline lysis I, was added, the tube shaken and left on ice for 30 minutes. The solution was then divided between two Ti60 centrifuge tubes and, after adding 10ml alkaline lysis II (0.2M NaOH, 1% (w/v) sodium dodecyl sulphate) to each tube, was mixed well and left on ice for 5 minutes before adding 7.5ml of alkaline lysis III (3M sodium acetate, pH4.8). The

tubes were again mixed thoroughly, left on ice for 1 hour and then centrifuged for 30 minutes at 30,000rpm in a Ti60 rotor in the Beckman L8 ultracentrifuge at 4°C (63,300xgav). The supernatants were collected into 30ml corex tubes, 0.6 volumes of isopropanol added, the tubes mixed and left for 10 minutes at room temperature. The resulting precipitate was then pelleted by centrifugation at 8,000rpm, for 15 minutes, in the SS-34 rotor for the Sorval RC5 centrifuge at room temperature (7,700xgav). The pellet was redissolved in a total volume of 30ml TE. (10mM Tris-HCl, pH8, 1mM EDTA) in a 50ml Falcon tube, 28.9g caesium chloride and 1.8ml of ethidium bromide (10mg/ml) added, and the tube shaken well. This gives a solution of density 1.59 g/ml. The solution was then clarified by centrifugation at 1,500rpm for 30 minutes in a Beckman Benchtop centrifuge. Avoiding the surface scum and pellet, the solution was transferred to an ultracentrifuge tube and centrifuged at 47,000rpm (179,000 xgav) for at least 20 hours in a VTi50 rotor in the Beckman L8 ultracentrifuge at 20°C.

The tube was then clamped and the top pierced. Using a long wavelength UV light two bands are seen. The upper band consists of nicked circular and linear DNA, including genomic DNA. The lower band consists of closed circular plasmid DNA. The plasmid band was collected by piercing the side of the tube with a 21g needle and drawing it off into a 5ml syringe. This was then further purified by re-centrifugation, in the same solution of density 1.5g/ml, in the VTi65 rotor at 56,000rpm (300,000 xgav) for at least 16 hours at 20°C in the Beckman L8 ultracentrifuge. The band, after this second spin, is visible in daylight and is collected as before but using a 2ml syringe and 22g needle.

Ethidium bromide was then extracted from the plasmid DNA by addition of an equal volume isoamylalcohol, mixing, and separating the phases in an eppendorf centrifuge for 2 minutes. The upper phase was discarded and the lower aqueous phase re-extracted three times more with an equal volume of isoamylalcohol.

The volume of the aqueous phase was estimated and, after transfer to a 30ml corex tube, 4 volumes of TE, pH8 added. Plasmid DNA was then precipitated by the addition of 2 volumes of absolute alcohol at -20°C overnight. The corex tube was then left on dry ice for 30 minutes before pelleting the DNA by centrifugation at 10,000rpm in the Sorvall HB4 rotor at 0°C (16,300 xgav). The pellet was dried and resuspended in 100µl TE, pH8, transferred to a 1.5ml eppendorf and the plasmid reprecipitated by adding 0.1 volumes 3M sodium acetate, pH5.8, 2.5 volumes of absolute alcohol and storing the tube on dry-ice for 10 minutes. The DNA was then pelleted by centrifugation for 15 minutes in an eppendorf centrifuge at 4°C, washed with cold ethanol, and centrifuged as before. The pellet was then vacuum dried and resuspended in 100µl TE, pH8. 2µl were taken to estimate the yield of plasmid DNA and, after making the solution 1mg/ml with respect to plasmid, a 1µg aliquot taken to run on an agarose gel. The remainder of the plasmid solution was then frozen in a dry-ice/ methanol bath and stored at -20°C until required.

4.2 Electrophoresis of Plasmid DNA

Plasmid DNA was routinely resolved in agarose gels in order to;

(a) check the purity of the plasmid prep.

(b) estimate the size of the plasmids, and their inserts.

(c) check the efficiency of restriction digests.

Gels were run in a Life-sciences mini-gel apparatus (gel volume: 25mls) in order to analyse the DNA rapidly, using TBE (0.089M Tris-borate, pH7.7, 0.089M Boric acid, 0.002M EDTA) as running buffer.

DNA samples, usually 0.5-1µg/track, in loading buffer (1 X TBE, 0.04% (w/v) bromophenol blue, 2.5% (w/v) Ficoll type 400) were resolved in 1% (w/v) agarose in 1 x TBE, containing 0.5µg/ml ethidium bromide. Electrophoresis was at 45V in 1 x TBE containing 0.5µg/ml ethidium bromide.

Gels were then transferred to a UV transilluminator (254nm) and photographed using polaroid type 667 film.

4.3 Restriction Digestion of Plasmid DNA

Type II restriction endonucleases, that is those which cut DNA within, or near to, a particular recognition sequence, were used. These were obtained from BRL and were used in either high, medium or low, ionic strength buffer (Table 4) depending on their designation in Maniatis et al.(1982).

When DNA was to be cut with more than one enzyme they were added together, provided both required the same ionic strength optimum. Otherwise the one requiring the lowest ionic strength was used first, and the salt concentration adjusted before adding the next.

20µg of plasmid DNA (1mg/ml) was transferred to an eppendorf tube and 5µl of the appropriate 10x restriction endonuclease buffer added. 50

Table 4

Buffers for restriction endonuclease digestion

From Maniatis et al., (1982)

Buffer	NaCl	Tris-HCl (pH 7.5)	$MgCl_2$	DTT
Low	—	10mM	10mM	1mM
Medium	50mM	10mM	10mM	1mM
High	100mM	50mM	10mM	1mM

units of the restriction enzyme was then added and the total volume brought up to 50µl with sterile water. The digestion was carried out at 37°C for 2 hours after which a 2µl aliquot was taken and analysed on a 1% agarose gel (Section 4.2) to check if the digestion was complete.

The restricted DNA was then purified by extracting it once with an equal volume of phenol/chloroform/isoamy alcohol (25:24:1), collecting the aqueous phase and precipitating the DNA with 2.5 volumes of absolute alcohol at -20°C overnight.

4.4 Recovery of DNA from Agarose Gels

Plasmid DNA, digested as described in 4.3, was electrophoresed as described in 4.2 except that Tris-acetate buffer (40mM Tris-HCl, pH7.4, 5mM sodium acetate, 1mM EDTA) was used instead of TBE, and the gel was run in the larger (gel volume = 60ml) BRL apparatus.

The DNA was electrophoresed at 60V for 45 minutes. The bands were then visualised using a long wavelength UV torch. The band representing the insert was then cut out using a scalpel, and placed in dialyses tubing with 200µl of tris-acetate buffer. The ends of the tubing were sealed with plastic clips and the bag placed in the electrophoresis tank, at right angles to the direction of the current. The DNA was then electrophoresed at 60V for 1 hour. The solution was then collected from the dialysis tubing and replaced with a further 200µl Tris-acetate buffer and electrophoreses continued for a further 15 minutes. The electrodes were then reversed for 2 minutes, in order to release any DNA stuck to the tubing, and the solution collected and added to the first 200 µl. The

pooled solution, in a 1.5ml eppendorf tube was centrifuged to pellet any fragments of agarose, the supernatant transferred to another eppendorf, and the DNA precipitated with 0.1 volumes 3M sodium acetate, pH5.8, and 2.5 volumes of absolute alcohol at -20°C overnight.

4.5 Nick-Translation of DNA

Nick-translations were carried out using E.Coli DNA polymerase I (BRL) which was found to contain enough contaminating DNase activity to give adequate incorporation of labelled nucleotide.

The nick-translation reaction was carried out in a total volume of 12µl, the final reaction conditions being:

4µM dCTP, 4µM dGTP, 4µM dTTP, 50µCi α-^{32}P-dATP (Amersham, 3000 Ci/mmole), 40µg/ml DNA, 50mM NaCl, 10mM Tris-HCl, pH7.4, 10mM Mg SO_4, 1mM dithiothrietol, 400 Units/ml DNA polymerase I.

The reaction mixture was incubated for 4 hours at 15°C. Nick translated DNA was then separated from unincorporated nucleotides by passing the mixture through a 1.5ml column of Biogel P-60 (Bio-rad) in a blue eppendorf tip, plugged with glass wool, and equilibrated with NE (50mM NaCl, 0.5mM EDTA, pH7.0). 100µl fractions were collected and screened with a Geiger counter. The peak fractions were pooled and a 1µl aliquot taken, to measure the radioactivity, by Cerenkov counting. The nick-translated DNA, which was generally of specific activity $5x10^7$ cpm/µg DNA, was stored frozen at -20°C.

5. Construction of a cDNA Library (in Collaboration with Dr.J.T. Knowler)

5.1 Synthesis of Single-Stranded Complementary DNA

12µg of poly A+RNA from the uteri of immature rats, which had been exposed to oestradiol-17β for 4 hours, was used as the template for the synthesis of single-stranded DNA.

The procedure used for the first and second strand synthesis and S1 nuclease digestion was essentially that described by Wickens et al, (1978) and involved carrying out these steps sequentially in the same reaction vessel.

Avian myeloblastosis virus (AMV) reverse transcriptase (Anglia) was used to synthesise the first strand under the following conditions: 240µg/ml poly A+RNA, 1mM dNTP's, 0.5 µCi α-^{32}P-dCTP (3000 Ci/mmole), 100µg/ml oligo $(dT)_{12-18}$ (BRL), 50mM Tris-HCl, pH8.3, 140mM NaCl, 10mM $MgCl_2$, 30mM β-mercaptoethanol and 2860 Units/ml AMV reverse transcriptase.

The reaction was carried out in a total volume of 50µl. The reaction mixture was incubated at 42°C for 1.5 hours before being boiled for 1.5 minutes and left on ice. This separates the RNA/first strand hybrids.

5.2 Second Strand Synthesis

The cold reverse transcription mixture was added to an equal volume

of second strand buffer (200mM Hepes, pH6.9, 200μM dNTPs, 1200 units/ml. E.Coli DNA polymerase I (BRL)) and incubated for a further 2.5 hours at 15°C.

5.3 SI Nuclease Digestion:

To the second strand synthesis reaction mixture was added 400μl of concentrated SI buffer such that the final reaction conditions were 300mM NaCl, 30mM sodium acetate, pH4.5, 3mM $ZnCl_2$, 5 Units/ml SI nuclease (New England Biochemicals). Digestions were incubated for 30 minutes at 37°C and the reaction then stopped by the addition of 10μl 0.5M EDTA, pH8. The reaction mixture was then extracted once, at room temperature, with an equal volume of saturated phenol (Section 1.3) followed by extraction with an equal volume of chloroform. 2.5 volumes of absolute alcohol were then added, to the collected aqueous phase, and the DNA precipitated overnight at -20°C.

5.4 Repair of SI Treated ds cDNA

SI nuclease treatment does not leave completely blunt ends on the cDNA, which was ensured by a further incubation with the Klenow fragment of DNA polymerase I. The DNA was pelleted, by centrifugation for 2 minutes in an eppendorf centrifuge, vacuum dried and resuspended in water. The repair reaction was carried out in a total volume of 25μl, the conditions being similar to those for second strand synthesis, that is 200mM Hepes, pH6.9, 25mM Tris, 10mM NaCl, 5mM $MgCl_2$, 15mM β-mercaptoethanol, 100 μM dNTPs and 400 units/ml Klenow fragment of E.Coli DNA polymerase I. The reaction mixture was incubated for 10 minutes at

room temperature and then stopped by the addition of 5µl 0.5M EDTA pH8. The mixture was then extracted once with an equal volume of phenol/chloroform and the aqueous phase collected and precipitated with 0.1 volumes 3M sodium acetate pH5.8, and 2.5 volumes absolute alcohol, overnight, at -20°C.

5.5 Preparation of the Plasmid Vector

The plasmid vector used was pUC8 (Vierra and Messing, 1982). This contains a multiple cloning site (MCS) which is in the correct reading frame to allow expression of a functional β-galactosidase. Cloning into any of the sites on the MCS linker leads to loss of a functional product and can be tested for by plating out transformants on X-gal plates: bacteria harbouring plasmids containing inserts will yield white colonies, whereas those with plasmids without inserts will yield blue colonies. This procedure, unlike that of using a drug resistance marker, does not require replica plating.

25µg of pUC8 were dissolved in buffer containing 20mM KCl, 10mM Tris-HCl, pH8, 10mM $MgCl_2$, 1mM DTT and 90 units Sma I restriction endonuclease added in a final volume of 100µl. The reaction mixture was then incubated at 30°C for 2 hours after which a 2µl sample was run on an agarose gel (Section 4.2) to check the digestion. The mixture was then extracted once at room temperature with an equal volume of phenol/chloroform, the aqueous phase collected and the DNA precipitated by addition of 0.1 volumes of 3M sodium acetate, pH5.8, and 2.5 volumes of absolute alcohol at -70°C for 1 hour. The DNA was then pelleted, by centrifugation for 10 minutes in an eppendorf centrifuge at 0°C, vacuum dried and resuspended in 20µl of 10mM Tris-HCl, pH8.

5.6 Alkaline Phosphatase Treatment of the Plasmid Vector

This procedure removes 5'phosphates from plasmid DNA and prevents self-ligation. Calf intestinal alkaline phosphatase (Boehringer) was used under the following conditions: 250µg/ml SmaI cut pUC8, 10mM Tris-HCl, pH9.2, 1mM $MgCl_2$, 0.1mM $ZnSO_4$, 0.1mM EDTA, 80 units/ml alkaline phosphatase in a total volume of 100µl. The reaction mixture was incubated for 15 minutes at 37°C followed by 15 minutes at 56°C. A further 8 units of alkaline phosphatase was then added and the mixture again incubated as above. The reaction was then stopped by extraction with phenol/chloroform, the aqueous phase being saved.

5.7 Ligation of the Plasmid

Three ligation reactions were carried out:

(i) ligation of the plasmid alone, to test the efficiency of the phosphatase treatment.

(ii) ligation of the plasmid to Hae III generated fragments of pAT153, to assess the ability of the plasmid to ligate

(ii) ligation of the plasmid to ds cDNA made as described in Section 5.1-4.

50ng of restricted, phosphatased pUC8 were used for each ligation reaction, in a total reaction volume of 20µl. The conditions were 50mM Tris-HCl, pH7.4, 10mM $MgCl_2$, 10mM DTT, 1mM dATP, 1mM spermidine, 0.1mg/ml

BSA and 25 units/ml T4 DNA ligase (BRL). The reaction was incubated for 24 hours at 12°C.

5.8 Transformation of E.Coli K12, Strain JM83

E.Coli JM83 cells (Messing, 1979) were made competent for transformation essentially according to Dagert and Ehlrich (1979). 1ml of an overnight culture of JM83 cells in L-Broth was used to inoculate 100ml of L-Broth in a conical flask. The cells were then grown in an orbital incubator, at 37°C, until they reached an OD_{600} of about 0.6 (indicative of log phase growth). The cells were then transferred to 2x50ml Falcon tubes and pelleted by centrifugation at 2,500rpm for 15 minutes in a Benchtop centrifuge. The pellets were then resuspended in 2x25ml of 10mM $MgCl_2$, and left on ice for 30 minutes before being pelleted as above. The bacterial pellets were then resuspended in 2x12.5ml 50mM $CaCl_2$ and left on ice for 15 minutes. The cells were again pelleted and finally resuspended in 2.0ml of 50mM $CaCl_2$ and left at 4°C.

The DNA for transformation was then made up to 100µl with TE, pH7.5 and 200µl of the competent cell suspension added. The suspension was mixed and left on ice for 30 minutes. The cells were then subjected to heat-shock at 42°C for 30 minutes and left at room temperature for 10 minutes. 0.7ml of pre-warmed L-Broth was then added and the mixture incubated, at 37°C, with shaking, for 1 hour.

200µl of transformation mix was spread per 15cm diameter L-agar plate (1% (w/v) bacto-tryptone, 1% (w/v) NaCl, 0.5% (w/v) yeast extract, 1.5% (w/v) bacto-agar (Difco)) containing 100µg/ml ampicillin and 0.5µl of

2% X-gal/ml. The plates were then inverted and incubated at 37°C overnight.

5.9 Transfer of Recombinants to Micro-Titre Plates

Bacteria harbouring plasmids with inserts show up as white colonies as they do not produce a functional β-galactosidase and cannot metabolise X-gal.

The white colonies were picked from the L-agar/X-gal plates with sterile toothpicks and transferred to wells in micro-titre plates. Each well contained 80µl of enriched L-Broth (1% (w/v) bacto-tryptone, 1% (w/v) NaCl, 0.5% (w/v) yeast extract, 0.18% (w/v) K_2HPO_4, 0.045% (w/v) Na citrate, 0.009% (w/v) Mg SO_4 $7H_20$, 0.09% (w/v) $(NH_4)_2$ SO_4, 4.4% (w/v) glycerol) containing 100µg/ml ampicillin.

The colonies were transferred to individual wells in the plates, the plates covered with plastic strips to stop cross-contamination, and incubated at 37°C overnight. The plates were then stored at -20°C until required.

About 650 recombinants were transferred in this way. The remainder of the ligation mixture was then transferred and stored, after a 50-fold amplification, in enriched L-Broth containing 100µg/ml ampicillin at -70°C.

6. Screening of the cDNA Library

The recombinants isolated and transferred to micro-titre plates, as

described in Section 5, were screened for oestrogen-responsive messenger sequences by two different techniques; firstly, by colony hybridisation, and secondly, by dot blotting plasmids isolated from individual colonies. In both cases the probes used were single-stranded cDNAs synthesised using poly A+ RNA from the uteri of untreated, and 4 hour oestrogen treated immature rats. Both procedures are described below.

6.1 Synthesis of a cDNA Probe Complementary to mRNA

The method described here is different from that used to synthesise the first strand of the ds cDNA used for cloning (Section 5.1) as the emphasis here is, not necessarily on obtaining full length transcripts, but on obtaining a high specific activity.

2µg of poly A+RNA, selected for by one passage through Oligo (dT) cellulose, from either four hour oestrogen stimulated or control immature rat uteri, were used as the template for the synthesis of a single-stranded cDNA probe.

The poly A+RNA (1mg/ml) was first denatured by heating to 65°C for 2 minutes and then cooled rapidly on ice. The synthesis of the cDNA was primed by incubating the poly A+RNA with 0.2µg of Oligo $(dT)_{12-18}$ in 0.25M Tris-HCl, pH8.3, 0.3M NaCl, 0.03M $MgCl_2$, 10mM DTT at 40°C for 10 minutes. The remaining reactants were then added, from concentrated stock solutions, such that the final reaction conditions were; 50mM Tris-HCl, pH8.3, 60mM NaCl, 6mM $MgCl_2$, 2mM DTT, 2mM dCTP, 2mM dGTP, 2mM dTTP, 50µCi α-^{32}P-dATP (3000 Ci/mmole), 0.1mg/ml actinomycin D (Sigma), 10ug/ml Oligo $(dT)_{12-18}$, 100µg/ml poly A+RNA, 250 units/ml AMV Reverse transcriptase (a

gift from Dr. C. Darnbourgh), in a total volume of 20µl. The reaction mixture was then incubated for 1.5 hours at 40°C, after which the reaction was stopped by the addition of 2µl of 0.5M EDTA, pH8.

The RNA template was then hydrolysed by making the solution 0.1M with respect to NaOH and incubating it at 65°C for 30 minutes. Tris-HCl, pH8, was then added to 0.1M and the mixture titrated to neutrality with 1M HCl, using phenol-red indicator.

The synthesised cDNA was then separated from unincorporated nucleotides by passing the mixture through a 1ml column of Sephadex G-100 (Pharmacia) equilibrated with TEN buffer (10mM Tris-HCl, pH8, 10mM, EDTA, 0.3M NaCl), in a 2ml siliconised glass pipette. The sample was washed through with TEN buffer and 50µl fractions collected into eppendorf tubes. The radioactivity of the fractions was assessed by Cerenkov counting. The fractions containing the cDNA were identified and pooled and the cDNA stored frozen at -20°C until required.

6.2 Sizing of Single-Stranded cDNA

Single-stranded cDNA was sized by electrophoresis through an alkaline agarose gel. Samples of cDNA, a few thousand cpm of each, in loading buffer (0.04% bromophenol blue, 1mM EDTA, 30mM NaOH, 50% glycerol) were fractionated in a 1.5% (w/v) agarose gel in 1X running buffer (1mM EDTA, 30mM NaOH). The cDNA, with ^{32}P- end labelled markers (for example, denatured end labelled Taq 1 digested pAT), was electrophoresed at 40V for 2-4 hours in 1X running buffer. The gel was then dried and auto-radiographed using Kodak X-omat S X-ray film and a fast-tungstate intensifier screen. The size range of the cDNA was then estimated from the positions of the markers on the film.

6.3 Growth of Recombinants on Nitrocellulose Filters

Duplicate copies of the recombinants in micro-titre plates were made using a Cookes multi-well dipper and the master copy stored away at -20°C. The duplicates were then used to grow the recombinants for screening.

Nitrocellulose filters were cut to fit a micro-titre plate, marked, and placed on 15cm diameter L-agar plates containing 100 µg/ml ampicillin. When the filters had become damp colonies were transferred from micro-titre plates using Cookes multi-well dippers. Four copies of each micro-titre plate were made in order that duplicates could be hybridised to each of the two probes used. The L-agar plates, with the nitrocellulose filters, were then inverted and incubated overnight at 37°C. The filters were then transferred to similar L-agar plates containing 165µg/ml chloramphenicol, and the plates again inverted and incubated at 37°C overnight.

6.4 Colony Lysis and Binding of DNA to Nitrocellulose Filters

The recombinant colonies grown on nitrocellulose filters were lysed essentially as described by Humphries et al. (1978). The following steps, which result in lysis of the bacteria, involve laying the nitrocellulose filters, colony sides up, on 3MM paper saturated with the appropriate solution.

The filters were first laid on filter paper saturated with 0.5M NaOH for 10 minutes (or until the colonies became glossy), blotted on dry

3MM paper, and laid on filter paper saturated with 1M Tris-HCl, pH7.4, for a further 10 mins. This last step was repeated twice more in order to neutralise the alkaline conditions. The filters were again blotted on dry 3MM paper and transferred to paper saturated with 1.5M NaCl, 0.5M Tris-HCl, pH7.4, for a further 10 minutes. The filters were blotted once more on dry 3MM paper and transferred to 15cm diameter petri-dishes to which 5ml of 1 x SSC (standard saline citrate: 1X = 0.15M NaCl, 0.015M trisodium citrate, pH7) containing Proteinase K (Sigma) at 1mg/ml. The petri-dishes were then taped together and shaken in an orbital incubator, at 150rpm and room temperature for 1 hour, to digest any bacterial debris which was still attached to the filter and could therefore mask the plasmid DNA from the probe.

The filters were then rinsed in 2 x SSC, air-dried, and baked in a vacuum oven for 2 hours at 80^{o}C to bind the DNA to the nitrocellulose. Filters were then stored in an air-tight box at room temperature until required.

6.5 Hybridisation Procedures

6.5.1 Prewashing

Filters, prepared as described in sections 6.2/6.3, were wetted in 3 x SSC and transferred to a plastic box containing 25ml prewashing buffer (50mM Tris-HCl, pH8, 1M NaCl, 1mM EDTA, 0.1% (w/v) sodium dodecyl sulphate) per 100cm^{2} nitrocellulose, and incubated for 1 hour at 42^{o}C in a shaking water bath.

6.52 Prehybridisation

The prewashing buffer was poured off and the filters sealed, in plastic bags (two filters back to back), with 8ml of prehybridisation buffer, that is a solution containing 10xDenhardts solution (50xDenhardts = 1% BSA, 1% Ficoll Type 400, 1% poly vinyl-pyrolidine, pH7.0), 50% (v/v) deionised formamide, 5 x SSPE (20 x SSPE = 3.6m NaCl, 200mM NaH_2PO_4, pH7.4, 20mM EDTA, pH7.4), 0.1% (w/v) sodium dodecyl sulphate, 10µg/ml poly A, 10µg/ml poly C, 100µg/ml denatured salmon sperm DNA. The bags were then placed in a plastic box filled with water and incubated, at 42°C, in a shaking water-bath overnight.

6.5.3 Hybridisation

The bags containing the filters were cut open, the prehybridisation buffer poured off, and the filters transferred to another plastic bag containing 4ml hybridisation buffer, that is prehybridisation buffer plus 10^6cpm of heat-denatured cDNA probe. The bag was sealed, placed in a plastic box filled with water, and incubated at 42°C in a shaking water bath for 24 hours.

6.5.4 Washing

After hybridisation, the hybridisation buffer was poured away, carefully, and the filters washed, in a plastic box, for 5 x 10 minutes at room temperature in 50ml/filter of low stringency wash buffer; 2 x SSC, 0.1% (w/v) sodium dodecyl sulphate, followed by 2 x 30 minutes at 60°C in high stringency wash buffer; 0.1 x SSC, 0.1% (w/v) sodium dodecyl

sulphate, in a shaking water bath. Filters were then blotted on dry 3MM paper and air-dried.

6.5.5 Autoradiography

As nitrocellulose filters are very brittle, they were sealed in plastic bags before autoradiography. Autoradiography was carried out for various times, at -70°C, using Kodak X-omat S X-ray film and Dupont Cronex lightning-plus intensifier screens.

6.5 Plasmid Dot-Blot Hybridisation

Plasmids were isolated from individual colonies using the plasmid mini-prep described in section 4.1.1. Up to 40 mini-preps could be handled at one time. The precipitate of plasmid DNA obtained from preps of this sort were pelleted by centrifugation for 15 minutes in an eppendorf centrifuge, at 4°C, the supernatant discarded and the pellet vacuum dried. The pellet was then resuspended in 20µl of sterile water, and the DNA denatured by boiling it for 5 minutes, and then cooling rapidly on ice. The condensation was then spun off the sides of the eppendorf by centrifugation for about 30 secs. in the eppendorf centrifuge.

Nitrocellulose filters, ruled into 1cm squares with a very clean ruler and a soft pencil, were then saturated with 20 x SSC and air-dried. 2 x 2µl aliquots of each plasmid was then spotted, in duplicate, onto two different filters, the spots allowed to dry and the filters baked, in vacuo, for 2 hours at 80°C.

The filters were then prehybridised, hybridised, washed and autoradiographed as described in sections 6.5.2-6.5.5.

The remaining 4µl of the plasmids were kept and run on mini-gels (section 4.2) in order to estimate the relative size of the inserts.

7. RNA Blotting and Hybridisation Procedures

7.1. Northern Blotting

7.1.1. Fractionation of RNA on Agarose Gels under Denaturing Conditions

Electrophoresis of RNA under denaturing conditions was carried out according to a modification of the procedure described by Ellis et al. (1982).

Samples of poly A+RNA, 0.5-15µg, were denatured by heating them to 65°C for 5 minutes in 1 x running buffer (20mM 3 -[N] - Morpholino propane sulfonic acid (MOPS)) (Sigma), 50% formamide, 2.2M formaldehyde and then cooling rapidly on ice. The samples were then made 0.04% with respect to bromophenol blue, and 2.5% with respect to Ficoll type 400 and loaded onto a 70ml, 0.8% agarose gel in 1 x running buffer containing 2.2M formaldehyde, in a BRL horizontal gel apparatus.

The RNA was then electrophoresed for 5 hours at 60V in 1 x running buffer.

5 and 10µg samples of rRNA were resolved simultaneously in order to provide size markers.

7.1.2. Transfer of RNA from Agarose Gels to:

(a) Nitrocellulose Filters.

RNA was transfered from agarose gels to nitrocellulose filters essentially as described by Thomas, (1980). Whatman 3MM paper, saturated with 20 x SSC, was laid over a glass plate such that two of its edges were dipping into reservoirs of 20 x SSC.

After electrophoresis of RNA (Section 7.1.1.) the gel was placed on the saturated 3MM paper, and cling-film placed round all four edges. A nitrocellulose filter, presoaked in 20 x SSC was then carefully laid over the gel so that no air was trapped between the gel and the filter. This was in turn overlaid with a piece of dry 3MM paper, a 6cm thickness of paper towels, and a glass plate. A 1kg weight was then placed on top of this and the assembly left overnight.

The nitrocellulose filter was then recovered, rinsed thoroughly in 2 x SSC in order to remove any contaminating agarose, air dried, and baked between two sheets of 3MM paper, at 80^{o}C for 2 hours, in a vacuum oven.

(b) Biodyne A membranes. (Pall filtration processes)

The procedure for the transfer of RNA from agarose gels to Biodyne A membranes was essentially the same as that described for transfer to nitrocellulose filters except for:

(i) Biodyne A membrane is hydrophilic and therefore does not require pre-wetting.

(ii) The membrane must not be washed after RNA transfer and before baking.

(iii) The membrane can be baked at 80°C for 1 hour in an ordinary air-circulating oven.

7.7.3 Positioning of rRNA Size Markers

rRNA samples were electrophoresed, in the last two tracks of all RNA gels, and blotted with the other samples onto either nitrocellulose filters, or Biodyne A membranes. After baking, the section of filter or membrane containing these samples was cut off and stained in 0.1% (w/v) Toluidine Blue (Sigma) in 10mM Tris-HCl, pH8 for 10-20 minutes. The filters were then destained in water until the 28S and 18S rRNA bands were clearly visible. The distance of these from the origin was noted and used later to estimate the size of specific mRNAs on the filters. This also provided a check that the RNA had actually blotted.

7.2. RNA Dot Blotting

Nitrocellulose filters were prepared as described for plasmid dot-blots (Section 6.5).

If xµg of polyA+ RNA was to be blotted then 2 x xµg were resuspended in sterile water in an eppendorf tube such that the

concentration was xμg/4μl. Five serial dilutions of this were then made. The RNA was then denatured by heating it to 65°C for 15 minutes and cooling it rapidly on ice. The condensation was then collected by centrifugation for 15-20secs. in an eppendorf centrifuge. 2 x 2μl aliquots of each dilution were then spotted onto the nitrocellulose such that one row of a filter consisted of: x, 1/2x, 1/4x, 1/8x, 1/16x, 1/32x. The filters were then air-dried and baked, in a vacuum oven, at 80°C for 2 hours and stored in an air-tight plastic box until required.

7.3. Hybridisation Procedures for Nitrocellulose Filters

7.3.1 Prehybridisation

Nitrocellulose filters were prehybridised as described in Section 6.4.2 except that the prehybridisation buffer was degassed under vacuum, the filters placed in a plastic box with enough degassed buffer to cover them, the box then placed in a desiccator, attached to a vacuum pump, and left until no air bubbles could be seen on the surface of the filter. Filters were then placed, singly, in plastic bags with 8ml prehybridisation buffer, as much air as possible removed from the bag, and the bag sealed and, as before, placed in a shaking water bath at 42°C overnight.

7.3.2 Hybridisation

Nitrocellulose filters were hybridised in 5ml hybridisation buffer, in this case prehybridisation buffer containing $0.5x10^6$ - $1x10^6$ cpm/ml of denatured, nick-translated, DNA. Hybridisation was carried out in plastic bags in a shaking water bath at 42°C for 24-48 hours.

7.3.3. Washing and Autoradiography

Nitrocellulose filters were washed and autoradiographed as described in Sections 6.4.4. and 6.4.5.

7.4. Hybridisation Procedures for Biodyne A Membranes

7.4.1. Prehybridisation

Due to the hydrophilic nature of Biodyne A membranes it was not necessary to subject them to the rigorous deaerating procedures used for nitrocellulose filters. Instead, Biodyne A membranes were sealed in plastic bags with 8ml of degassed prehybridisation buffer B, a solution containing 50% (v/v) deionised formamide, 5xDenhardts solution, 4xSET (20 x SET = 3M NaCl, 20mM EDTA, 0.4M Tris-HCl, pH7.8), 0.1% (w/v) sodium dodecyl sulphate, 10µg/ml poly A, 10µg/ml poly C, 100µg/ml denatured salmon sperm DNA. The bags were then placed in a water-filled plastic box at 42^{o}C, in a shaking water bath, overnight.

7.4.2. Hybridisation

Hybridisation to Biodyne A membranes was as described for nitrocellulose filters (Section 7.3.2) except that prehybridisation buffer B was used.

7.4.3 Washing and Autoradiography

At no point during the washing and autoradiography procedures were the membranes allowed to dry.

After hybridisation, hybridisation buffer was carefully poured off and the membranes rinsed in low stringency wash buffer (2xSSC, 0.1% (w/v) sodium dodecyl sulphate). The membranes were then placed in flasks containing low stringency wash buffer. (2.5ml/cm^2 of membrane) and agitated vigorously (200rpm) for 5 minutes at room temperature. The wash buffer was then discarded and this step repeated twice more. The membranes were then transferred to plastic boxes containing high stringency wash buffer (2.5ml/cm^2 of membrane) and washed for 2x15 minutes at 50°C in a shaking water bath. The membranes were then sealed, damp, in plastic bags and autoradiographed as described in Section 6.4.5.

7.5 Rehybridisation of Biodyne A Membranes

A procedure for the removal of probe from Biodyne A membrane was obtained which allowed the membrane to be rehybridised and therefore give more efficient use of RNA.

Biodyne A membranes were removed from the bags in which they had been sealed, damp, for autoradiography, and placed in a second plastic bag containing unhybridisation buffer (10mM sodium phosphate, pH6.5, 50% (v/v) deionised formamide), 1ml/cm^2 membrane, and the bag incubated at 65°C for 1 hour in a shaking water bath.

The membrane was then placed in a flask containing low stringency wash buffer, 2.5ml/cm^2 membrane, and the flask shaken vigorously (200rpm) for 15 minutes at room temperature. The membrane was then ready to prehybridise and hybridise to the new probe as described in Sections 7.4.

RESULTS

1. Extraction of PolyA+RNA from the Immature Rat Uterus

1.1 Introduction

Uterine polyA+RNA was required for:

(i) the construction of a cDNA library.

(ii) the synthesis of single-stranded cDNA probes with which to screen the library.

(iii) use in northern and RNA dot blotting.

All of the procedures require intact, biologically active, poly A+RNA.

The immature rat uterus is a smooth muscle tissue whose size and shape make it difficult to homogenise efficiently using a conventional glass/teflon homogeniser. This, together with the fact that it has a high endogenous level of ribonuclease (Greenman and Kenney, 1964), make it a difficult tissue from which to obtain intact mRNA. A number of isolation procedures were therefore tried in order to optimize the yield of intact, biologically active polyA+RNA.

The difficulty in homogenisation was decreased by immediately freezing excised uteri and grinding them to a fine powder, in liquid nitrogen, in a mortar and pestle before using them for the extraction of RNA.

RNA was isolated from the uteri of immature rats which had been exposed to oestradiol for 4 hours before death, by the four procedures

described in materials and methods. Rats that had been given oestradiol 4 hours before death were used to test the different isolation procedures as this time corresponds to the first peak of oestrogen-induced RNA synthesis of 7-10 fold control levels (Waters and Knowler, 1981).

The integrity of the RNAs obtained was assessed by northern blotting samples of polyA+RNA from each preparation onto nitrocellulose filter paper, and hybridising these to nick-translated plasmid p749, a rat skeletal muscle actin cDNA clone in plasmid pBR322 (a gift from Dr D.P. Leader). An actin clone was chosen as, the uterus being a smooth muscle tissue, it was thought that actin mRNA would be relatively abundant. This is supported by the polypeptide profile of in vitro translation of uterine mRNA (Beaumont and Knowler, 1983). The intactness of the mRNA preparations was assessed by examination of the resulting autoradiographs. Tight bands representing actin mRNA indicated that the RNA was intact, whereas streaking below the bands indicated degradation of the message. The extent of degradation of actin mRNA was taken as indicative of that of the total mRNA population.

The biological activity of the various RNA preparations was also assessed by the ability of the polyA+ fraction obtained from them to function as a template for the synthesis of single-stranded cDNA. The conditions used were as described in materials and methods 6.1, except that 10μCi of α-^{32}P-dATP (3000Ci/mmole) and 20μM dATP were used. The single-stranded cDNA's synthesised in these reactions were resolved on alkaline-agarose gels (materials and methods 6.2) and the gels autoradiographed in order to determine the length of the transcripts obtained. As the polyA+RNA was to be used to construct a cDNA library, the ability of a preparation to give long transcripts was of importance.

The following methods for the preparation of RNA were compared:

(1) Extraction of RNA from polysomes (materials and methods 3.1). Polysomes were purified by centrifugation through sucrose (Merryweather and Knowler, 1980). Poly A+ RNA was obtained by extraction of the polysomes with phenol and precipitation of the RNA followed by Oligo (dT) cellulose chromatography (materials and methods 3.3).

The main advantages of this procedure over the others tried were that it was quick, did not require much manipulation of the RNA, and cellular DNA was removed, easily, at an early stage in the procedure.

The major disadvantages were that:

(i) the tissue, even when ground to a powder, required homogenisation in a glass/teflon homogeniser, during which the polysomes were susceptible to attack by endogenous ribonucleases.

(ii) only mRNAs which were being translated at the time of homogenisation were obtained.

(2) Using guanidinium thiocyanate and lithium chloride (materials and methods 3.2.1.)

This method attempted to avoid degradation of RNA by homogenising the tissue in a buffer designed to cause denaturation of all cellular proteins, including RNase. The buffer used contained 5M guanidinium thiocyanate, a salt in which both the anion and cation are chaotropic agents. It also contained β-mercaptoethanol, which breaks the disulphide

bonds necessary for RNase activity. The half-life of RNase in this buffer is estimated to be less than 5 secs. (Von Hippel and Wang, 1964), and the extent of degradation of RNA in the early stage of this extraction procedure was, therefore, dependent on the speed with which the tissue could be efficiently homogenised.

RNA was recovered from the homogenate by selective precipitation with 4M LiCl (Auffrey and Rougeon, 1980). This step involved leaving the RNA at $4^{o}C$ for long periods during which time any contaminating RNase could have its effect.

(3) Using guanidinium thiocyanate and caesium chloride (materials and methods 3.2.2).

This method is similar to that described above in that it again involved homogenisation in 5M guanidium thiocyanate, β-mercaptoethanol and, in this case, a detergent, and was therefore also dependent on the speed and efficiency with which the tissue was homogenised. The RNA was then purified by centrifugation through a high density caesium chloride cushion (Glisin et al., 1974). The protein, in this system, formed a pellicle at the top of the tube, the DNA banded at the interface. Both of these could then be carefully removed before recovering the RNA pellet.

(4) Using hot phenol/chloroform, sodium dodecyl sulphate and caesium chloride (materials and methods 3.2.3.)

The fourth method again involved trying to denature all cellular proteins, including RNase, this time using hot phenol/chloroform and

sodium dodecyl sulphate. The method was suggested by Dr D.P. Leader who had found it the most effective in extracting RNA from mouse skeletal muscle. Uteri were ground to a fine powder in liquid nitrogen using a mortar and pestle, as the only form of homogenisation. The powder was then added to an ice-cold mixture of phenol/chloroform/sodium dodecyl sulphate buffer and this then plunged into a 60°C water bath and shaken vigorously for 3 minutes. The mixture was further agitated during this and subsequent extractions by drawing it up and down in a pasteur pipette. After precipitation of the extracted nucleic acids, the RNA was purified by centrifugation through a high density caesium chloride cushion (Glisin et al, 1974).

1.2 Comparison of Extraction Procedures

The extraction of RNA from uterine polysomes was attempted first as this was the preferred method in the laboratory at that time. A sample of the RNA obtained was resolved in a 2% agarose gel (materials and methods 3.5) and appeared to be of reasonable quality; tight bands representing rRNA and a smear representing mRNA. However, when the mRNA was used as a template for the synthesis of single-stranded cDNA very little incorporation of labelled nucleotide was observed. This was not due to the method, or enzyme preparation, used for the synthesis as this was tested with a sample of T-cell mRNA, also obtained from polysomes. In identical reactions using uterine and T-cell mRNA, and 10uCi α-^{32}P- dATP, 20μM dATP, 2.66×10^4 cpm and 1.4×10^6 cpm were incorporated into the respective cDNAs. This implied that the fault was with the uterine mRNA preparation and prompted the investigation of the alternative procedures for the extraction of uterine mRNA.

As can be seen from Table 5 no procedure had a major advantage over the others as regards yield of RNA, each gave approximately 40µg RNA/uterus. The other three parameters tested, however, showed that although the poly A+RNA from polysomes was the most intact, it did not reverse transcribe as well as that obtained using the modified Kirby procedure and that, overall, the later method was best suited to extracting RNA from the immature rat uterus. This method was therefore used for all subsequent RNA preparations.

2.1 Strategy for the Construction of a Uterine cDNA Library

12µg of poly A+RNA isolated from the uteri of immature female rats, which had been exposed to oestradiol for 4 hours, was used to construct a cDNA library as described in materials and methods 5. The general scheme followed is shown in Fig.5

The cloning vector used was pUC8 (Vierra and Messing, 1982), a plasmid containing the ampicillin resistance gene and a multiple cloning site (MCS) in the correct reading frame of the lac operon DNA to allow the expression of a functional β-galactosidase. A physical map of pUC8 is given in Fig.6. Cloning into any of the sites on the MCS linker results in loss of a functional β-galactosidase and failure of the host cells to metabolise X-gal, a chromogenic substrate for β-galactosidase. Recombinants can therefore be selected by growing the transformed host cells on L-agar plates containing X-gal and ampicillin; cells containing plasmids with inserts will yield white colonies while those without inserts give blue colonies (Gronenbom and Messing, 1978).

Table 5

Comparison of rat uterine RNA extraction procedures

Groups of 15-20, 21 day old female rats, weighing 30-35g, received 1μg of oestradiol-17β in saline by intraperitoneal injection 4 hours before death. RNA was then prepared from the uteri by:

1) isolation from polysomes (materials and methods 3.1)
2) guanidinium thiocyanate and lithium chloride method (materials and methods 3.2.1)
3) guanidinium thiocyanate and caesium chloride method (materials and methods 3.2.2)
4) phenol/chloroform, sodium dodecylsulphate and caesium chloride method (materials and methods 3.2.3)

The yield of RNA was taken as the mean value obtained from at least four different preparations in each group.

The integrity of the mRNA from each preparation was assessed as described in Results 1.2.

The viability of the mRNA from each preparation was assessed by measuring the incorporation of α-^{32}-dATP into single-stranded cDNA, by Cerenkov counting, using a sample from each preparation as template. The reaction conditions were as described in materials and methods, except that 10uCi α-^{32}P-dATP and 20μM dATP were used.

The sizes of the single-stranded cDNAs obtained were estimated by resolving a 20,000 cpm aliquot from each preparation on an alkaline agarose gel (materials and methods 6.2) using ^{32}P end-labelled markers, and autoradiography.

Method of RNA preparation.	Yield of RNA (µg/rat uterus)	Integrity of mRNA (% of hybridisation as discreet bands)	Viability of mRNA: a) cpm α-^{32}PdATP incorporated into single-straned cDNA.	b) size range of single-standed cDNAobtained.
1	37.0	93.7	47,200	400-1300 nucleotides
2	42.6	59.8	26,600	400-1400 "
3	40.2	44.1	30,100	200-900 "
4	46.6	65.9	80,000	300-1400 "
T-cell mRNA	-	-	388,000	350-1600 "

Figure 5

Scheme for the construction of a cDNA library

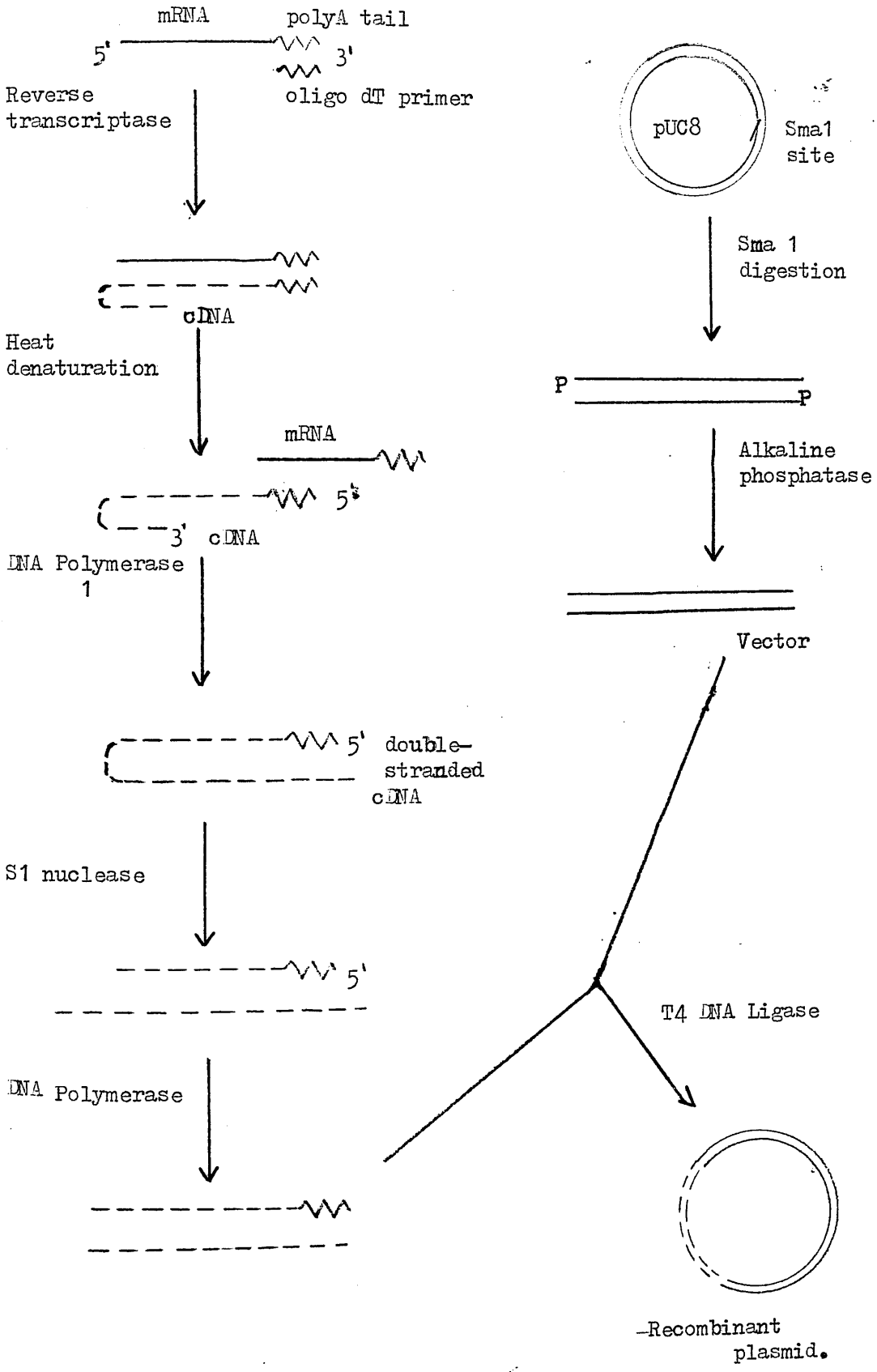
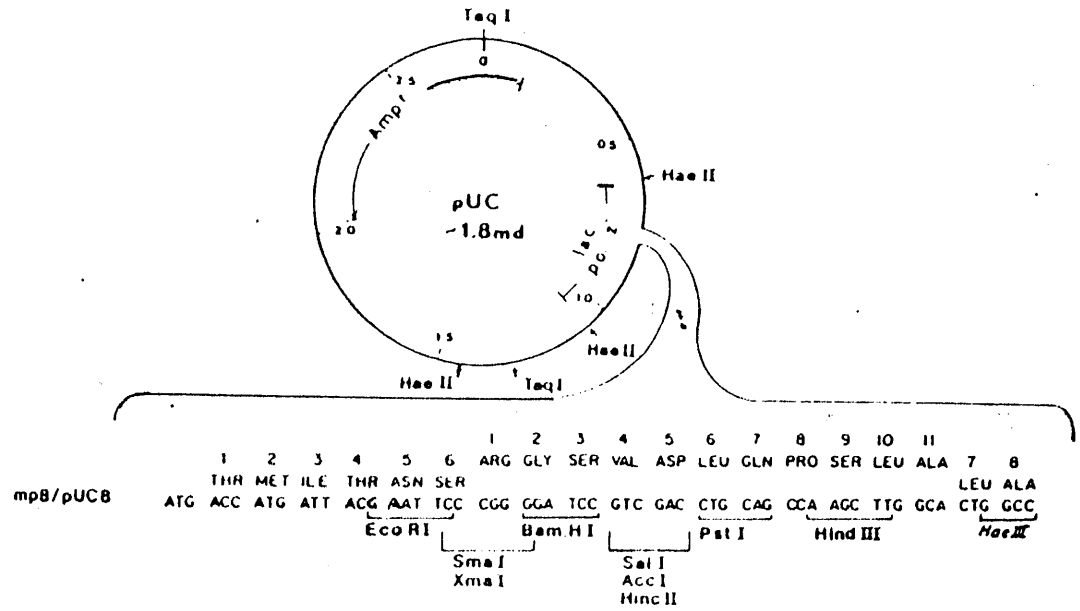

mRNA
polyA tail
5'
3'
oligo dT primer
Reverse transcriptase
cDNA
Heat denaturation
mRNA
5'
3'
cDNA
DNA Polymerase 1
5'
double-stranded cDNA
S1 nuclease
5'
DNA Polymerase
pUC8
Sma1 site
Sma 1 digestion
P
P
Alkaline phosphatase
Vector
T4 DNA Ligase
Recombinant plasmid.

Figure 6

A genetic map of the pUC8 plasmid


Taq I
0
25
Amp r
05
Hae II
pUC
-1.8md
20
lac Z
10
15
Hae II
Taq I
Hae II
1 2 3 4 5 6 1 2 3 4 5 6 7 8 9 10 11 7 8
THR MET ILE THR ASN SER ARG GLY SER VAL ASP LEU GLN PRO SER LEU ALA LEU ALA
mp8/pUC8
ATG ACC ATG ATT ACG AAT TCC CGG GGA TCC GTC GAC CTG CAG CCA AGC TTG GCA CTG GCC
EcoRI
BamHI
PstI
HindIII
HaeIII
SmaI
XmaI
SalI
AccI
HincII

2.2 Result of Cloning

The double-stranded cDNA, synthesised using the 4 hour oestrogen induced mRNA was cloned into the SmaI site of plasmid pUC8 and the resulting recombinants used to transform E.Coli K12 strain JM83 cells (Messing, 1979) to ampicillin resistance. A fraction of the transformation mixture was plated out on L-agar plates, containing 0.001% X-gal and 100µg/ml ampicillin, and, after incubating overnight at 37°C, resulted in approximately 650 white colonies. These were picked and transferred to micro-titre plates containing rich L-broth (Gergen et al., 1979) made 100µg/ml with respect to ampicillin, grown overnight at 37°C, and the plates stored at -20°C until required. The recombinants in the remainder of the transformation mixture, approximately 2000, were amplified and stored in rich L-Broth containing 100µg/ml ampicillin at -70°C.

A random sample of colonies were then picked from the micro-titre plates and their plasmids isolated by the plasmid mini-prep (materials and methods 4.1.1). Aliquots of these plasmid preparations were then resolved on a 1% agarose gel (materials and methods 4.2) and the gel photographed under UV illumination. The majority of the plasmids isolated were found to run slower, and therefore were larger, than intact pUC8 (Fig.7). That is, they consisted of pUC8 plus a fragment of double-stranded cDNA and were therefore true recombinant molecules.

3. Screening the cDNA Library

The main purpose in constructing a cDNA library was to isolate

Figure 7

Analysis of recombinant plasmids from the rat uterine cDNA library

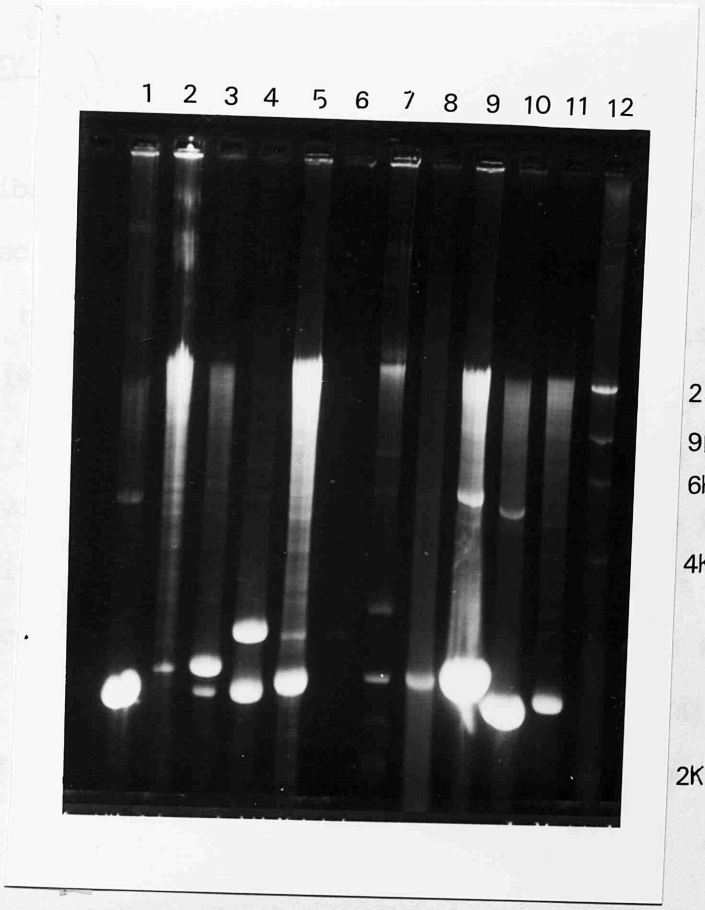
Plasmids were isolated from recombinant colonies by the plasmid mini-prep procedure (materials and methods 4.1.1). Samples of these were then resolved on 1% agarose gel (materials and methods 4.2), with intact pUC8, the plasmid vector used to construct the library.

Track

1 – pUC8

2 – 11 random recombinants

12 – Hind III digested λDNA


1 2 3 4 5 6 7 8 9 10 11 12
23Kb
9Kb
6Kb
4Kb
2Kb

clones of oestrogen responsive mRNAs for further study. These clones could be identified by screening the recombinants with cDNA probes made to poly A+ RNA populations from control (unstimulated) and 4 hour oestrogen stimulated immature rat uteri. Recombinants representing mRNAs regulated by oestrogen within this 4 hour period should bind one or other of the probes preferentially.

3.1 Primary Screen

The library was initially screened by a technique which, although not very discriminating, can be readily applied to a large number of clones. The technique of colony hybridisation was devised by Grunstein and Hogness (1975), and a modification by Humphries et al., (1978) was used to screen the complete library. The procedure, described in materials and methods 6, involved transferring the recombinant colonies from micro-titre plates onto nitrocellulose filters where their growth was continued. The plasmid DNA content of the bacteria was then amplified by treating the filters with chloramphenicol, after which the bacterial colonies were lysed and their DNA fixed to the filters by baking in vacuo at 80^{o}C for 2 hours.

The recombinant DNA, immobilised on nitrocellulose filters was then hybridised to a ^{32}P-labelled cDNA probe. After hybridisation the filters were washed and autoradiographed. The resulting autoradiographs show which recombinants are complementary to sequences represented in the mRNA population used to make the probe. The extent of hybridisation to a given recombinant should also reflect the abundance of its complementary message in the mRNA population used to synthesise the probe. By using two probes,

synthesised from different mRNA populations, recombinants representing messenger sequences which differ in their relative abundance in each mRNA population can be isolated.

The original method employed to lyse the bacterial colonies did not involve proteinase K treatment, but simply involved lysing the bacteria with alkali, neutralisation, and washing the filters, before fixing the DNA and hybridisation. This led to clear patches on the autoradiographs where the colonies had been and, in some cases, these were surrounded by 'haloes' of exposed film (Fig.8A). This suggested that:

(1) the bacteria were not being completely lysed in the centre of the colonies. Thus, washing removed these centre portions leaving only a halo of DNA fixed to the filter.

(ii) the bacterial debris resulting from cell lysis was not being efficiently removed from the filter by the 2 x SSC wash and was masking the plasmid DNA on the filter from exposure to the probe.

A series of recombinants were selected with which to resolve these problems (Fig-8). They included two recombinants which, on the basis of a preliminary screen, hybridised strongly to the 4 hour oestrogen stimulated cDNA population. They also included two clones which did not appear to hybridise, and a control colony containing only intact pUC8.

Growing the colonies for a shorter time so that they were smaller, and varying the lysing procedure using lysozyme and/or detergent treatments for various durations did improve the results slightly.

Figure 8

Comparison of colony lysing procedures

Four recombinants were selected on the basis of a primary screen because they bound (F2 and D7), or did not bind (E3 and H6), cDNA to 4 hour oestrogen-induced mRNA. They, and a control colony containing intact pUC8, were grown on two nitrocellulose filters (materials and methods 6.3). The filters were then placed, colony side up, on 3MM paper saturated with:

Filter A: 0.5M NaOH, for 10 minutes

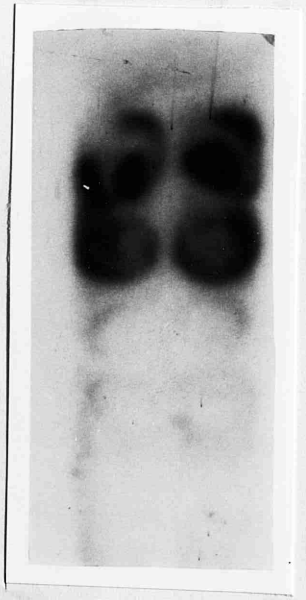
1M Tris-HCl, pH 7.4, for 10 minutes

1.5M NaCl, 0.5M Tris-HCl, pH 7.4, for 10 minutes

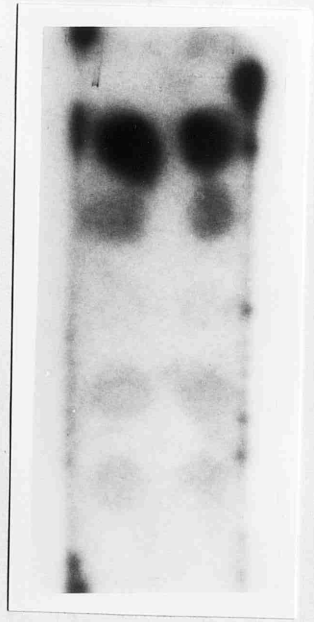
Filter B: as for filter A plus 1 hour in 5ml of proteinase K solution (1mg/ml in 2xSSC)

The filters were then baked, prewashed, prehybridised and then hybridised to $1x10^6$cpm of cDNA to 4 hour oestrogen-induced mRNA (materials and methods 6.5). The filters were then washed and exposed to Kodak X-omat S X-ray film, using an intensifier screen, for 36 hours.

A.



B.



Recombinant code.	Hybridisation to 4hour oestrogen cDNA.
F2	+ve
D7	
A1 (pUC8)	control
E3	-ve
H6	

However, when a proteinase K digestion step was introduced, complete discs as opposed to 'haloes' were seen (Fig. 8B). This implied that the problem had been caused by a masking of the DNA by bacterial debris. All filters thereafter were treated with proteinase K.

Another major problem encountered during the screening of the library by this method was the amount of background binding of the probe to the nitrocellulose filter. This could be negligible on one filter and high on the duplicate filter, and there appeared to be no way of predicting which filters would give the high background. As the duplicate filters were treated in the same way, at the same time, using the same solutions and probes, the fault appeared to be in the actual filters.

Various washing procedures, before and after hybridisation, had little effect. A number of screens, using different filters, of each plate were carried out to try and overcome this problem. A method for drastically reducing the amount of background binding to nitrocellulose filters was devised (materials and methods 7.3.1), which involved submerging the filters in degassed prehybridisation buffer and then further deaerating the filters themselves. Apparently the fault arose when air trapped within the nitrocellulose filter came to the surface when the filter was placed at $42^{\circ}C$ and thus prevented efficient prehybridisation. Unfortunately, this problem was not resolved until after the initial screening of the library.

The duplication between filters, even on 'clean' filters, was not very good and was the result of differing efficiencies at a number of steps in the method. That is, duplicate colonies could grow to different

extents on different nitrocellulose filters and, as a result, were more or less susceptible to the effect of chloramphenicol, the lysing procedure, and the proteinase K treatment.

The effectiveness of the colony hybridisation method is dependent, to a large extent, on the specificity of the probes used. On the basis of previous Rot curve analysis (Aziz et al., 1979) and in vitro translations (Beaumont and Knowler, 1983), it was reasonable to hope that the cDNA library would contain species complementary to mRNAs that were moderately increased or decreased in abundance by oestrogen. It was much less likely however that species would be detected that changed in abundance so dramatically as to be undetected in one or other mRNA populations. Thus the changes induced by oestrogen could be masked by the poor duplication between filters. This was clearly not satisfactory. However, as no other methods were available for screening large numbers of recombinants, the poor discrimination could only be compensated for by repetition of screening. After careful comparison of the resulting autoradiographs, a few probable and a larger number of possible positives were identified (Fig. 9).

3.2 Secondary Screening of the cDNA Library

The low sensitivity and lack of reliability of the colony hybridisation technique described above, meant that the selected colonies had to be rescreened by more sensitive techniques.

About 160 colonies picked for further analysis. Plasmid mini-preps (materials and methods 4.1.1) were made from all the colonies of

Figure 9

Screening of the uterine cDNA library by colony hybridisation

Recombinant colonies were transferred from 96-well micro-titre plates and grown on nitrocellulose filters, lysed and the DNA 'fixed' to the filters by baking (materials and methods 6.3, 6.4). The filters were prehybridised and hybridised to reverse transcribed mRNA from unstimulated, or 4 hour oestradiol-treated, rat uteri. The filters were washed and exposed for 1 week to Kodak X-omat S X-ray film, using an intensifier screen (materials and methods 6.5).

Symbols on figure:

Film I: Colony hybridisation of plate 2 to 4 hour oestrogen-induced cDNA

Film II: Colony hybridisation of plate 2 to control cDNA

Film III: Colony hybridisation of plate 4 to 4 hour oestrogen-induced cDNA.

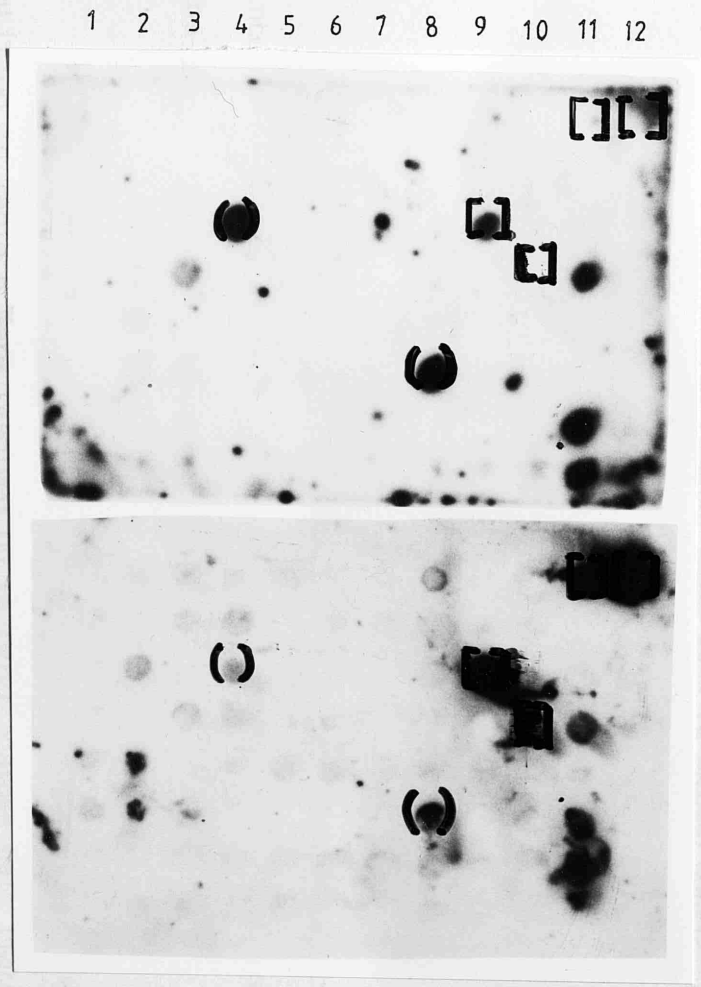
Film IV: Colony hybridisation of plate 4 to control cDNA.

Symbols on overlay:

O - Colonies which preferentially bind 4 hour cDNA

[] - Colonies which preferentially bind control cDNA

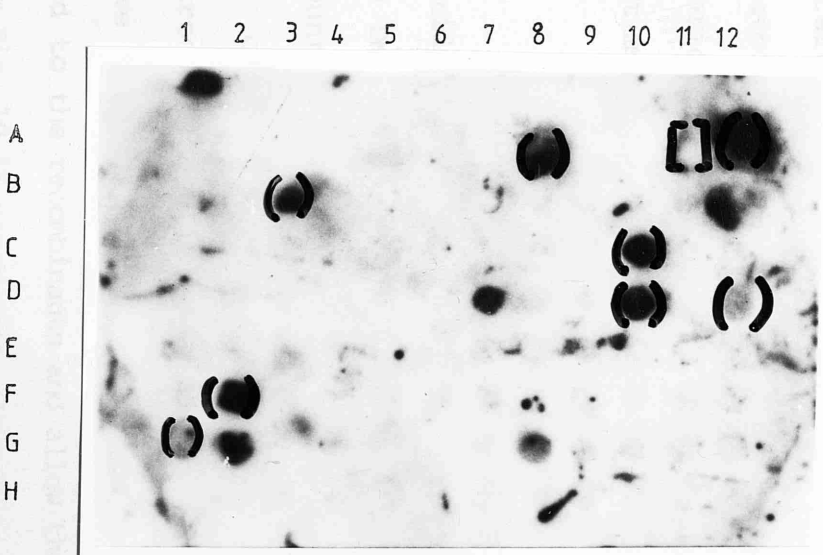
I.


1 2 3 4 5 6 7 8 9 10 11 12
A
B
C
D
E
F
G
H

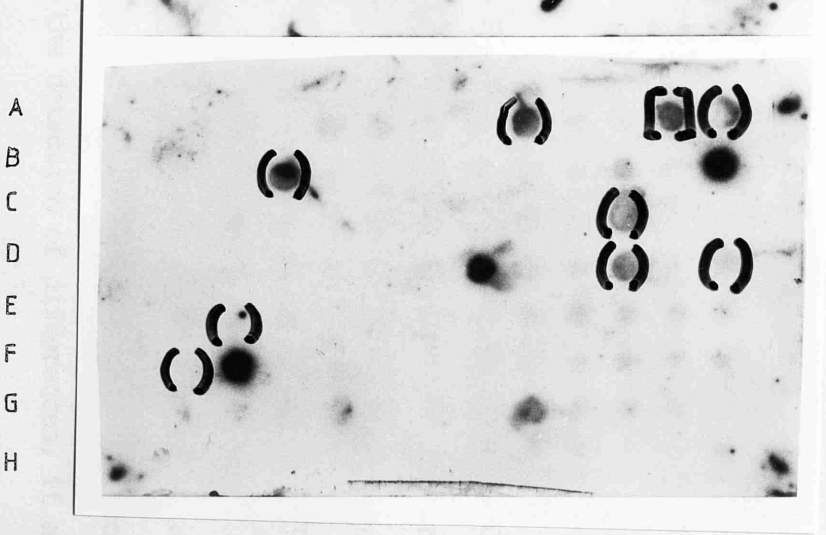
II.

A
B
C
D
E
F
G
H

III.


1 2 3 4 5 6 7 8 9 10 11 12
A
B
C
D
E
F
G
H

IV.


A
B
C
D
E
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G
H

interest. Aliquots of these were spotted, in duplicate, onto two nitrocellulose filters ruled into 1cm squares. These filters were then hybridised to ^{32}P-labelled cDNA probes made by reverse transcribing unstimulated, and 4 hour oestrogen-stimulated, mRNA populations (Fig. 10).

This method avoids most of the problems besetting colony hybridisations; that is there are no other macromolecules present to mask the DNA from the probe, and duplication is much better since the same amount of the same solution is used. Also the duplicates within a screen are on the same filter thereby avoiding differences between the nitrocellulose filters. There is also much more plasmid DNA available to drive the hybridisation, therefore a larger amount of the probe should bind to the recombinants and allow the detection of differences, if any. From Fig. 10 it can be seen that:

(1) the background binding is much lower than in colony hybridisation.

(2) the duplication is very good.

(3) a number of recombinants bind the probes differentially.

Table 6 presents the relative intensity of 12 selected recombinants of interest. Their identification codes are derived from their positions on the culture plate as shown on Fig. 10.

The remaining aliquots of the plasmid mini-preps of the plasmids identified in Table 6 were resolved on 1% (w/v) agarose gels (materials and methods 4.2) and the relative size of the plasmids, and therefore of their inserts, estimated (Fig. 11).

Figure 10

Secondary Screening of the Uterine cDNA Library

Recombinant plasmids were isolated by the mini-prep procedure described in materials and methods 4.1.1. They were then dotted onto nitrocellulose filters and hybridised to:

a) reverse transcribed mRNA from unstimulated rat uteri; Filters I and III.

b) reverse transcribed mRNA from 4 hour oestradiol-treated rat uteri; Filters II and IV.

The filters were then washed and autoradiographed for 3 days using an intensifier screen.

Intact pUC8 was used as a control: Position H4 on filters III and IV.

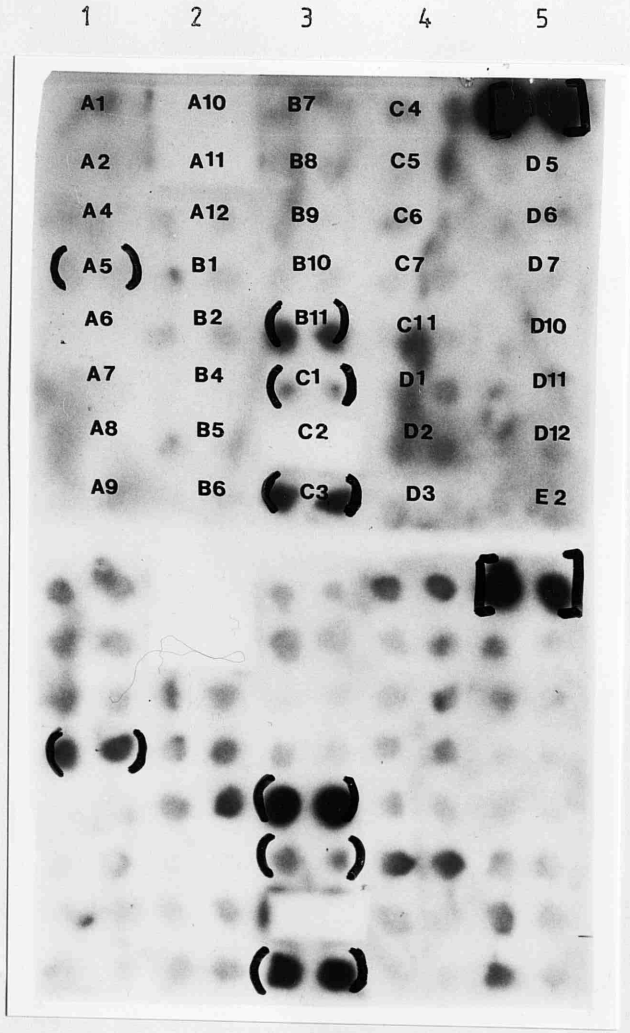
Symbols on Overlay:

[] - plasmids preferentially binding control cDNA.

O - plasmids preferentially binding 4 hour oestrogen-induced cDNA

The letters and numbers represent the position in the original micro-titre plate from which the recombinant was selected (plasmid code name). Filters I and II have the same plasmids in the same positions as do filters III and IV

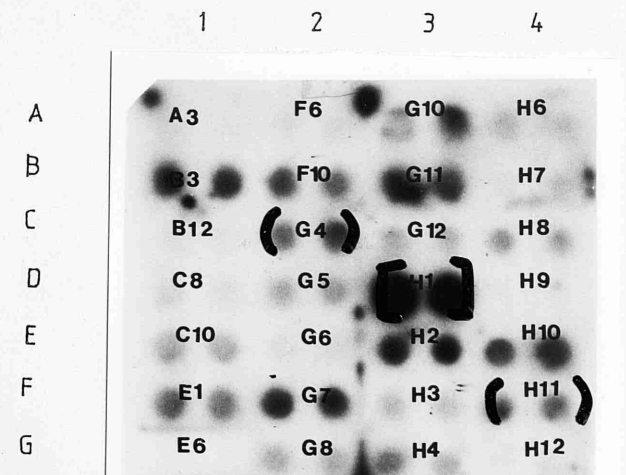
I.


1 2 3 4 5
A A1 A10 B7 C4
B A2 A11 B8 C5 D5
C A4 A12 B9 C6 D6
D A5 B1 B10 C7 D7
E A6 B2 B11 C11 D10
F A7 B4 C1 D1 D11
G A8 B5 C2 D2 D12
H A9 B6 C3 D3 E2

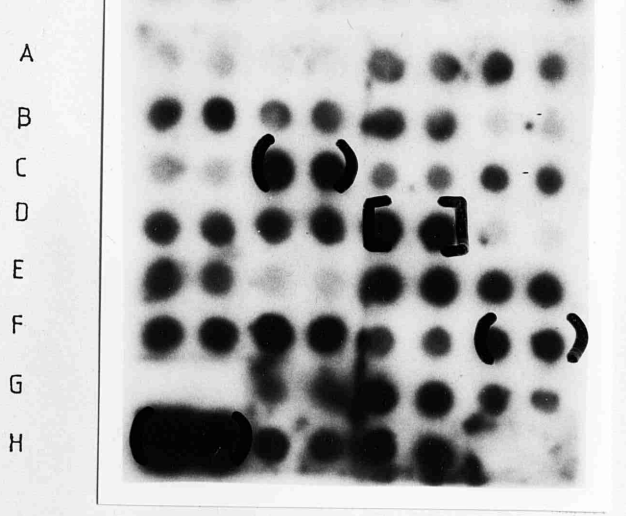
II.

A
B
C
D
E
F
G
H

III.


1 2 3 4
A A3 F6 G10 H6
B B3 F10 G11 H7
C B12 G4 G12 H8
D C8 G5 H1 H9
E C10 G6 H2 H10
F E1 G7 H3 H11
G E6 G8 H4 H12
H E10 G9 H5

IV.


A
B
C
D
E
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G
H

Table 6

The relative differences in the extent of hybridisation of twelve recombinant plasmids to control and four hour oestrogen-stimulated cDNAs

The plasmids are named according to their positions in the original micro-titre plates (see Fig.10). The relative intensities of hybridisation (subjectively determined) were estimated from the autoradiographs of the secondary screen of the library (Fig. 10).

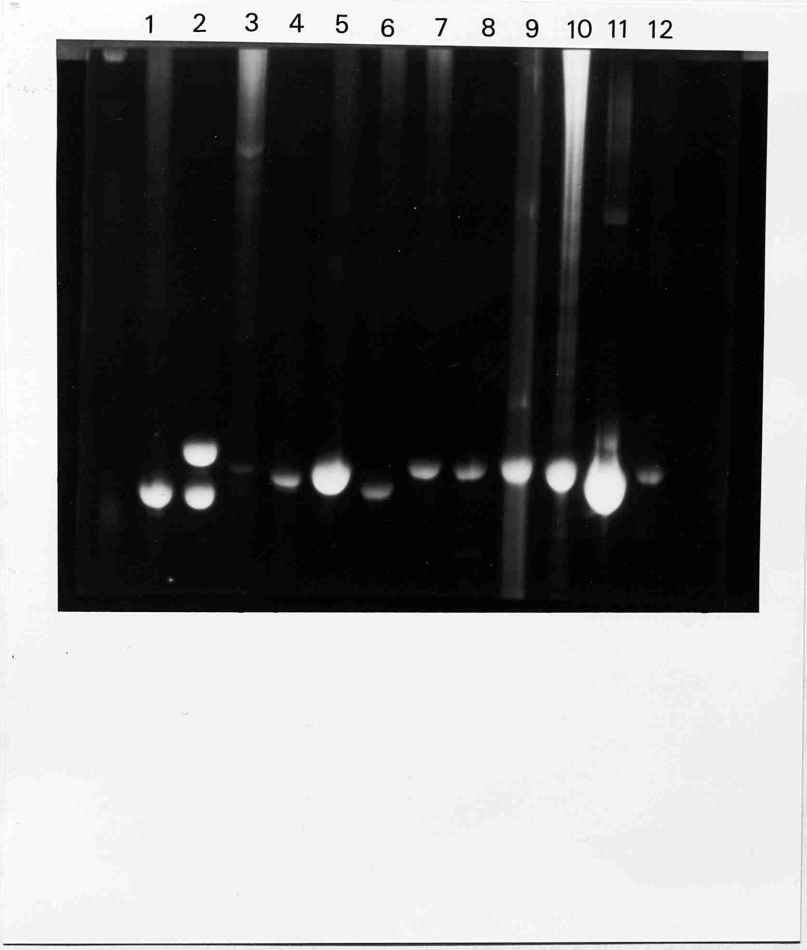
Plasmid	Relative Extent of Hybridisation to: a) control cDNA	b) 4hour oestrogen-stimulated cDNA
B11	++	++++
E10	+++	+++++
F4	++	++++
A5	+	+++
D4	++++	++
C1	+	++
G4	++	++++
B2F	+	+++
B2H	+	+++
C3	+	+++
H1	++++	++
H11	+	+++

Figure 11

Estimation of Relative sizes of the 12 Selected Recombinant Plasmids

Aliquots of mini-preps of the 12 plasmids were resolved, with Hind III digested λ DNA and pUC8, on a 1% agarose gel (materials and methods 4.2). The sizes of the inserts were then calculated from digests of the plasmids using the Hind III λ fragments and pUC8 as markers.

Track No.	Plasmid Code	Estimated Size
1	pUC8	380
2	G4	230
3	H1	216
4	D4	199
5	B2H	162
6	C1	280
7	A5	295
8	B2F	347
9	F4	260
10	C3	200
11	H11	235
12	E10	


1 2 3 4 5 6 7 8 9 10 11 12
23Kb
9Kb
6Kb
4Kb
2Kb

For further analysis and preservation, the clones of interest were picked from the micro-titre plate wells and grown, in 5ml of rich L-broth containing 100 µg/ml ampicillin, at 37°C overnight in an orbital incubator. The cultures were then divided into 1ml aliquots and stored frozen at -20°C.

The plasmids from these clones were then isolated using the plasmid midi-prep procedure (materials and methods 4.1.2) and these also stored, frozen in water at 1mg/ml, at -20°C.

The response of some of these clones to oestrogen and antioestrogen in both the immature rat uterus and MCF-7 human breast cancer cell line was studied and is discussed later.

4. The Effect of Oestrogen and Tamoxifen on RNA Levels in the Immature Rat Uterus

The effect of oestrogen and tamoxifen on the levels of expression of specific messenger sequences encoded in the DNA of the clones isolated from the cDNA library, or obtained from other laboratories, were studied using the techniques of northern and RNA dot blotting. This required the extraction of RNA from the immature rat uterus at various times after oestrogen and tamoxifen treatment, the selection of mRNA from this, and the quantitation of rRNA contamination of these mRNA preparations.

4.1. Extraction of Uterine RNA from Oestrogen and Tamoxifen Treated immature Rats

The time-courses chosen for oestrogen and tamoxifen treatment, that is, the points at which RNA was extracted from treated rats, were based on

previous work carried out in this laboratory, particularly that of A.P. Waters (see Introduction 5.2). This work showed that administration of oestradiol to immature rats leads to a rapid increase in the synthesis of RNA in the uterus to 7-10 fold control levels after 2-4 hours. This is followed by a smaller increase between 12-24 hours. Administration of tamoxifen resulted in a broad peak of RNA synthesis between 12-36 hours, with a maximum at 24 hours. This difference in the stimulation of RNA synthesis by oestradiol and tamoxifen was thought to be due to:

(1) different serum half-lives of these two compounds, oestradiol having a half-life of a few hours whereas tamoxifen has a half-life of a few days (Adam et al., 1980).

(2) the fact that tamoxifen is slowly metabolised in the liver to its more active monohydroxylated form (Borgna and Rochefort, 1980; Borgna, 1981).

(3) the slower uptake of tamoxifen, compared to that of oestradiol, by uterine nuclei (Makku et al., 1981; Clark et al., 1973).

Also, DNA synthesis and cell division in the uterus occur between 20-28 hours after administration of oestrogen (Katzenellenbegen and Gorski, 1975). Administration of tamoxifen however leads to DNA synthesis between 40-50 hours, but does not induce cell division (Cowan and Leake, 1979; Jordon and Dix, 1980). These facts implied that any difference in the regulation of specific mRNAs by oestradiol and tamoxifen should be found prior to hyperplasia, that is, within 28 hours after oestradiol administration, and within 48 hours of tamoxifen administration. RNA was

therefore extracted from immature rat uteri at 0, 2, 4, 8, 12, 16, 20, 24, 26 and 28 hours after oestradiol treatment, and 0, 6, 12, 18, 24, 30, 36, 42 and 48 hours after tamoxifen treatment, by a modification of the Kirby (1956) procedure (materials and methods 3.2.3.)

The amount of RNA per rat uterus obtained at each of these times, averaged over a number of preparations, is shown in Fig. 12. This shows that both oestradiol and tamoxifen cause an increase in the amount of total RNA extracted from the uterus, but the increase begins very soon after oestradiol treatment and continues until 26 hours, whereas with tamoxifen it does not start to increase until after 12 hours and plateaus after 24 hours. The increased recovery was not due to easier homogenisation of the stimulated tissue as, in all cases, the uteri were ground to a fine powder and remained frozen throughout.

4.2 The Effect of Oestradiol and Tamoxifen on polyA+RNA levels in the Immature Rat Uterus

PolyA+RNA was isolated from total RNA by Oligo (dT) cellulose chromatography (materials and methods 3.3). As mRNA only makes up 1-5% of total cellular RNA, whereas rRNA constitutes greater than 75%, one passage through a column of Oligo (dT) cellulose leaves the mRNA contaminated, to about 50%, with rRNA. Running the preparation through the column again reduces, but does not remove completely, rRNA contamination, and also increases the risk of degradation of the mRNA. It was decided that it was better to avoid repeated passage through oligo (dT) cellulose. Rather, an alternative approach was adopted in which the rRNA contamination of mRNA recovered from one oligo (dT) cellulose fractionation was quantitated and taken into account in subsequent assays.

Figure 12

The Effect of Oestrogen and Tamoxifen on the amount of RNA in the Immature Rat Uterus

Groups of 20-40, 21 day old female rats, weighing 30-35g received:

A. 1µg of oestradiol

B. 30µg of tamoxifen

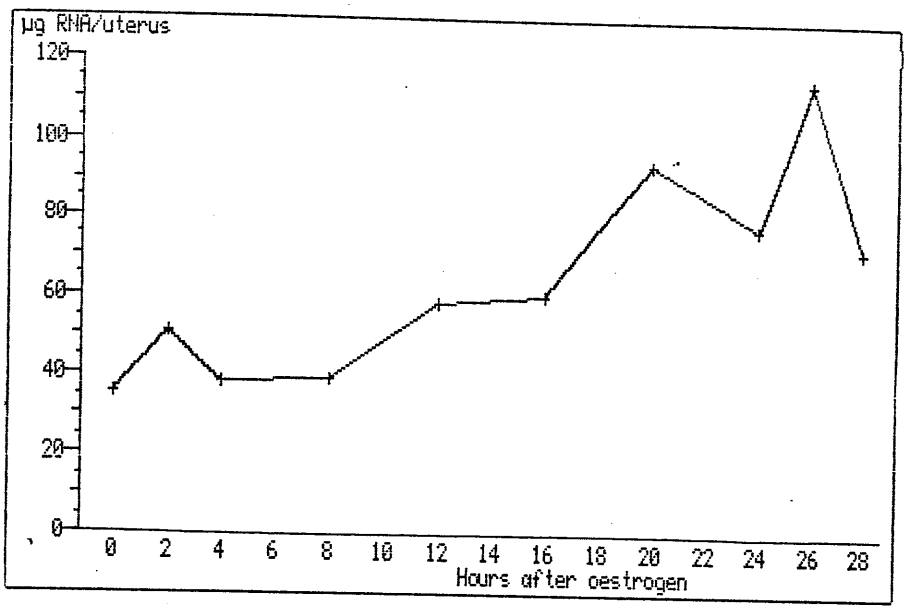
Control animals received carrier alone.

The animals were killed at the times indicated and RNA prepared from the uteri as described in materials and methods 3.2.3.

The amount of RNA obtained per uterus at each time point was averaged over a number of preparations and plotted against duration and type of treatment.

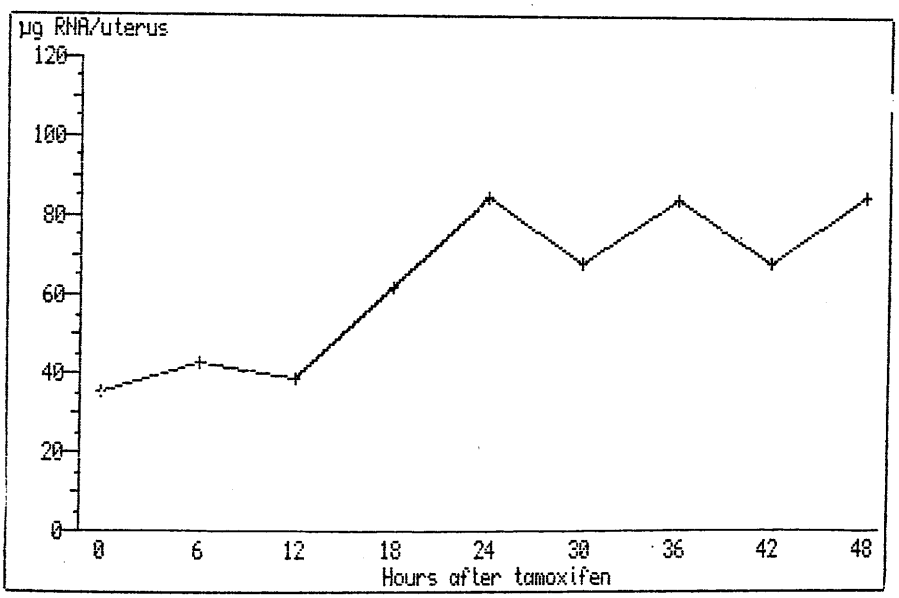
The figures below each graph indicated the number of preparations contributing to each point and the standard deviation for each point as a percentage of the mean value.

A



Hours after oestrogen.	0	2	4	8	12	16	20	24	26	28
Number of preparations.	9	4	8	4	4	5	3	3	3	2
%S.D.	38.5	27.2	22.9	16.6	21.4	30.0	21.6	21.6	7.6	31.4

B



Hours after tamoxifen.	0	6	12	18	24	30	36	42	48
Number of preparations	9	2	3	3	3	4	3	2	2
%S.D.	38.5	11.0	36.4	12.6	21.1	13.7	29.1	9.3	18.8

4.2.1. Quantitation of rRNA Contamination of PolyA+RNA Preparations

Strategy: Quantitation was obtained by hybridising a 28S rDNA probe to a northern blot of samples of pure 28S rRNA and the uterine polyA+RNA preparations. Densitometric scanning of the resulting autoradiograph allowed a standard curve to be constructed from which values were obtained for the amount of 28S rRNA in the unknowns. Assuming a molar ratio of 28S: 18S rRNA of 1:1, the total amount of rRNA and, by subtraction, the amount of polyA+RNA, in each preparation was estimated.

Method: Pure 28S rRNA was obtained by fractionating the poly A-RNA obtained from oligo (dT) cellulose chromatography on a linear 10-25% sucrose density gradient (materials and methods 3.4). The gradients were then scanned by pumping them through the flow cell of a Gilford 240 spectrophotometer and monitoring the OD_{260}. The fraction corresponding to the 28S rRNA peak was collected and the concentration of RNA estimated. The integrity and purity of the 28S rRNA was checked by running a sample on a 2% agarose gel (materials and methods 3.5) Fig. 13.

Standard amounts of pure 28S rRNA, ranging from 50ng to 500ng, were resolved on a formaldehyde/agarose gel, with 500ng and 250ng of two uterine mRNA preparations. They were then blotted onto either nitrocellulose filter paper or Biodyne A membrane (materials and methods 7.1) and hybridised to nick-translated p28S, a 7Kb genomic 28S rDNA sequence in pBR322 (a gift from R. Fulton) (materials and methods 7.3, 7.4). The filter was then autoradiographed and the resulting film scanned using an LKB laser densitometer.

Figure 13

Purification of 28S rRNA

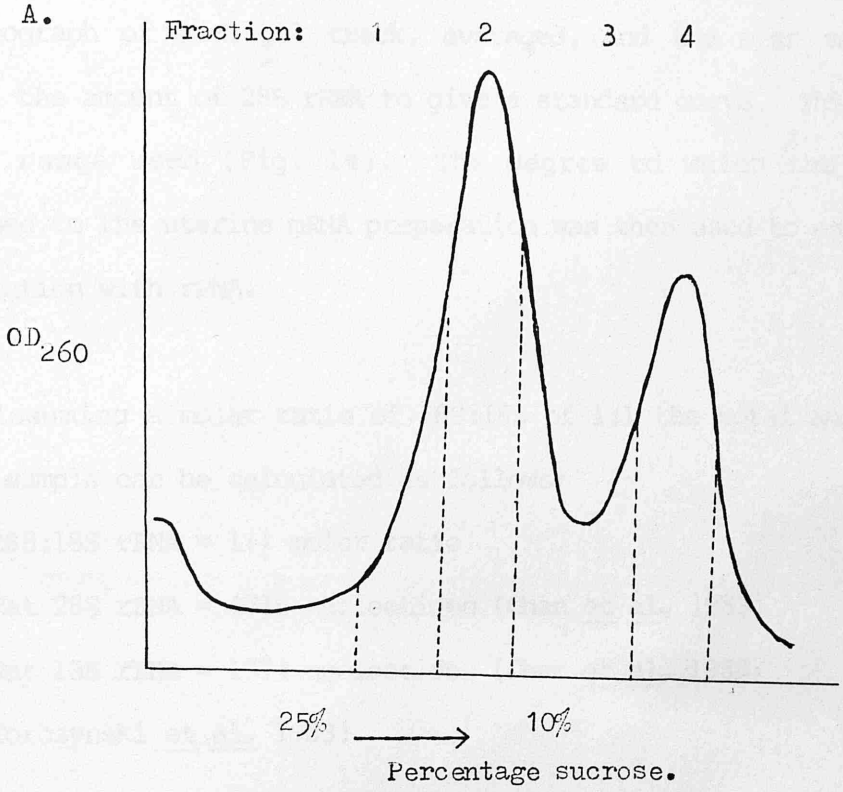
A. 2mg of polyA-RNA was resolved on a linear 10-25% sucrose gradient (materials and methods 3.4). This was then fractionated using the flow-through cell of a Gilford 240 spectrophotometer and the OD_{260} monitored.

Four fractions were collected as indicated.

B. Aliquots of the RNA from the four fractions obtained as shown in A were resolved on a 2% agarose gel (materials and methods 3.5).

Fractions 1-4 in A correspond to tracks 1-4 in B.

A.



B.

Track: 1 2 3 4

origin.

A number of densitometric readings were taken down the autoradiograph of each gel track, averaged, and the mean value plotted against the amount of 28S rRNA to give a standard curve. This was linear in the range used (Fig. 14). The degree to which the rDNA probe hybridised to the uterine mRNA preparation was then used to estimate their contamination with rRNA.

Assuming a molar ratio of 28S:18S of 1:1 the total amount of rRNA in each sample can be calculated as follows:

28S:18S rRNA = 1:1 molar ratio

Rat 28S rRNA = 4718 nucleotides (Chan et al., 1983)

Rat 18S rRNA = 1874 nucleotides (Chan et al., 1983; Torczynski et al., 1983).

therefore the mass ratio of the two molecules is:

18S:28S = 1874:4718

= 2.5176:1

that is, there is 2.52 times more, by weight, 28S rRNA than 18S rRNA.

From the standard curve 500ng of the unknown contains xng of 28S rRNA, it therefore contains

$$\frac{x}{2.52} \text{ ng of 18S rRNA}$$

The total amount of rRNA is then:

$$x + \frac{x}{2.52} \text{ ng}$$

Figure 14

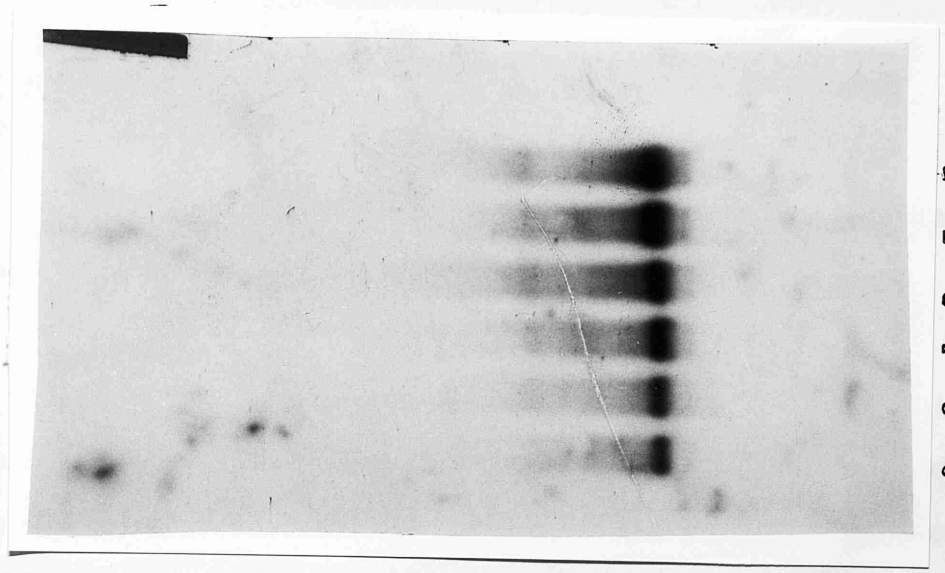
Hybridisation of cloned 28S rDNA to 28S rRNA

A. Samples of 28S rRNA were resolved on a 0.8% agarose/2.2M formaldehyde gel, blotted onto nitrocellulose and hybridised to a nick-translated 28S rDNA probe. The filter was then washed and autoradiographed (materials and methods 7.3)

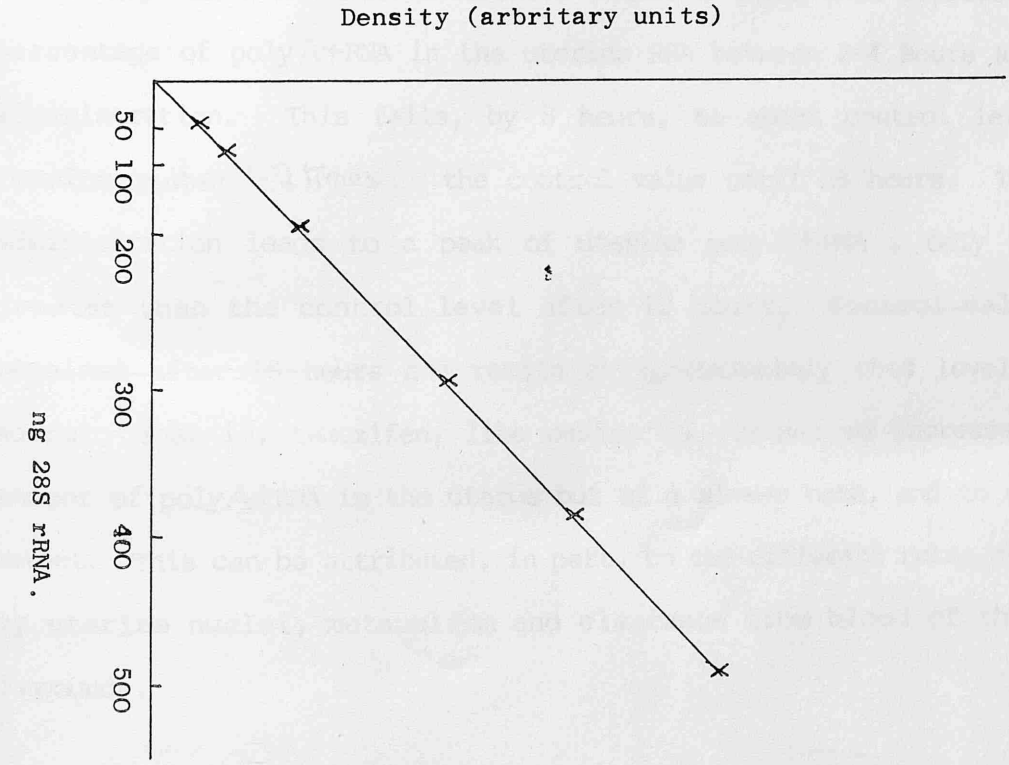
Track 1	-	500ng	28S rRNA
2	-	400ng	28S rRNA
3	-	300ng	28S rRNA
4	-	200ng	28S rRNA
5	-	100ng	28S rRNA
6	-	50ng	28S rRNA

B. The autoradiograph in Figure 14A was scanned using an LKB laser densitometer. Five scans of each track were obtained and the average value for each plotted against the amount of 28 rRNA in each track.

A.


1
2
3
4
5
6

B.


Density (arbritary units)
50
100
200
300
400
500
ng 28S rRNA.

the amount of polyA+RNA is therefore:

$$500 - (x + \frac{x}{2.52}) \text{ ng}$$

mRNA preparations which had been quantitated against rRNA standards could in turn be used to quantitate other preparations.

Analysis: The above calculations achieve two ends. Firstly, they permit hybridisation with a standard quantity of mRNA which has been corrected for its contamination with rRNA. Secondly, they allow an accurate estimation of the effect of oestrogen or tamoxifen on the percentage messenger content of uterine RNA.

Fig. 15 shows that oestradiol causes a three-fold increase in the percentage of polyA+RNA in the uterine RNA between 2-4 hours after its administration. This falls, by 8 hours, to about control levels and remains within 1-2 times the control value until 28 hours. Tamoxifen administration leads to a peak of uterine polyA+RNA, only two-fold greater than the control level after 12 hours, ~~Control values are regained after 18 hours~~ and remain at approximately this level for 42 hours. That is, tamoxifen, like oestradiol, causes an increase in the amount of polyA+RNA in the uterus but at a slower rate, and to a lesser extent. This can be attributed, in part, to the different rates of uptake by uterine nuclei, metabolism and clearance from blood of these two compounds.

5. Strategy for Quantitation of the Effects of Oestrogen and Tamoxifen on the Expression of Specific Genes

Figure 15

The Effect of Oestrogen and Tamoxifen on the amount of PolyA+RNA/Rat Uterus

Groups of 20-40, 21 day old rats of 30-35g, each received:

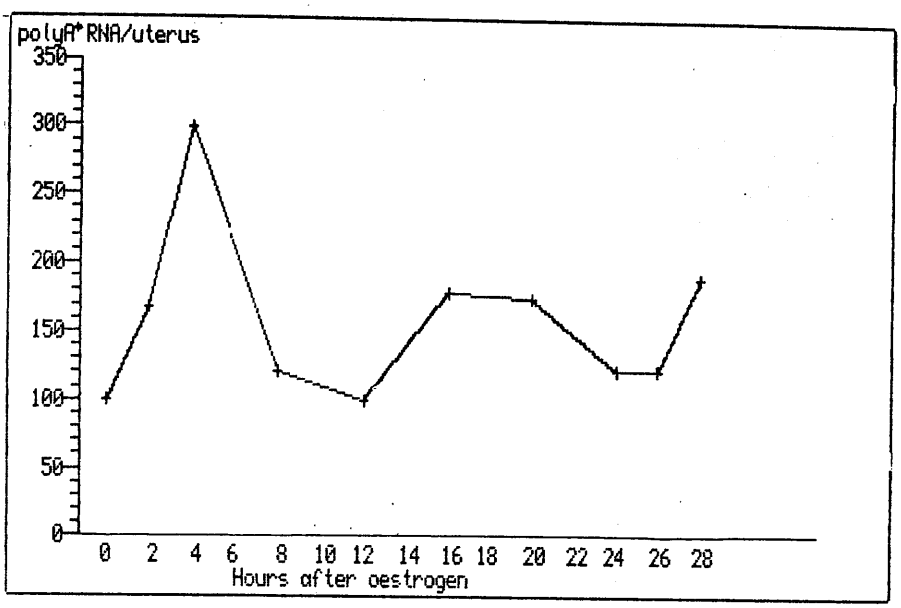
A: 1µg of oestradiol

B: 30µg of tamoxifen

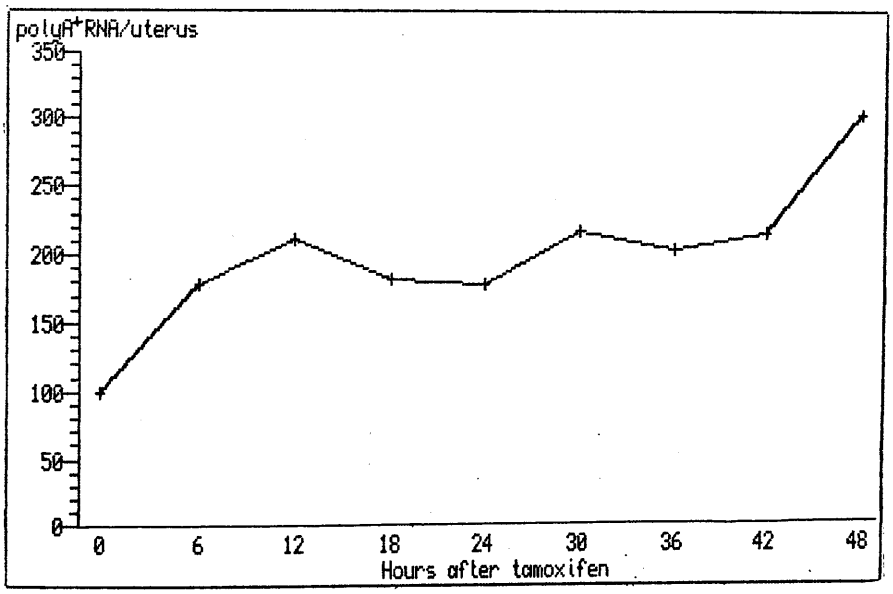
Control animals received carrier alone

The animals were killed at various times after treatment and RNA extracted from their uteri and enriched in polyA+RNA by one passage through Oligo (dT) cellulose (materials and methods 3.3). The amount of rRNA contamination, and therefore of polyA+RNA, in each preparation was estimated as described in Results 4.2. The amount of polyA+RNA/rat uterus was then calculated at each time point and plotted, as a percentage of the amount per control uterus, against duration and type of treatment.

A.


polyA+RNA/uterus
350
300
250
200
150
100
50
0
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28
Hours after oestrogen

B.


polyA+RNA/uterus
350
300
250
200
150
100
50
0
0 6 12 18 24 30 36 42 48
Hours after tamoxifen

RNA was isolated from the uteri of immature rats which had been treated with carrier alone, oestradiol for 2, 4, 8, 12, 16, 20, 24, 26 and 28 hours, and with tamoxifen for 6, 12, 18, 24, 30, 36, 42 and 48 hours. The reasons for using these particular time-courses of treatment were discussed previously (Section 4.1). PolyA+RNA was then enriched in each RNA preparation by oligo (dT) cellulose chromatography (materials and methods 3.) and the amount of contamination by rRNA of each preparation estimated as described in Section 4.2.1. The amount of each rRNA preparation equivalent to 1μg of pure polyA+RNA was then calculated in order that the same amount of polyA+RNA could be analysed at each time point of treatment.

The effects of oestradiol and tamoxifen on the expression of a specific sequence were assessed by both northern and RNA dot blotting by hybridisation to nick-translated plasmid containing the sequence of interest (materials and methods 7).

Northern blots were used in order to determine the size of the mRNAs complementary to the inserted sequence in the plasmid. The resulting autoradiographs could also be scanned with the laser densitometer and, as the same amount of polyA+RNA was resolved per track, the relative amounts of the specific messenger in each population could be compared, as could the relative amounts of each size of complementary sequence within one population.

Northern blots were however time consuming and subject to a number of problems. The major problem was that, with large filters, the amount of non-specific binding of the radioactive probe to the filter was

sometimes quite high, and could obscure bands, or interfere with scanning of the autoradiograph. Also, as the messenger sequences being looked for were of the rare or mid-abundant class, either a lot of RNA had to be used and/or the films exposed for a relatively long time. Long exposure increases the background on films and can result in them being difficult to scan and photograph. The problems were less with Biodyne A filters than with nitrocellulose filters, which varied greatly between batches. Deaerating the solutions used for hybridisation and the nitrocellulose filters themselves, decreased the amount of non-specific binding.

Dot blots of the different mRNA preparations were faster and simpler to do than northern blots. In this case the RNA was concentrated in a small area; therefore less was needed. Also shorter exposure times and smaller filters were used which led to lower background on the films. These facts, together with the fact that five serial dilutions of each sample were dotted, ensured that each filter yielded a number of tracks on a film which were scanned, compared and averaged. Northern blots were however necessary in order to estimate the size, of and number of, mRNA species which contributed to the binding on the dot blots.

5.1 The Effect of Oestrogen and Tamoxifen on the Level of Expression of the Oestrogen-regulated Recombinants Isolated from the Uterine cDNA Library

Time did not permit an analysis of the effects of oestrogen and tamoxifen on the expression of all twelve of the recombinants that were initially selected and sized (Table 6). Only three of the selected recombinants were fully analysed, these being B11, E10 and F4. These

three recombinants were analysed by both northern and RNA dot blotting, using the same amount of uterine polyA+ RNA from each time-course of oestrogen and tamoxifen treatment. The results are described below.

In a preliminary control experiment, intact pUC8, the plasmid vector in which the library was constructed, was nick-translated and $2.5x10^6$ cpm hybridised to five serial dilutions of 2µg polyA+ RNA of control and 4 hour oestrogen-induced mRNA. After washing, the filters were autoradiographed. Exposure of the films for one week resulted in nothing being detected on the film, after two weeks faint signals were detected from the first three dilutions (result not shown). This implied that very little binding of pUC8 to uterine RNA occurred and showed that whole plasmids could be nick-translated as probes without producing artifactual results due to the pUC8 sequence.

5.1.1. The Effect of Oestrogen and tamoxifen on the Expression of Bll Specific Sequences in the immature Rat Uterus

The analysis of plasmid Bll has been shown in detail in order to demonstrate the sources of, and extent of, the errors produced using the methods of quantitation described above. The sources of error also apply to the analysis of all the other recombinants studied in both the immature rat uterus and in the MCF-7 human breast cancer cell line, and must be considered when trying to draw any conclusions from the data presented.

Plasmid Bll consistently bound more of the 4 hour oestrogen-stimulated cDNA probe, that is, it was induced by oestrogen. It did not however, when compared to some of the other plasmids, have a very large

insert, the insert being 250 bp. Samples of uterine mRNA from oestrogen-stimulated rats were resolved on a formaldehyde/agarose gel and blotted onto Biodyne A membrane. These were then hybridised to nick-translated B11, washed at medium stringency and autoradiographed (materials and methods 7). The probe hybridised to three specific bands, and less specifically elsewhere on the tracks. The non-specific hybridisation suggested that the B11 insert contained a poly T tract which was long enough to allow hybridisation to all polyA+RNA. Higher stringency washes would probably decrease this. The remainder of the insert caused binding more specifically to three bands, the sizes of which were estimated from the position of the 28S and 18S rRNA markers (Fig. 16). B11 hybridised mainly to an RNA of 4.5Kb and, to a lesser extent, to two higher molecular weight RNAs of 12.6Kb and 11.2Kb. Since total mRNA, as opposed to polysomal mRNA was used, these higher molecular weight RNAs could represent precursors.

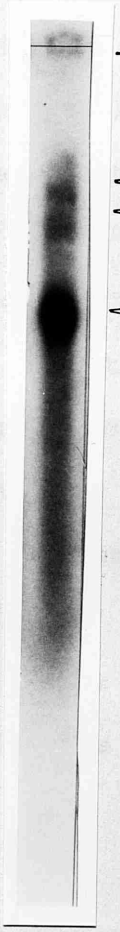
The background binding to all tracks in the northern blot hindered densitometric scanning of the autoradiograph, therefore RNA dot blots were used to quantitate the induction of B11 sequences by oestrogen and tamoxifen. Serial dilutions, from 1µg - 0.0625µg, of each oestrogen-stimulated mRNA preparation were made, denatured and dotted onto a nitrocellulose filter. The filter was then baked, hybridised to nick-translated B11, washed and autoradiographed (materials and methods 7.2). Dot blots of 1µg - 0.0625µg of the polyA+RNAs from the tamoxifen treated rats were also prepared and hybridised, back to back with the filter containing control and oestrogen-stimulated polyA+RNAs, to the B11 probe. The filters were washed and autoradiographed for the same duration. This allowed direct comparison between the samples.

Figure 16

Northern Blot Analysis of 4 hour Oestrogen-Induced Uterine PolyA+RNA for Sequences Complementary to Plasmid B11 Insert

2.5µg of polyA+RNA, quantitated as described in Results 4.2, from the uteri of immature rats given 1µg of oestradiol, intraperitoneally, 4 hours before death, was resolved on a 0.8% agarose/2.2M formaldehyde gel, blotted onto Biodyne A membrane and hybridised to plasmid B11 labelled with ^{32}P by nick-translation. The membrane was then washed, sealed in a plastic bag and autoradiographed (materials and methods 7.1, 7.4)

The sizes of the bands were estimated using the position of 28S and 18S rRNA markers.


-origin
12.6Kb
11.2Kb
28S
4.3Kb
18S

The result of a 36 hour exposure of these filters is shown in Fig. 17, which illustrates an induction of B11 sequences over the time-course used. In order to quantitate this the films were scanned using an LKB laser densitometer and integrator. This gives a plot of density versus distance and integrates the area under the curve. It also gives the total area under the curve the value of 100% and estimates the percentage of this represented by each distinct peak. Therefore, by scanning the top row of the autoradiograph in Fig. 17(B), 10 peaks are obtained and values derived for these with respect to the total area (Fig. 18).

The results obtained with plasmid B11 were used to assess the accuracy of the method of quantitation. Each row, that is each mRNA concentration, of the films investigating the effect of oestrogen and tamoxifen on B11 expression (Fig. 17) were scanned separately with the densitometer. These scans are compared in Fig. 19. On both films the higher concentrations of RNA lead to overexposure which masks the small differences between successive time points. However, as the concentration of RNA decreases, the differences between the time points become more pronounced.

Due to the variation observed between rows on the B11 films it was decided that, rather than plot the values obtained from scanning a particular RNA concentration, to scan every row on each film of each sequence studied, estimate the mean value for each time point and plot this against the duration and type of treatment. The percentage standard deviation for each point was also calculated. This value represents the combined error from a number of sources, for example, in the original estimation of the quantity of polyA+RNA/preparation, in pipetting, both

Figure 17

The Effect of Oestrogen and Tamoxifen on the Level of RNA Sequences Complementary to the Insert of Plasmid B11 in the Immature Rat Uterus

Samples of polyA+RNA isolated from the uteri of 21 day-old rats weighing 30-35g which had received:

A. 1μg of oestradiol in saline, or saline alone

B. 30μg of tamoxifen in corn-oil

At various times before death, by the procedure described in materials and methods 3.2.3, and corrected for contamination with rRNA, were denatured and serial dilutions prepared. The RNA was then dotted onto nitrocellulose filters and hybridised to plasmid B11, labelled with ^{32}P by nick-translation (materials and methods 4.5). The filters were then washed and autoradiographed.

A.

µg polyA$^+$RNA

Hours after oestrogen.

0 2 4 8 12 16 20 24 26 28

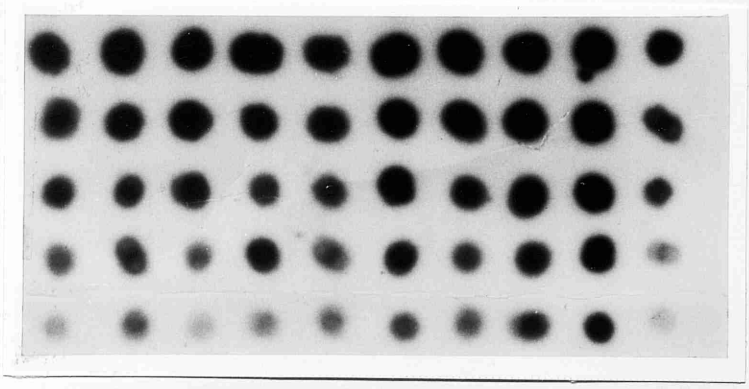
1

0.5

0.25

0.125

0.0625



B.

µg polyA$^+$RNA

Hours after tamoxifen.

6 12 18 24 30 36 42 48

1

0.5

0.25

0.125

0.0625

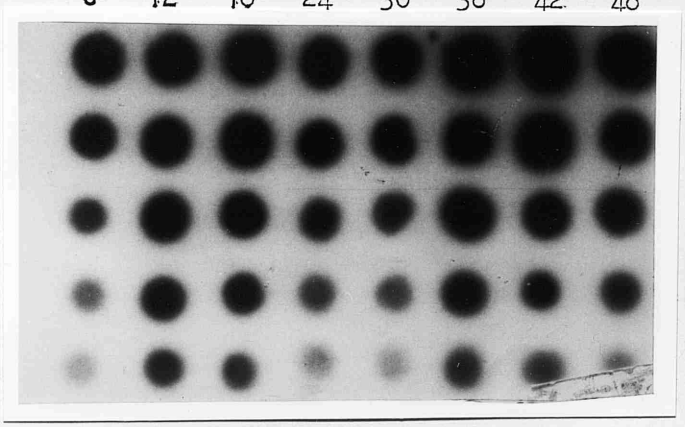


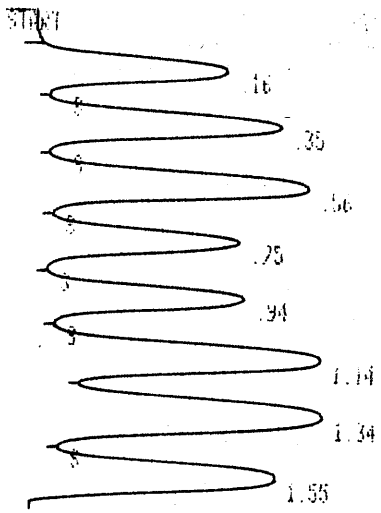
Figure 18

An Example of a Densitometric Scan of an Autoradiograph

The top row of Figure 17B was scanned using an LKB Bromma 2202 Ultrascan laser densitometer and recording integrator 2220.

Hours after tamoxifen

6
12
18
24
30
36
42
48



STOP

RUN # 489

AREA%

RT	AREA	TYPE	AR/HT	AREA%
0.16	1.5011E+07	SBB	0.087	8.875
0.35	1.9753E+07	SPB	0.091	11.678
0.56	2.4155E+07	SPB	0.100	14.280
0.75	1.4328E+07	SPB	0.081	8.471
0.94	1.5401E+07	SPB	0.085	9.105
1.14	2.6718E+07	SPH	0.107	15.796
1.34	3.0507E+07	SHB	0.121	18.035
1.55	2.3276E+07	TSPP	0.107	13.761

TOTAL AREA= 1.6915E+08
MUL FACTOR= 1.0000E+00

Figure 19

Method of Analysis of the Effect of Oestrogen and Tamoxifen on the Level of Specific Sequences

Example : B11 sequences in the immature rat uterus.

Each row of the autoradiographs in Fig. 17 A and B were scanned separately using a densitometer, and the values obtained for the percentage of the total scan represented by each dot plotted against the duration and type of treatment.

The mean value (x), in arbitrary units of density, and the percentage standard deviation (% SD) were also calculated for each point.

A. Scan of 0.0625µg of 0-28 hour oestrogen-induced polyA+RNA

B. Scan of 0.125µg of 0-28 hour oestrogen-induced polyA+RNA

C. Scan of 0.25µg of 0-28 hour oestrogen-induced polyA+RNA

D. Scan of 0.50µg of 0-28 hour oestrogen-induced polyA+RNA

E. Scan of 1.00µg of 0-28 hour oestrogen-induced polyA+RNA

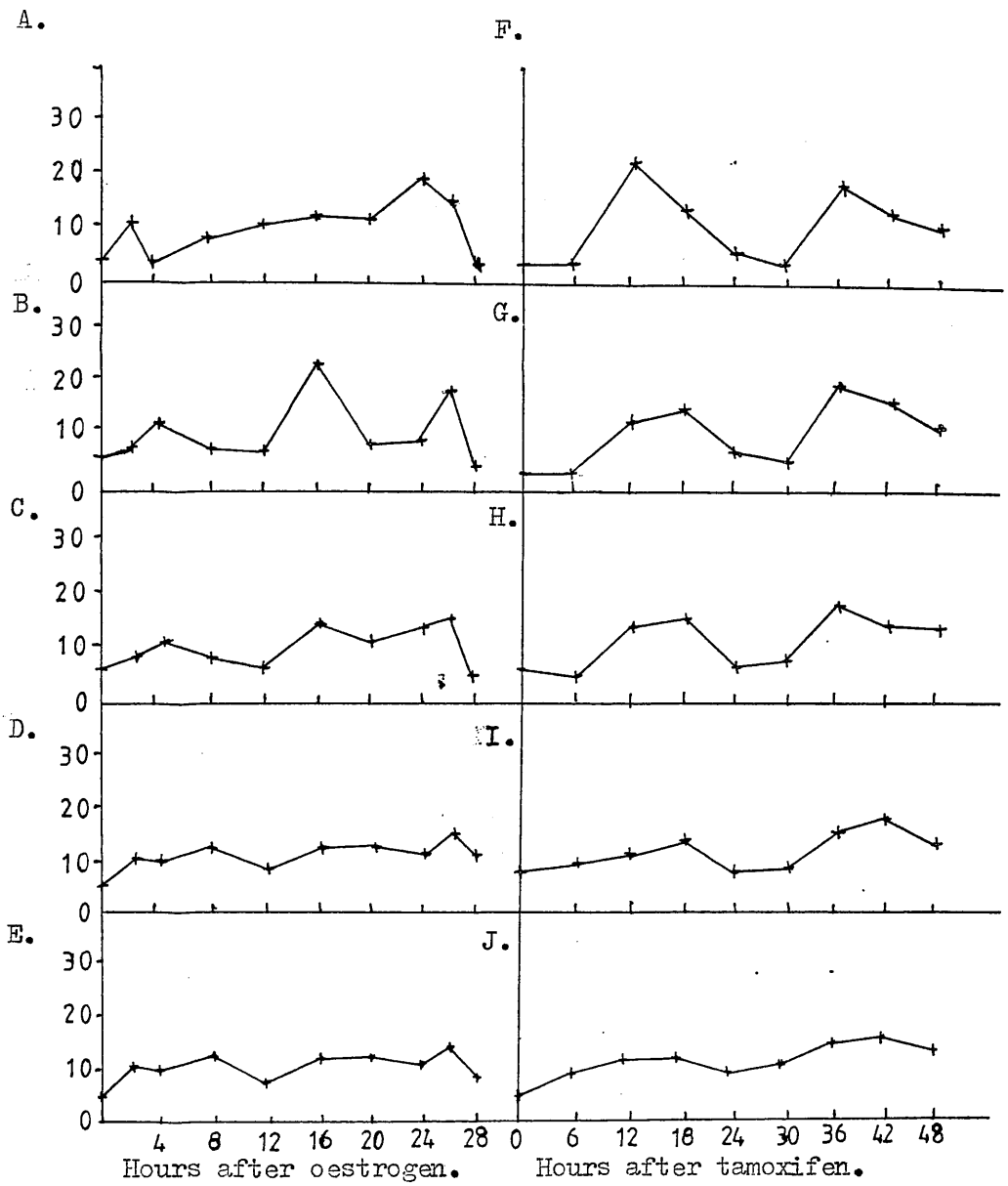
F. Scan of 0.0625µg of 6-48 hour tamoxifen-induced polyA+RNA

G. Scan of 0.125µg of 6-48 hour tamoxifen-induced polyA+RNA

H. Scan of 0.25µg of 6-48 hour tamoxifen-induced polyA+RNA

I. Scan of 0.50µg of 6-48 hour tamoxifen-induced polyA+RNA

J. Scan of 1.00µg of 6-48 hour tamoxifen-induced polyA+RNA



Hours after oestrogen.	0	2	4	8	12	16	20	24	26	28
$\bar{X}$	5.5	9.8	8.4	10.3	7.9	15.7	10.4	13.6	17.9	4.9
%S.D.	28.7	14.7	46.9	32.9	16.4	29.4	21.9	27.0	20.4	42.9
Hours after tamoxifen.	6	12	18	24	30	36	42	48		
$\bar{X}$	6.2	15.6	14.9	7.7	7.9	18.2	16.7	17.1		
%S.D.	37.6	31.5	9.3	13.4	28.0	11.4	5.3	17.0		

in diluting the samples and in applying them to the filters, and from the densitometer, both in itself and in the positioning of the track to be scanned (whether it passed through the centre of all the dots or not).

The percentage error in each case must be considered when determining whether the difference in the level of a particular mRNA between time points is real or not.

The level of B11 mRNA at each point in the time courses of oestrogen and tamoxifen treatment was estimated as a percentage of the value obtained using control RNA. It was then plotted against the length of treatment (Fig. 20). This shows that the level of B11 mRNA increases, somewhat erratically, to a maximum four times control level at 26 hours after oestrogen, a smaller peak, three times control level, being observed after 16 hours. Tamoxifen also causes two maxima of B11 RNA; three times the control level at 12-18 hours and 3.3 times control levels at 36-42 hours after administration.

5.1.2. The Effect of Oestrogen and Tamoxifen on the Expression of E10 Specific Sequences in the Immature Rat Uterus

Plasmid E10 consistently bound more 4 hour oestrogen-stimulated cDNA probe than control cDNA probe, implying that it was induced by oestrogen within 4 hours. The insert in this case was, like B11, of the order of 250 bp and, again like B11, probably contained a poly T tract, as there was some non-specific binding to all poly A+RNA on northern blots (Fig. 21).

Figure 20

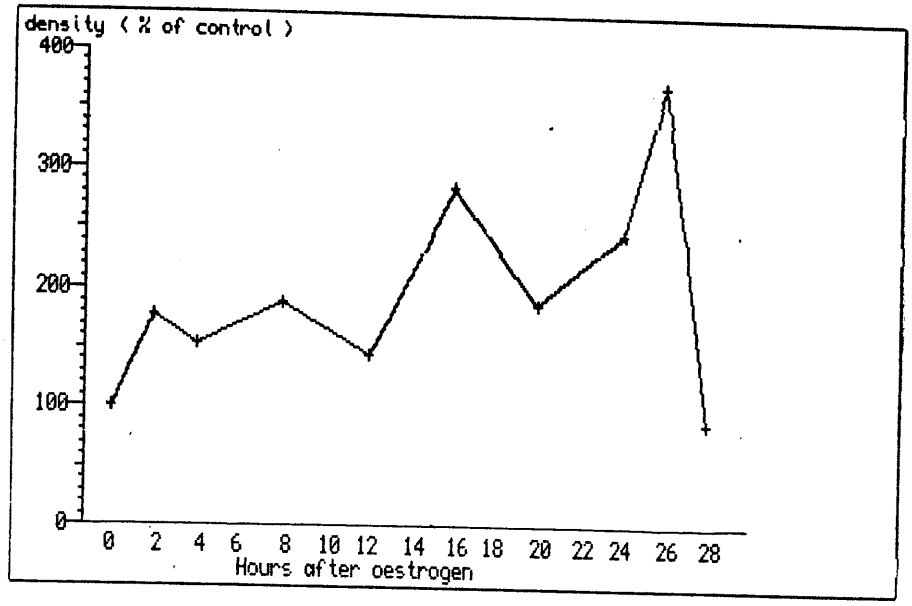
Quantitation of the Effect of Oestrogen and Tamoxifen on the Level of Bll RNA in the Immature Rat Uterus

Each row of the autoradiographs shown in Figure 17 A and B was scanned using a laser densitometer and the mean and percentage standard deviation (% S.D.) for each polyA+RNA preparation used calculated as described in Results 4.2.1. The mean values were then calculated as a percentage of the control value (0 hours) and plotted against:

A. The duration of oestrogen treatment

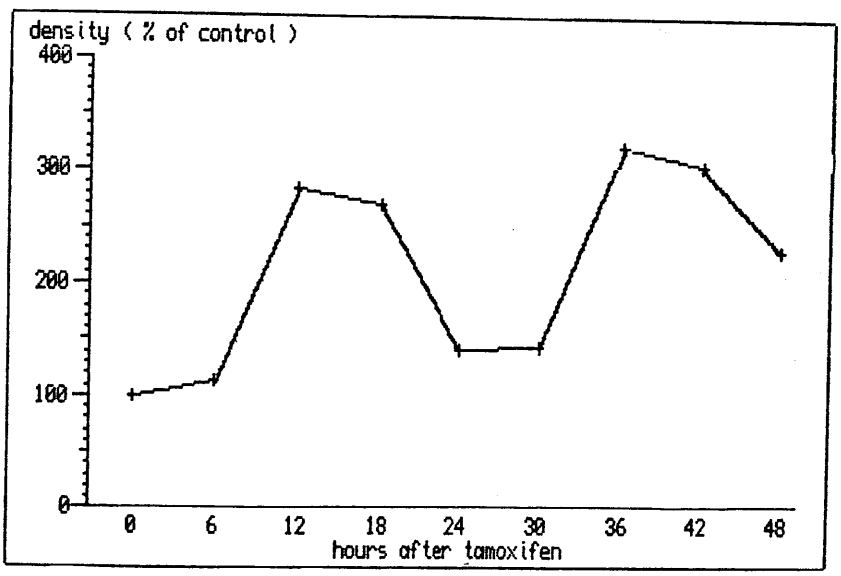
B. The duration of tamoxifen treatment

A.



Hours after oestrogen.	0	2	4	8	12	16	20	24	26	28
%S.D.	28.7	14.7	46.9	32.9	16.4	29.4	21.9	27.0	20.4	42.9

B.



Hours after tamoxifen.	0	6	12	18	24	30	36	42	48
%S.D.	28.7	37.6	31.5	9.3	13.4	28.0	11.4	5.3	17.1

Figure 21

Northern Blot Analysis of the Effect of Oestrogen and Tamoxifen on Sequences Complementary to Plasmid E10 in the Immature Rat Uterus

2.5µg of polyA+RNA, quantitated as described in Results 4.2.1, from the uteri of 21 day old rats which had received either:

a) 1µg of oestradiol in saline

b) 30µg of tamoxifen in corn-oil

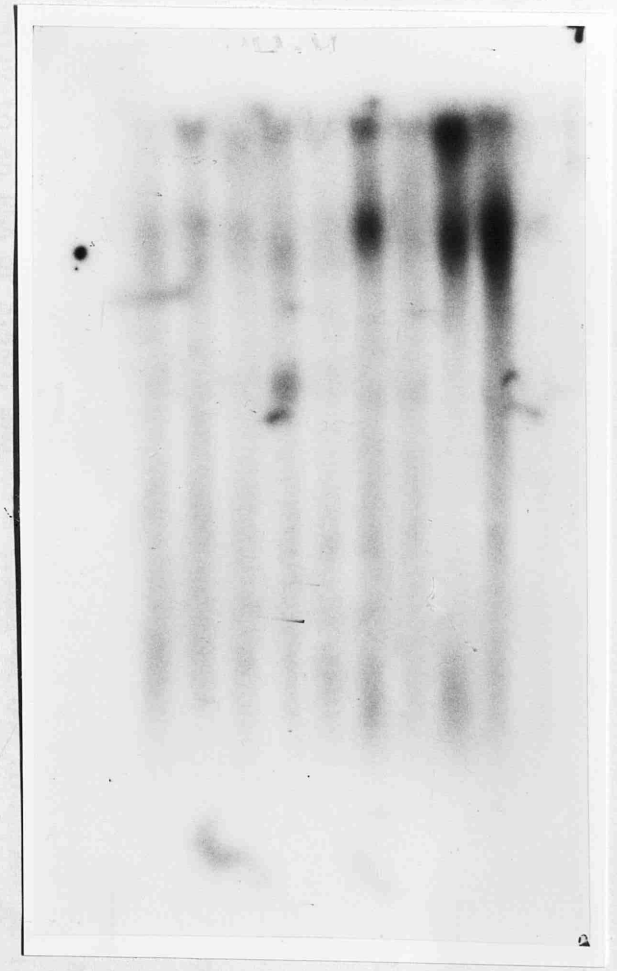
c) carrier alone

For the times indicated, were resolved on 0.8% agarose/2.2M formaldehyde gels, blotted onto Biodyne A membranes and hybridised to plasmid E10 labelled with ^{32}P by nick-translation (materials and methods 4.5, 7.1, 7.4). The membranes were then washed, sealed in a plastic bag and autoradiographed.

The sizes of the bands were estimated from the position of 28S and 18S rRNA markers.

Hours after oestrogen.

0 2 4 8 12 16 20 24 26 28



origin

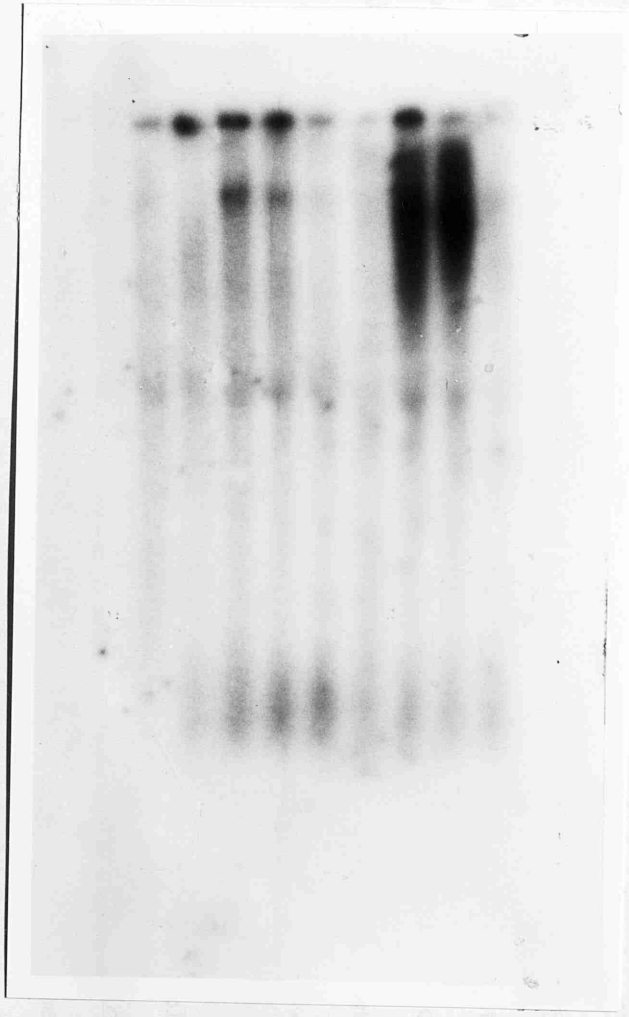
~14.6Kb

5.0Kb

2.0Kb

Hours after tamoxifen.

0 6 12 18 24 30 36 42 48



origin

~14.6Kb

5.0Kb

2.0Kb

Samples of control, oestrogen and tamoxifen stimulated poly A+RNA from the immature rat uterus were resolved on agarose/formaldehyde gels, blotted onto Biodyne A membranes and hybridised to intact plasmid E10 and the filters autoradiographed (Fig. 21). These showed that plasmid E10 hybridised mostly to a RNA of 14.6Kb, to a lesser extent to one of 5Kb, and faintly to a 2Kb RNA. These also showed a pattern of induction with oestrogen and tamoxifen but the background on the films made them unsuitable for scanning. The induction of E10 RNA was therefore quantitated using RNA dot blots, the results being calculated as for B11.

Administration of oestrogen to immature rats resulted in a two-fold increase in the amount of E10 RNA/unit poly A+RNA within 2 hours, which was maintained until 12 hours. A seven-fold increase was observed between 12-16 hours which decreased to 3.5 times control levels between 20-24 hours. A second peak, six-times control, was observed at 26 hours after which, by 28 hours, levels had returned to normal (Fig. 22). Tamoxifen also resulted in two peaks of E10 RNA levels; a six-fold increase between 6-12 hours which was maintained until 18 hours after which it fell to control level by 24 hours, and an eight-fold increase between 30-36 hours which declined steadily to normal by 48 hours (Fig. 23).

5.1.3 The Effect of Oestrogen and Tamoxifen on the Expression of F4 Specific Sequences in the Immature Rat Uterus

Plasmid F4 also appeared to be induced within 4 hours of oestrogen administration to immature rats. The insert in this plasmid was approximately 350bp. Northern blot analysis showed that this insert bound specifically to an RNA of 3.4Kb, and to a lesser extent to an RNA of 1.3Kb (Fig. 24).

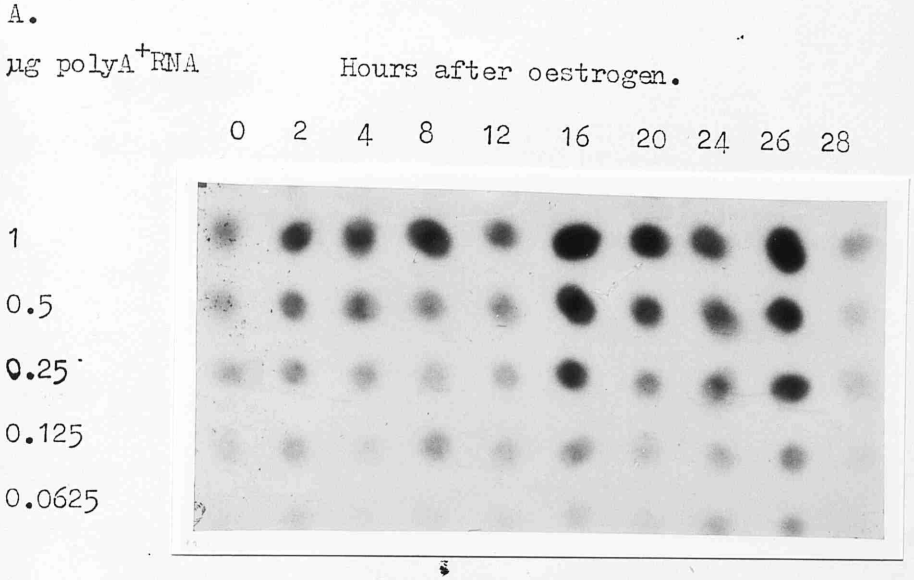
Figure 22

The Effect of Oestrogen on the Level of RNA Sequences Complementary to Plasmid E10 in the Immature Rat Uterus

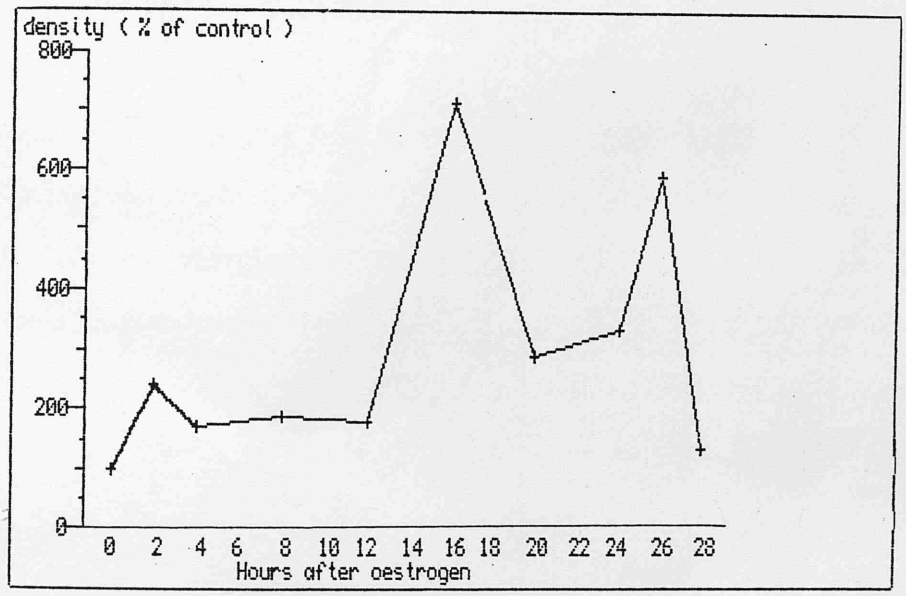
A. Samples of polyA+RNA isolated from the uteri of 21 day old rats, weighing 30-35g, which had received either 1µg of oestradiol in saline, or saline alone, at various times before death by the procedure described in materials and methods 3.2.3, 3.3, and corrected for contamination with rRNA, were denatured and serial dilutions prepared. The RNA was then dotted onto a nitrocellulose filter and hybridised to plasmid E10 labelled with ^{32}P by nick-translation (materials and methods 7.2, 7.3). The filter was then washed and autoradiographed.

B. Each row of the autoradiograph shown in A was scanned using a laser densitometer and the mean and percentage standard deviation (% SD) for each polyA+RNA preparation used was calculated as described in Results 4.2.1. The mean values were then calculated as a percentage of the control value (0 hours) and plotted against the duration of treatment.

A.



B.



Hours after oestrogen.	0	2	4	8	12	16	20	24	26	28
%S.D.	40.9	21.0	22.5	56.0	19.8	31.0	39.6	35.5	21.8	47.1

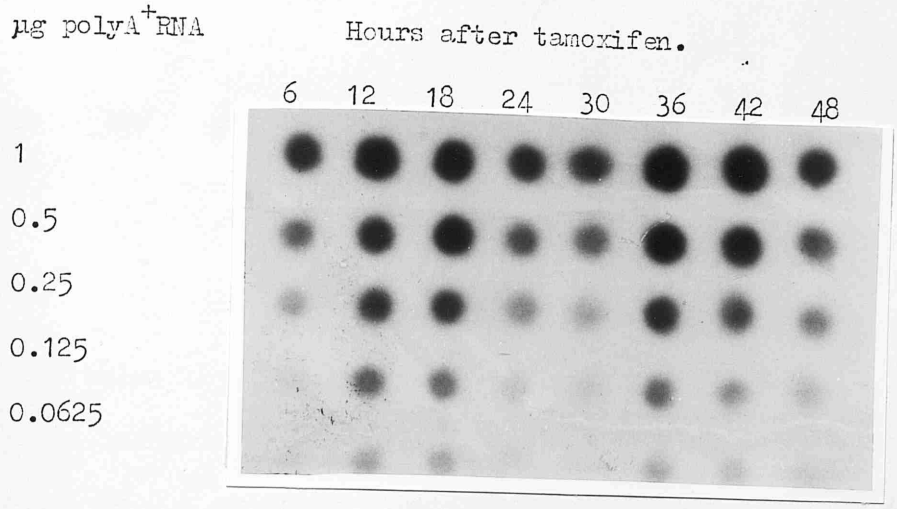
Figure 23

The Effect of Tamoxifen on the Level of RNA Sequences Complementary to Plasmid E10 in the Immature Rat Uterus

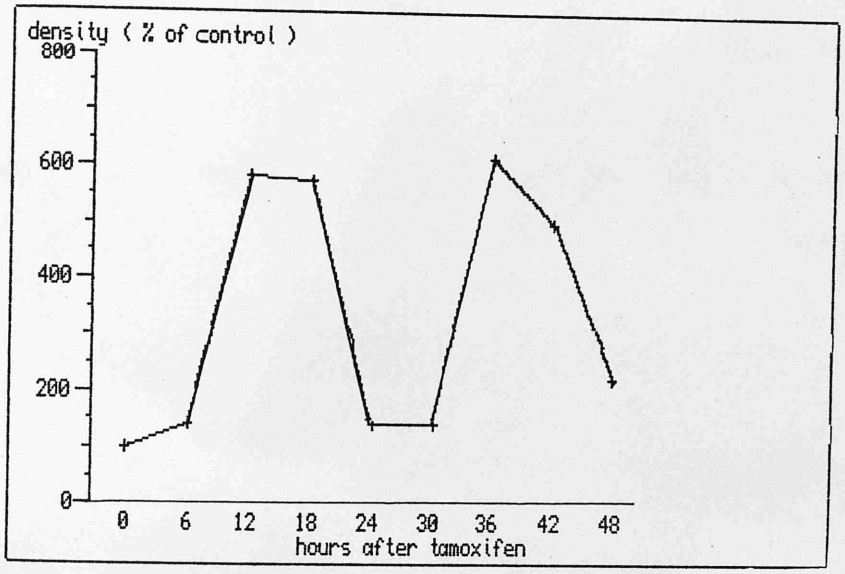
A. Samples of polyA+RNA isolated from the uteri of 21 day old rats weighing 30-35g which had received 30μg of tamoxifen in corn-oil at various times before death by the procedure described in materials and methods 3.2.3, 3.3 and corrected for contamination with rRNA, were denatured and serial dilutions prepared. The RNA was then dotted onto a nitrocellulose filter and hybridised to plasmid E10 labelled with ^{32}P by nick-translation (materials and methods 7.2, 7.3). The filter was then washed and autoradiographed.

B. Each row of the autoradiograph shown in A was scanned using a laser densitometer and the mean and percentage standard deviation (% SD) for each polyA+RNA preparation used calculated as described in Results 4.2.1. The mean values were then calculated as a percentage of the control value (0 hours) on the oestrogen filter Fig. 23A) and plotted against the duration of treatment.

A.



B.



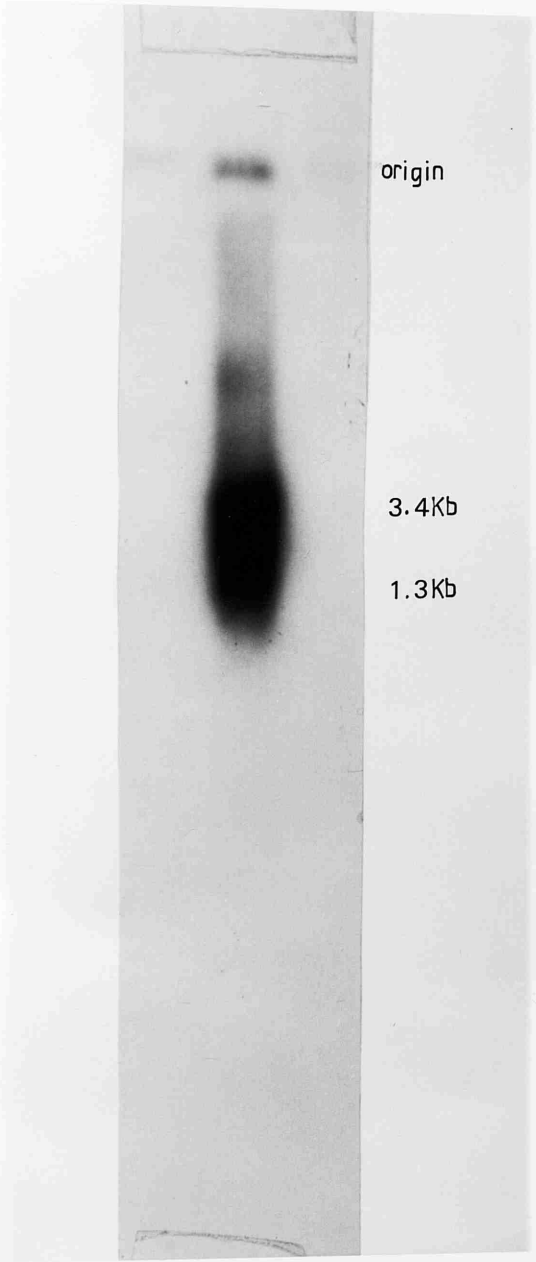
Hours after tamoxifen.	0	6	12	18	24	30	36	42	48
%S.D	40.9	42.9	31.4	14.1	48.1	40.0	16.9	11.3	40.5

Figure 24

Northern Blot Analysis of 4 hour Oestrogen-Induced Uterine PolyA+RNA for Sequences Complementary to Plasmid F4 Insert

A 2.5µg sample of polyA+RNA, obtained as described in materials and methods 3.2.3, 3.3, from the uteri of 21 day old rats which had received 1µg oestradiol in saline for four hours, was resolved on a 0.8% agarose/ 2.2M formaldehyde gel, blotted onto a nitrocellulose filter, and hybridised to plasmid F4 labelled with ^{32}P by nick-translation (materials and methods 7.1, 7.3). The filter was then washed, sealed in plastic bags and autoradiographed.

The size of the band was estimated using the position of 28S and 18S rRNA markers.


origin
3.4Kb
1.3Kb

The induction of F4 RNA was quantitated using RNA dot blots, the results being calculated as for B11. The effect of oestrogen on the level of F4 mRNA was to cause a steady increase to seven-fold control levels within 8 hours of treatment. This increase continued to a maximum of nine-fold control by 20 hours, after which a slight fall, to six-fold control by 26 hours was observed (Fig. 25). Tamoxifen also caused a steady increase in F4 mRNA to a maximum, seven-times control, in the first 12 hours after treatment. This high level of F4 mRNA was maintained from 12-24 hours, after which it declined to four-fold control at 48 hours (Fig. 26).

Figures 20, 22, 23, 25 and 26 represent the amounts, relative to that in unstimulated rat uteri, of B11, E10 and F4 specific RNA per unit of polyA+RNA at each time point, and do not take into account the different number of units of polyA+RNA available per rat uterus at each time point (Fig. 15). An estimate of the amount of each specific RNA per rat uterus after the various treatments was obtained by multiplying the amount of each specific RNA per unit of polyA+RNA at each point by the number of units of polyA+RNA per uterus at the respective points (Fig. 15). This was calculated for B11, E10 and F4 specific sequences and the results are shown in Fig. 27.

Fig. 27 showed that the pattern of increases and decreases in B11, E10 and F4 RNAs in response to oestrogen were very similar, with a peak at 4 hours, a trough at 12 hours and a second peak at 16-20 hours after administration, but that the extent of these changes were different.

Tamoxifen administration resulted in a similar pattern of response in B11 and E10 RNA levels per uterus as was observed in response to

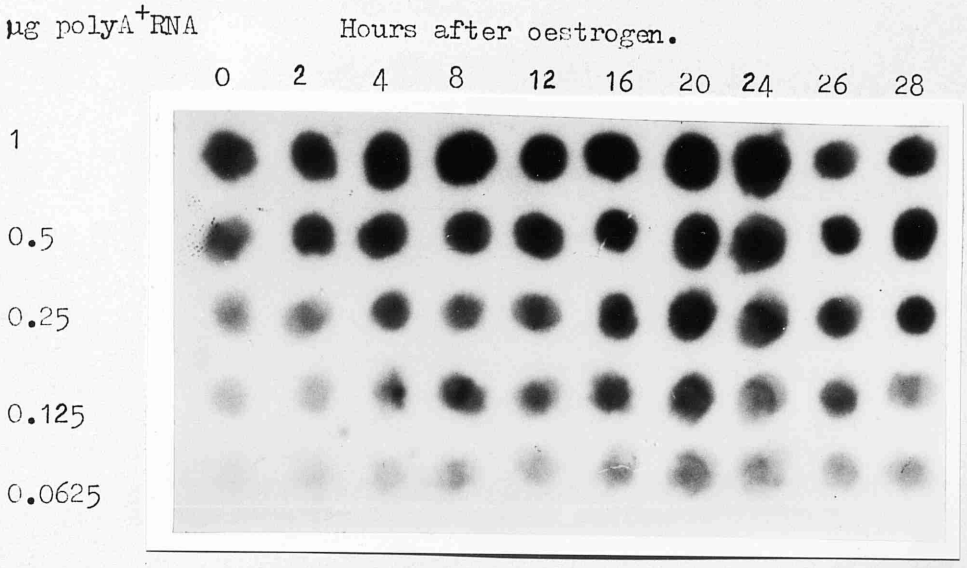
Figure 25

The Effect of Oestrogen on the Level of RNA Sequences Complementary to Plasmid F4 in the Immature Rat Uterus

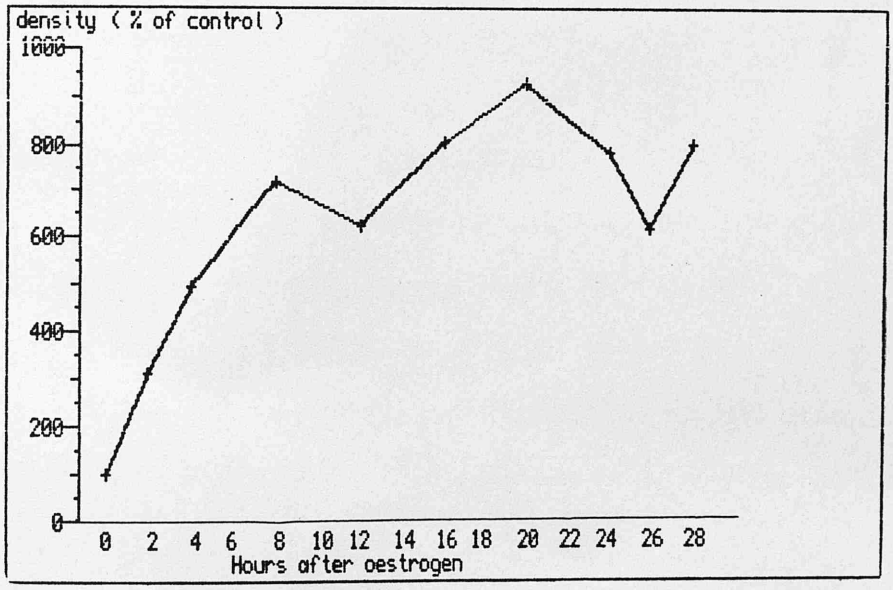
A. Samples of polyA+RNA isolated from the uteri of 21 day old rats weighing 30-35g which had received either 1µg of oestradiol in saline, or saline, at various times before death by the procedure described in materials and methods 3.2.3, 3.3. The amount used was corrected for contamination with rRNA, denatured and serial dilutions prepared before the RNA was blotted onto a nitrocellulose filter and hybridised to plasmid F4 labelled with ^{32}P by nick-translation (materials and methods 7.2, 7.3). The filter was then washed and autoradiographed.

B. Each row of the autoradiograph shown in A was scanned using a laser densitometer and the mean and percentage standard deviation (% S.D.) for each polyA+RNA preparation used calculated as described as a percentage of the control value (0 hours) and plotted against the duration of treatment.

A.



B.



Hours after oestrogen.	0	2	4	8	12	16	20	24	26	28
%S.D.	38.4	40.6	22.2	32.1	10.6	11.0	17.8	19.9	23.4	19.5

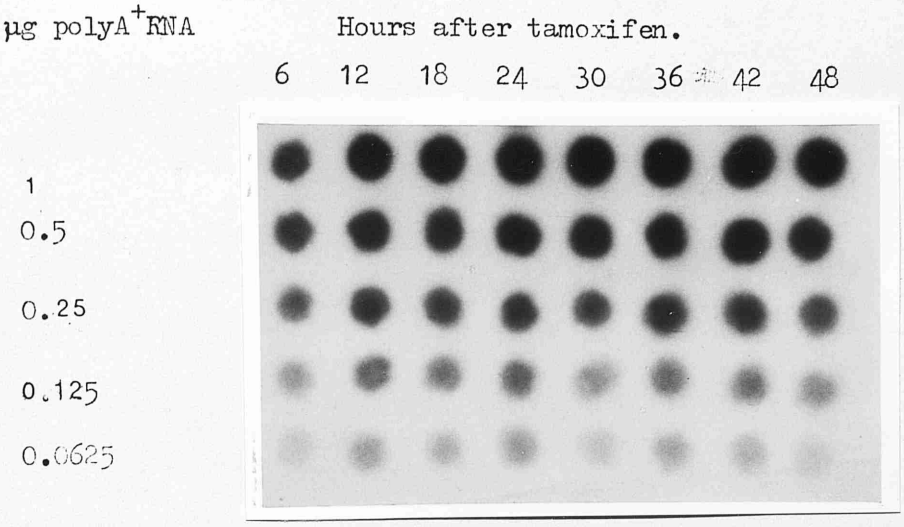
Figure 26

The Effect of Tamoxifen on the Level of RNA Sequences Complementary to Plasmid F4 in the Immature Rat Uterus

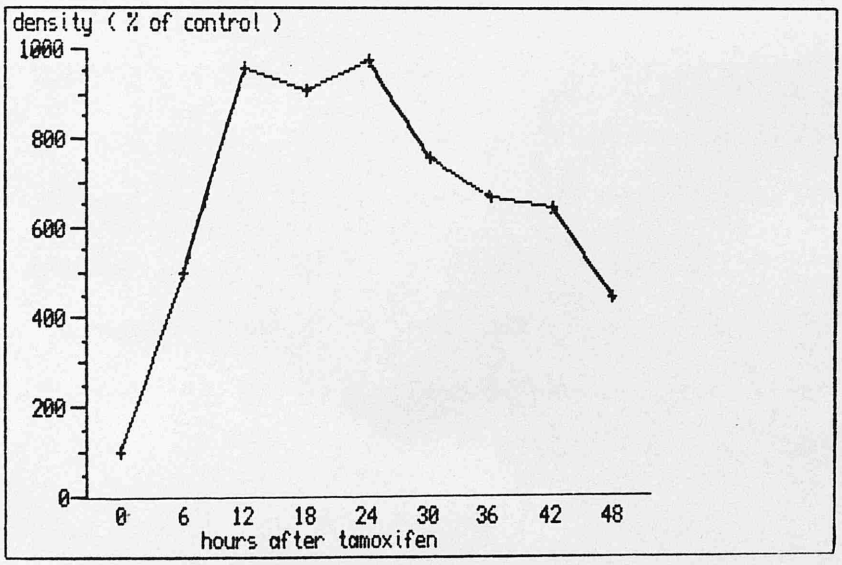
A. Sample of polyA+RNA isolated from the uteri of 21 day old rats weighing 30-35g which had received 30µg of tamoxifen in corn-oil at various times before death by the procedure described in materials and methods 3.2.3, 3.3 and corrected for contamination with rRNA, were denatured and serial dilutions prepared. The RNA was then dotted onto a nitrocellulose filter and hybridised to plasmid F4 labelled with ^{32}P by nick-translation (materials and methods 7.2, 7.3). The filter was then washed and autoradiographed.

B. Each row of the autoradiograph shown in A was scanned using a laser densitometer and the mean and percentage standard deviation (% SD) for each polyA+RNA preparation used calculated as described in Results 4.2.1. The mean values were then calculated as a percentage of the control value (0 hours on the oestrogen filter, Fig. 25A) and plotted against the duration of treatment.

A.



B.



Hours after tamoxifen.	0	6	12	18	24	30	36	42	48
%S.D.	38.4	28.6	9.8	18.1	19.9	13.0	11.0	18.4	39.9

Figure 27

The Effect of Oestrogen and Tamoxifen on the Amount of B11, E10 and F4 RNA per Rat Uterus

These figures were derived by multiplying the amount of B11, E10 and F4 RNA per unit of uterine polyA+RNA from oestrogen and tamoxifen treated rats, 21 days and weighing 30-35g (Figs. 20, 22, 23, 25, 26), by the amount of polyA+RNA available per uterus after each treatment (relative to control uteri) (Fig. 15). The results are expressed as a percentage of the value obtained for the amount of each RNA per control uterus.

A. Effect of oestrogen on the level of B11 RNA per uterus.

B. Effect of tamoxifen on the level of B11 RNA per uterus.

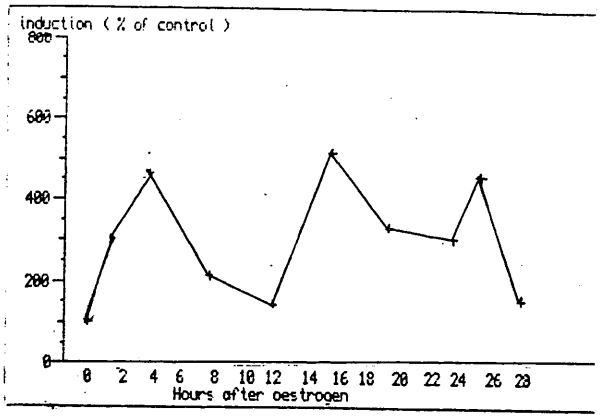
C. Effect of oestrogen on the level of E10 RNA per uterus.

D. Effect of tamoxifen on the level of E10 RNA per uterus.

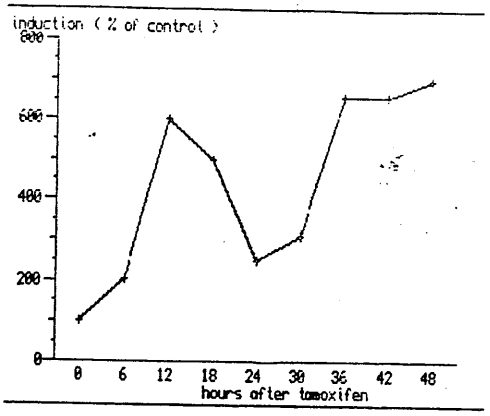
E. Effect of oestrogen on the level of F4 RNA per uterus.

F. Effect of tamoxifen on the level of F4 RNA per uterus.

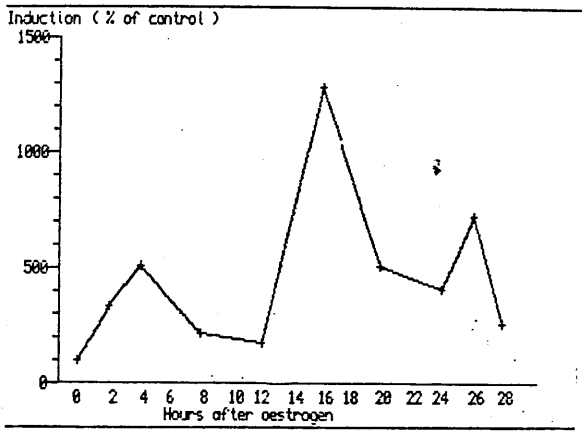
A.


induction (% of control)
800
600
400
200
0
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28
Hours after oestrogen

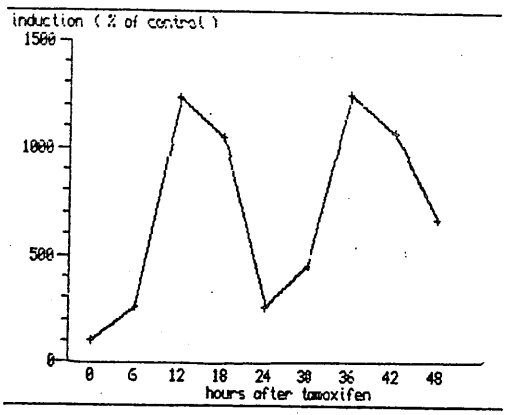
B.


induction (% of control)
800
600
400
200
0
0 6 12 18 24 30 36 42 48
hours after tamoxifen

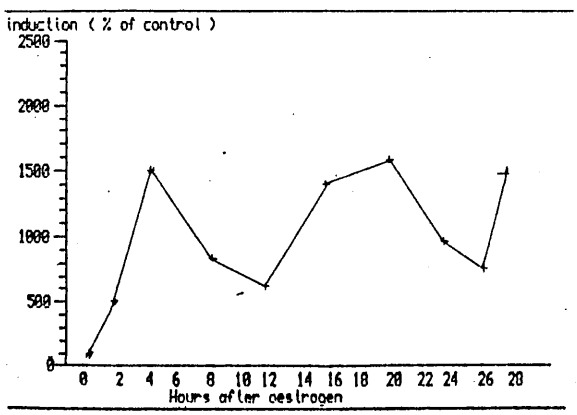
C.


Induction (% of control)
1500
1000
500
0
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28
Hours after oestrogen

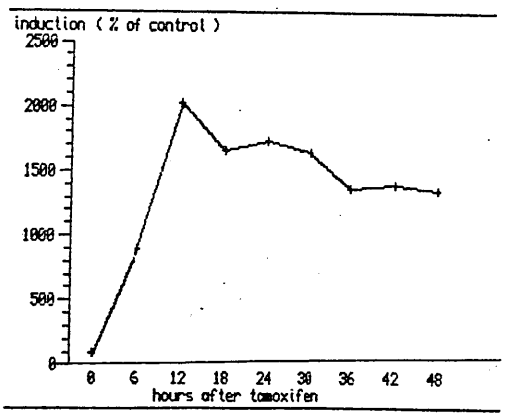
D.


induction (% of control)
1500
1000
500
0
0 6 12 18 24 30 36 42 48
hours after tamoxifen

E.


induction (% of control)
2500
2000
1500
1000
500
0
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28
Hours after oestrogen

F.


induction (% of control)
2500
2000
1500
1000
500
0
0 6 12 18 24 30 36 42 48
hours after tamoxifen

oestrogen but with the peaks occurring at 12 and 36 hours and being more drawn out. The increases in response to tamoxifen were also of approximately the same order as those induced by oestrogen except in that the initial increase in E10 RNA induced by oestrogen was only six-fold control whereas that induced by tamoxifen was twelve-fold.

Taking into account the slower uptake and metabolism of tamoxifen compared to oestrogen in the immature rat uterus (Adam et al., 1981) and the Rot hybridisation data linking 12 hours of tamoxifen treatment with 4 hours of oestrogen treatment (Waters et al., 1983), the peaks at 4 and 16 hours after oestrogen probably correspond to those at 12 and 36 hours after tamoxifen and therefore the effect of oestrogen and tamoxifen on E10 and B11 RNA levels would appear to be very similar.

The F4 recombinant was somewhat different from B11 and E10 in that although oestrogen administration resulted in peaks at 4 and 16 hours, control values were not regained, possibly implying a longer half-life for this message than for the others. Also tamoxifen administration resulted in only one peak of F4 RNA, after 12 hours, which then declined slowly. This suggested that tamoxifen and oestrogen have different effects on F4 RNA.

5.2. The Effect of Oestrogen and Tamoxifen on the Level of Expression of Oncogenes in the Immature Rat Uterus

A number of recombinant plasmids, containing inserts of specific sequences, were obtained from other laboratories and their expression and possible regulation by oestrogen and tamoxifen in the immature rat uterus

studied. Among these were a number of recombinants encoding viral oncogenes (v-oncs). These retroviral sequences cause the transformation of cells from a normal to a neoplastic state (Weinberg, 1982). They are not necessary for the replication of the retrovirus harbouring them (Sheiness et al., 1980; Hughes et al., 1979) and this, together with the fact that cellular homologues of almost all v-oncs so far identified have been found in all vertebrate species so far examined (Bishop 1983; Weiss et al., 1982) and in Drosophila and yeast (Shilo and Weinberg, 1981; Gallwitz et al., 1983; Ze'ev et al., 1984) has led to the suggestion that v-oncs have arisen by transduction of cellular genes (c-oncs). (Review: Bishop, 1983). The extent of conservation of oncogene sequences among different species implies a fundamental role for the proteins encoded by them in normal cells (Stehelin et al., 1976; Bishop, 1981).

The ability of both viral and cellular oncogenes to cause uncontrolled cellular proliferation has been shown to be due to over expression, amplification, point mutations (Schwab et al., 1983; Kozbor and Croce, 1984) and, possibly, to loss of their cell-cycle dependent regulation (Campisi et al., 1984; Kelly et al., 1983; Robertson, 1984).

The fact that these genes are expressed in proliferating cells and that administration of oestrogen causes the cells of the immature rat uterus to proliferate suggested they would be interesting to study in this system.

Cloned sequences of v-Ha-ras, v-Ki-ras and v-myc were obtained as these oncogenes have been shown to be expressed in a wider variety of both normal and tumour tissues than other oncogenes (Robertson, 1984). A recombinant containing a c-sis sequence was also obtained. The results of these studies are shown below.

5.2.1. Quantitation of the Effect of Oestrogen and Tamoxifen on the Expression of c-myc Specific Sequences in the Immature Rat Uterus

The plasmid p-myc-2 consists of a 500bp insert encoding the v-myc sequence of avian myeloblastosis virus in pBR322 and was obtained from the Beatson Institute, Glasgow, as were all the oncogene clones.

Intact nick-translated plasmid pBR322 was hybridised to samples of oestrogen-induced mRNA and the filter autoradiographed. No binding of pBR322 to uterine RNA was observed (result not shown) and therefore intact plasmids, as opposed to purified inserts, were nick-translated and used as probes.

Samples of oestrogen-stimulated polyA+RNA were resolved on agarose/formaldehyde gels, blotted onto nitrocellulose and hybridised to nick-translated p-myc-2. The resulting autoradiograph demonstrated the presence of myc specific sequences in the immature rat uterus. The sizes of these were estimated, using 18S and 28S markers, to be 6.6, 4.9, 3.6 and 2.5Kb, the major band being 4.9Kb (Fig. 28A). As the same amount of polyA+RNA had been resolved per time point, and the background on the film was very low, each track was scanned five times using the densitometer, the values obtained averaged and plotted against the time of oestrogen treatment (Fig. 28B). This showed a considerable increase in the amount of the major myc specific RNA between 2-4 hours after oestrogen which declined to control level by 8 hours. A second peak occurred after 12 hours which declined to about zero by 20 hours. This was followed by a third increase at 24-28 hours.

Figure 28

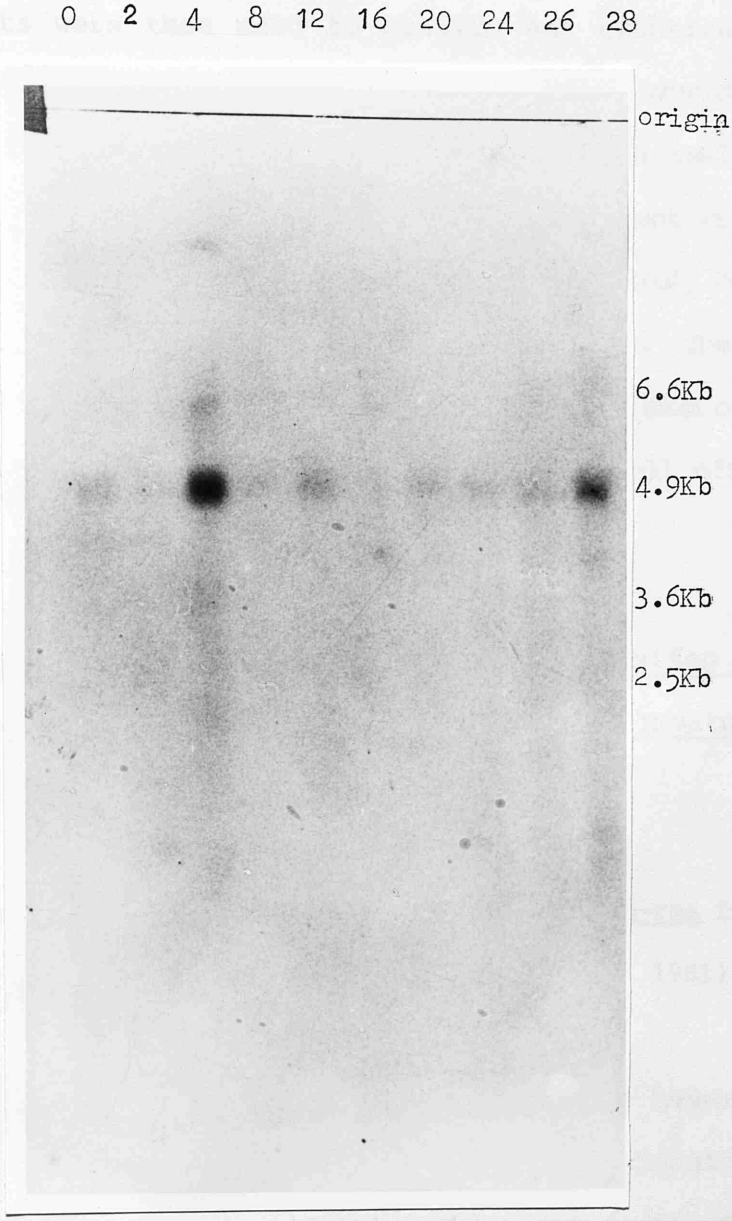
Northern Blot Analysis of the Effect of Oestrogen on Sequences Complementary to v-myc Sequence in the Immature Rat Uterus

A. 2.5µg samples of polyA+RNA obtained as described in materials and methods 3.2.3, 3.3, from the uteri of 21 day old rats which had received 1µg oestradiol in saline, or saline alone, for the times indicated, were resolved on a 0.8% agarose/2.2M formaldehyde gel, blotted onto a nitrocellulose filter and hybridised to plasmid p-myc-2 labelled with ^{32}P by nick-translation (materials and methods 7.1, 7.3). The filter was then washed, sealed in a plastic bag and autoradiographed.

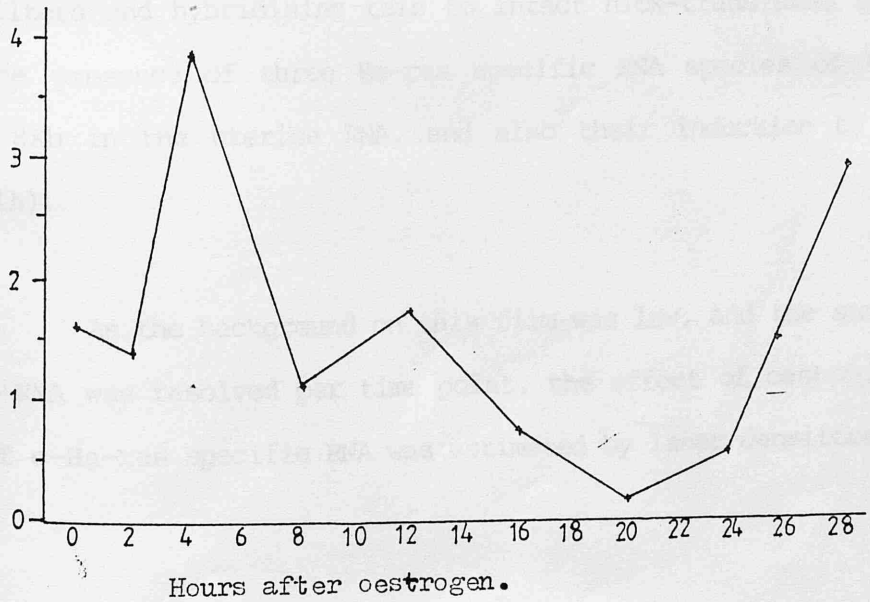
The sizes of the bands were estimated using the position of 28S and 18S rRNA markers.

B. The above autoradiograph was scanned using a laser densitometer and the average value over five scans of each track, for the major myc RNA species plotted against the duration of oestrogen treatment.

A.



B. Density(arbitrary units).



RNA dot blots were then used to confirm and quantitate the induction of c-myc by oestrogen. In this case the amount of hybridisation is due to all four hybridising RNA species. This showed a twelve-fold increase in c-myc RNA within four hours of oestrogen treatment which had returned to normal by 8 hours. A second peak, six-times control, occurred at 12 hours and fell to control levels by 24 hours (Fig. 29). The effect of tamoxifen (FIg 30) was to cause a steady increase to a maximum of about five-fold between 18-36 hours after treatment which then fell off until control levels were regained by 48 hours.

5.2.2 Quantitation of the Effect of Oestrogen and Tamoxifen on the Expression of c-Ha-ras Specific Sequences in the Immature Rat Uterus

The BS9 clone consisted of a 500bp insert of the v-Ha-ras fragment of cloned Harvey Murine Sarcoma virus in pBR322 (Ellis et al., 1981).

The possible expression of the c-Ha-ras gene in the immature rat uterus was examined by resolving samples of oestrogen-stimulated poly A+RNA on agarose/formaldehyde gels, blotting these onto nitrocellulose filters and hybridising this to intact nick-translated BS9. This showed the presence of three Ha-ras specific RNA species of 4.6Kb, 2.7Kb and 1.8Kb in the uterine RNA, and also their induction by oestrogen (Fig. 31A).

As the background on this film was low, and the same amount of poly A+RNA was resolved per time point, the effect of oestrogen on the amount of c-Ha-ras specific RNA was estimated by laser densitometry of the film.

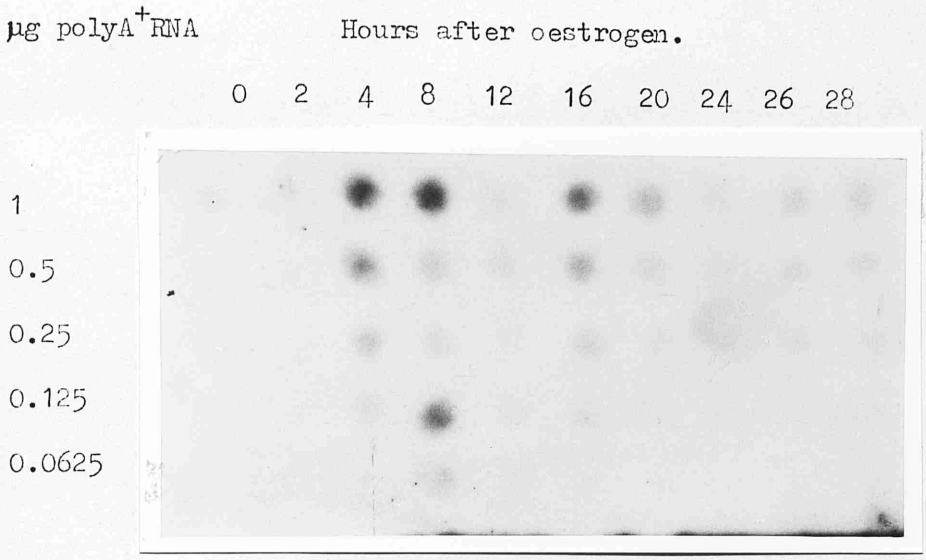
Figure 29

The Effect of Oestrogen on the Level of RNA Sequences Complementary to v-myc in the Immature Rat Uterus

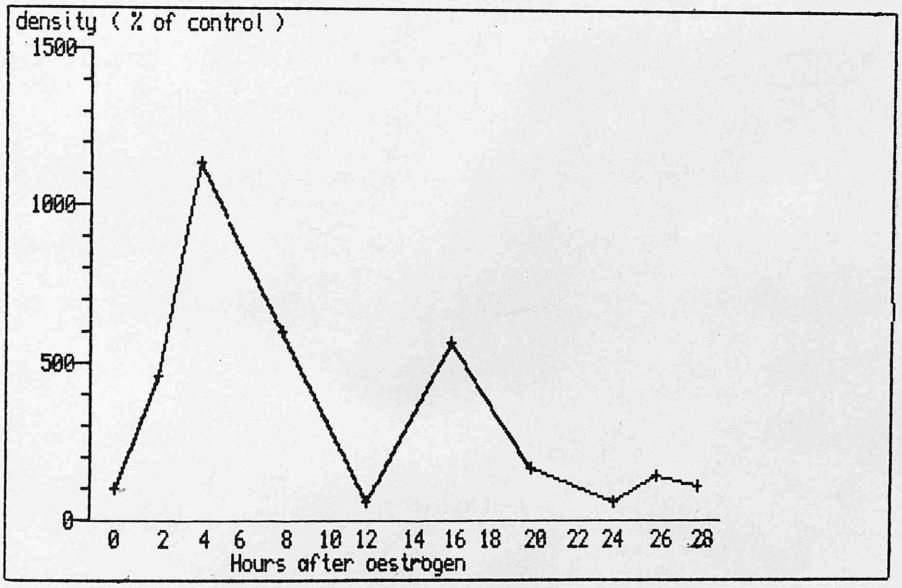
A. Samples of polyA+RNA isolated from the uteri of 21 day old rats weighing 30-35g which had received either 1μg of oestradiol in saline, or saline alone, at various times before death by the procedure described in materials and methods 3.2.3, 3.3 and corrected for contamination with rRNA, were denatured and serial dilutions prepared. The RNA was then dotted onto nitrocellulose filter and hybridised to plasmid p-myc-2 labelled with ^{32}P by nick-translation (materials and methods 7.2, 7.3). The filter was then washed and autoradiographed.

B. Each row of the autoradiograph shown in A was scanned using a laser densitometer and the mean and percentage standard deviation (% SD) for each polyA+RNA preparation used calculated as described in Results 4.2.1. The mean values were then calculated as a percentage of the control values (0 hours) and plotted against the duration of treatment.

A.

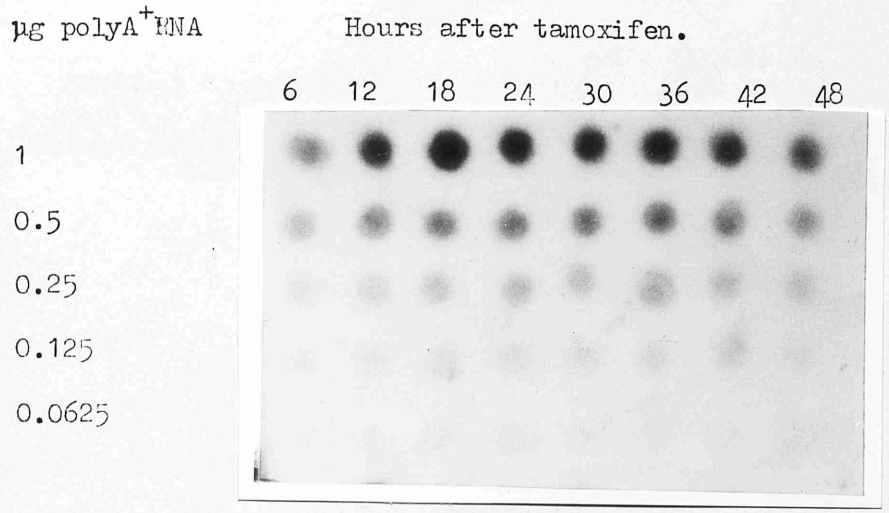


B.

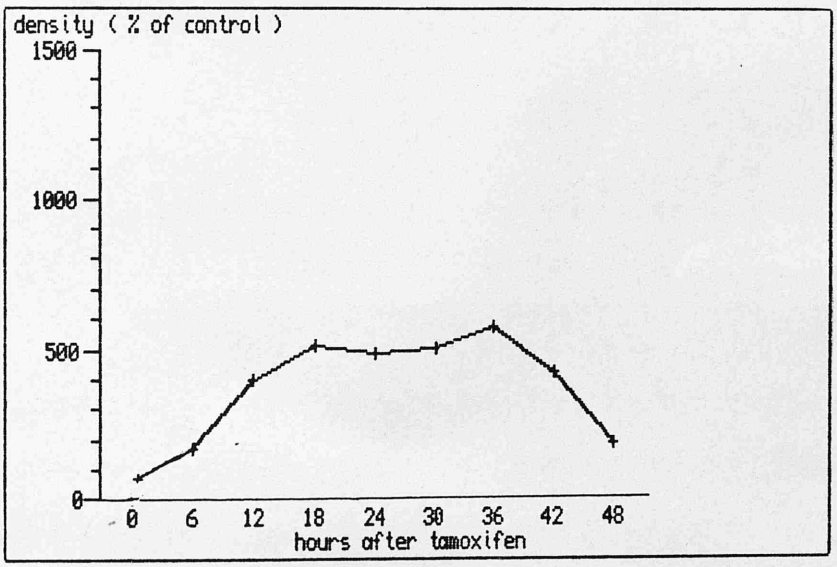


Hours after oestrogen.	0	2	4	8	12	16	20	24	26	28
%S.D.	6.1	5.9	2.6	44.3	83.3	17.7	25.1	77.1	43.1	13.9

A.



B.



Hours after tamoxifen.	0	6	12	18	24	30	36	42	48
%S.D.	6.1	24.0	19.9	13.9	10.2	2.5	5.1	9.8	58.4

Figure 30

The Effect of Tamoxifen on the Level of RNA Sequences Complementary to v-myc in the Immature Rat Uterus

A. Samples of polyA+RNA isolated from the uteri of 21 day old rats weighing 30-35g which had received 30μg of tamoxifen in corn-oil at various times before death by the procedure described in materials and methods 3.2.3, 3.3 and corrected for contamination with rRNA, were denatured and serial dilutions prepared. The RNA was then dotted onto a nitrocellulose filter and hybridised to plasmid p-myc-2 labelled with ^{32}P by nick-translation (materials and methods 7.2, 7.3). The filter was then washed and autoradiographed

B. Each row of the autoradiograph shown in A was scanned using a laser densitometer and the mean and percentage standard deviation (% SD) for each polyA+RNA preparation used calculated as described in Results 4.2.1. The values were then calculated as a percentage of the control values (0 hours) and plotted against the duration of treatment.

Figure 31

Northern Blot Analysis of the Effect of Oestrogen on Sequences Complementary to v-Ha-ras Sequence in the Immature Rat Uterus

A. 2.5µg samples of polyA+RNA, obtained as described in materials and methods 3.2.3, 3.3, from the uteri of 21 day old rats which had received 1µg oestradiol in saline, or saline alone, for the times indicated, were resolved on a 0.8% agarose/2.2M formaldehyde gel, blotted onto nitrocellulose filter, and hybridised to plasmid BS9 labelled with ^{32}P by nick-translation (materials and methods 7.1, 7.3). The filters were then washed, sealed in plastic bags and autoradiographed.

The sizes of the bands were estimated using the position of 28S and 18S rRNA markers.

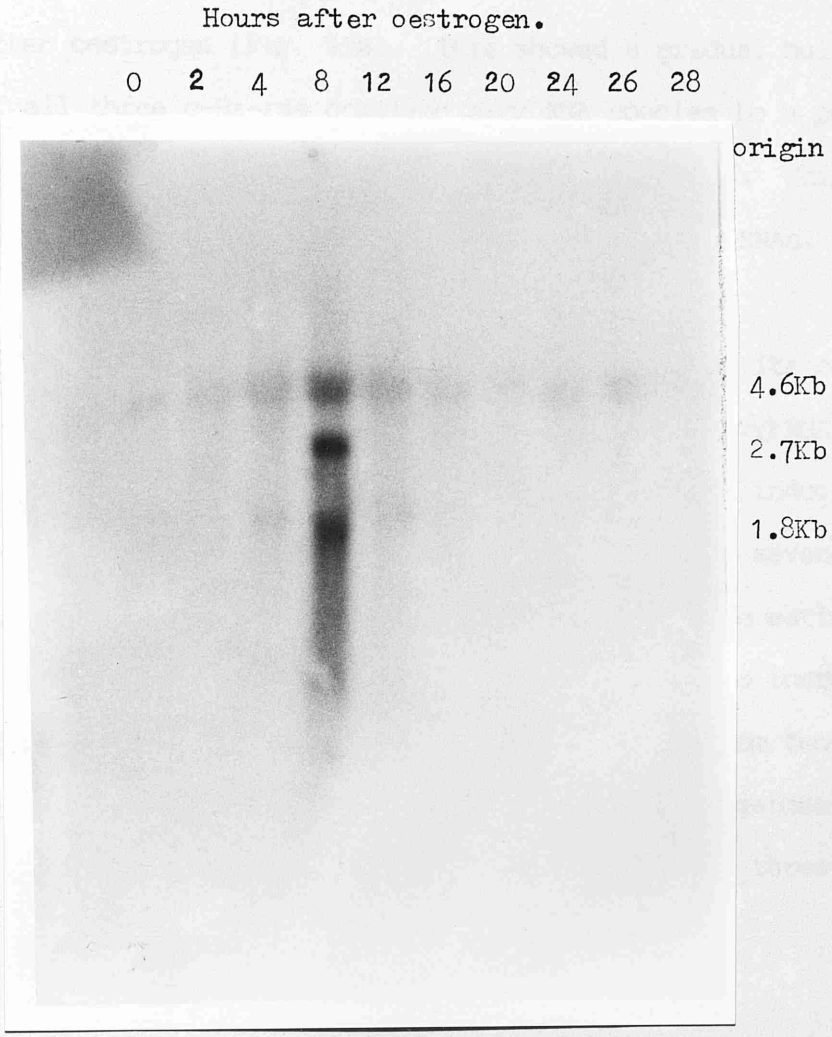
B. The autoradiograph in A was scanned using a laser densitometer and the average value, over five scans of each track, for the major Ha-ras species, plotted against the duration of oestrogen treatment.

—·—·— 4.6Kb

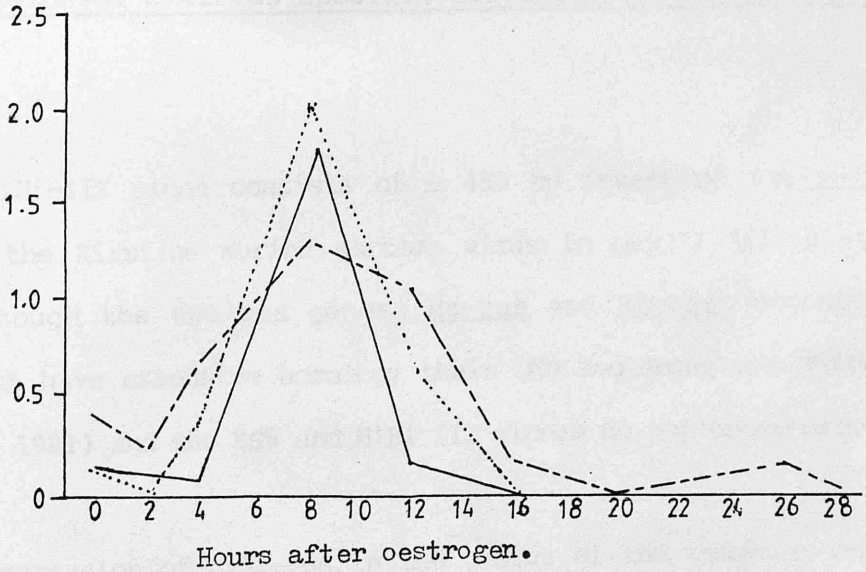
····· 2.7Kb

——— 1.8Kb

A.



B. Density(arbritary units).



Each track was scanned five times, the readings averaged and plotted against time after oestrogen (Fig. 31B). This showed a gradual build up of the level of all three c-Ha-ras complementary RNA species to a peak 8 hours after hormone administration, after which levels fell off. This was confirmed with a number of different batches of uterine polyA+RNAs.

RNA dot blots were then used in order to check and quantitate this induction. These were carried out on one batch of uterine polyA+RNA and, unlike the results from the northern blots, gave two peaks of induction, one nine-fold control levels between 4-8 hours, and a second, seven-fold control at 16 hours (Fig. 32). The effect of tamoxifen was also estimated using RNA dot blots (Fig. 33). This showed that tamoxifen also increased the amount of c-Ha-ras RNA/unit of polyA+RNA. An increase from two-fold to six-fold control levels occurred between 6-12 hours after treatment and was maintained until 42 hours, after which it declined to about three-fold control.

5.2.3 Quantitation of the Effect of Oestrogen and Tamoxifen on the Expression of c-ki-ras Specific Sequences in the Immature Rat Uterus

The HiHi-III clone consists of a 450 bp insert of the v-Ki-ras fragment of the Kirstine murine sarcoma virus in pBR322 (Ellis et al., 1981). Although the two ras genes, Ha-ras and Ki-ras, encode 21Kd proteins which have extensive homology their DNA sequences are different (Ellis et al., 1981) and the BS9 and HiHi III clones do not cross-react.

The expression of c-Ki-ras in the uterus of the immature rat was demonstrated by northern blot analysis of 4 hour oestrogen-induced poly

Figure 32

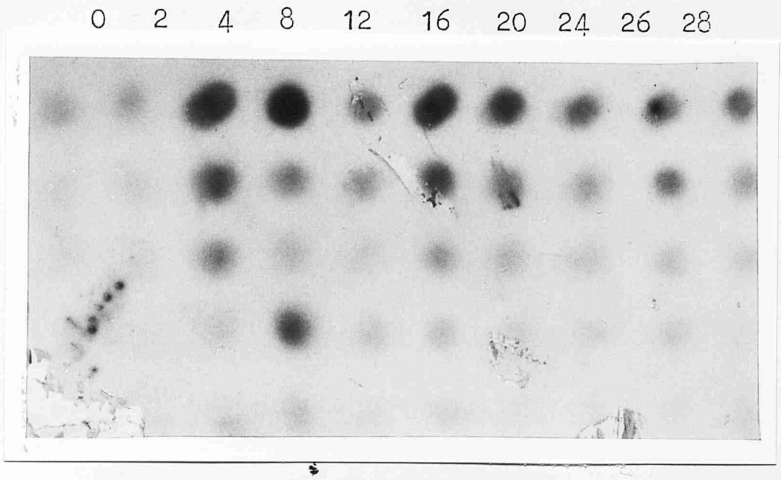
The Effect of Oestrogen on the Level of RNA Sequences Complementary to v-Ha-ras in the Immature Rat Uterus

A. Samples of polyA+RNA isolated from the uteri of 21 day old rats weighing 30-35g which had received either 1μg of oestradiol in saline, or saline alone, at various times before death by the procedure described in materials and methods 3.2.3, 3.3 and corrected for contamination with rRNA, were denatured and serial dilutions prepared. The RNA was then dotted onto a nitrocellulose filter and hybridised to plasmid BS9 labelled with ^{32}P by nick-translation (materials and methods 7.2, 7.3). The filter was then washed and autoradiographed.

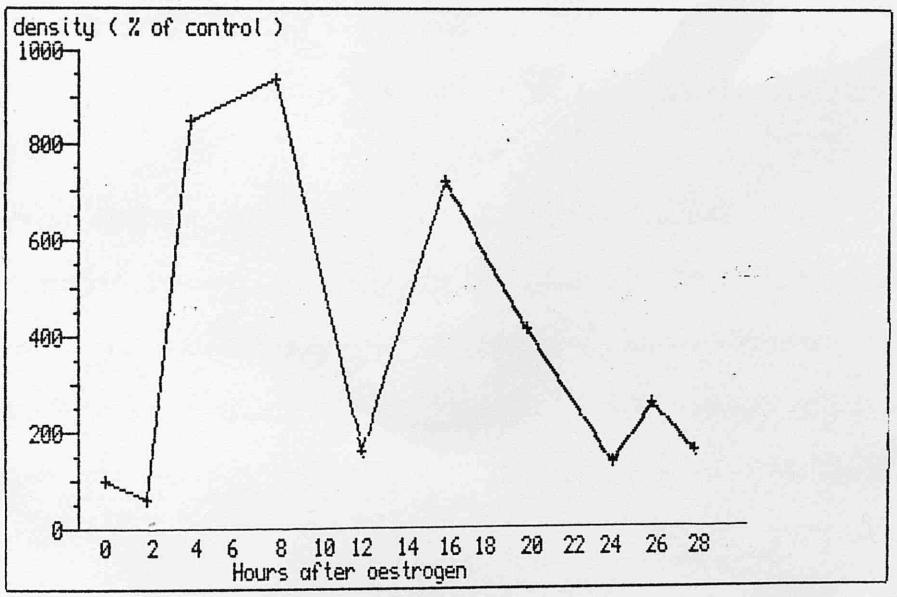
B. Each row of the autoradiograph shown in A was scanned using a laser densitometer and the mean and percentage standard deviation (% SD) for each polyA+RNA preparation used calculated as described in Result 4.2.1. The mean values were then calculated as a percentage of the control value (0 hours) and plotted against the duration of treatment.

A.

µg polyA$^+$RNA Hours after oestrogen.



B.



Hours after oestrogen.	0	2	4	8	12	16	20	24	26	28
%S.D.	7.3	49.9	33.7	38.9	28.0	19.0	6.5	19.7	44.0	33.7

Figure 33

The Effect of Tamoxifen on the Level of RNA Sequences Complementary to v-Ha-ras in the Immature Rat Uterus

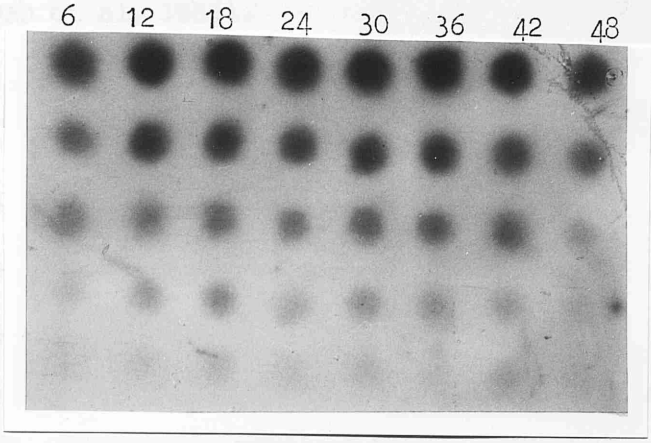
A. Samples of polyA+RNA isolated from the uteri of 21 day old rats weighing 30-35g which had received 30μg of tamoxifen in corn-oil at various times before death by the procedure described in materials and methods 3.2.3, 3.3 and corrected for contamination with rRNA, were denatured and serial dilutions prepared. The RNA was then dotted onto a nitrocellulose filter and hybridised to plasmid BS9 labelled with ^{32}P by nick-translation (materials and methods 7.2, 7.3). The filter was then washed and autoradiographed.

B. Each row of the autoradiograph shown in A was scanned using a laser densitometer and the mean and percentage standard deviation (% SD) for each polyA+RNA preparation used calculated as described in Results 4.2.1. The values were then calculated as a percentage of the control values (0 hours) and plotted against the duration of treatment.

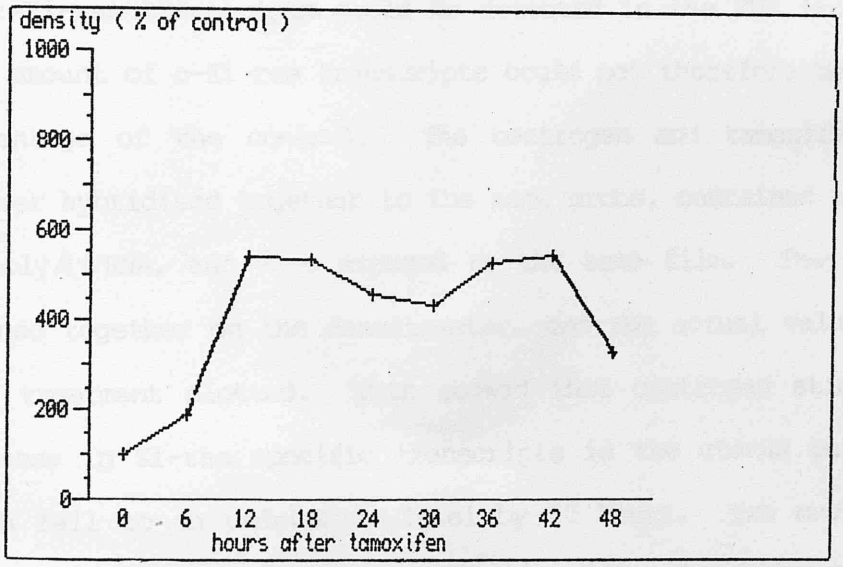
A.

μg polyA$^+$RNA

Hours after tamoxifen.



B.



Hours after tamoxifen.	0	6	12	18	24	30	36	42	48
%S.D.	7.3	27.8	17.2	10.4	9.6	17.0	11.9	14.9	5.5

A+RNA. This showed the presence of complementary RNAs of 22Kb, 5Kb and 1.2Kb (Fig. 34). The 5Kb and 1.2Kb species have been reported in other tissues (Schwab et al, 1983).

The background binding of this probe to all the filters used was very high, even though the plasmid preparation itself, when resolved on a 1% agarose gel (materials and methods 4.2) appeared uncontaminated. This made the resulting films unsuitable for scanning and RNA dot blots were used to determine the effect of oestrogen and tamoxifen on the level of c-Ki-ras transcripts.

Figure 35 shows that, at the level of detection of this method, no c-Ki-ras transcripts could be detected in the RNA from control rats. The amount of c-Ki-ras transcripts could not therefore be expressed as a percentage of the control. The oestrogen and tamoxifen filters were however hybridised together to the same probe, contained the same amounts of polyA+ RNA, and were exposed to the same film. They were therefore scanned together on the densitometer, and the actual values obtained for each treatment plotted. This showed that oestrogen stimulated a rapid increase in Ki-ras specific transcripts in the uterus between 2-4 hours which fell to an undetectable level by 12 hours. Two much smaller peaks occurred at 16 hours and 26 hours (Fig. 35). Tamoxifen caused a smaller sustained increase in c-Ki-ras RNA, levels rising by 6 hours and not falling until 36 hours after treatment (Fig. 36).

5.2.4 Quantitation of the Effect of Oestrogen and Tamoxifen on the Expression of c-sis Specific Sequences in the Immature Rat Uterus

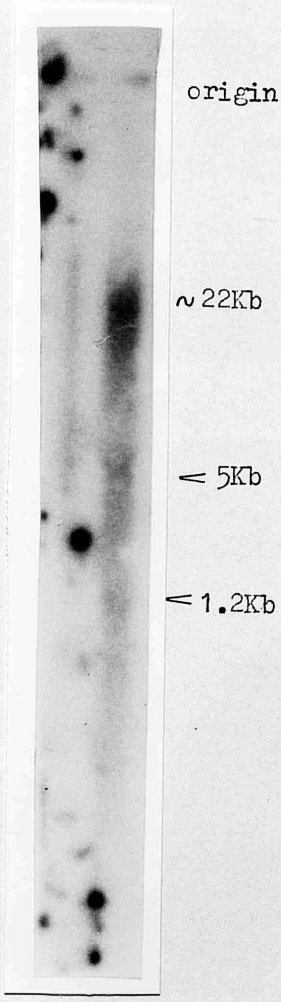
The c-sis clone consisted of a 1700bp insert of the human c-sis gene in pBR322

Figure 34

Northern Blot Analysis of 4 hour Oestrogen-Induced Uterine PolyA+RNA for Sequences Complementary to v-Ki-ras Sequence

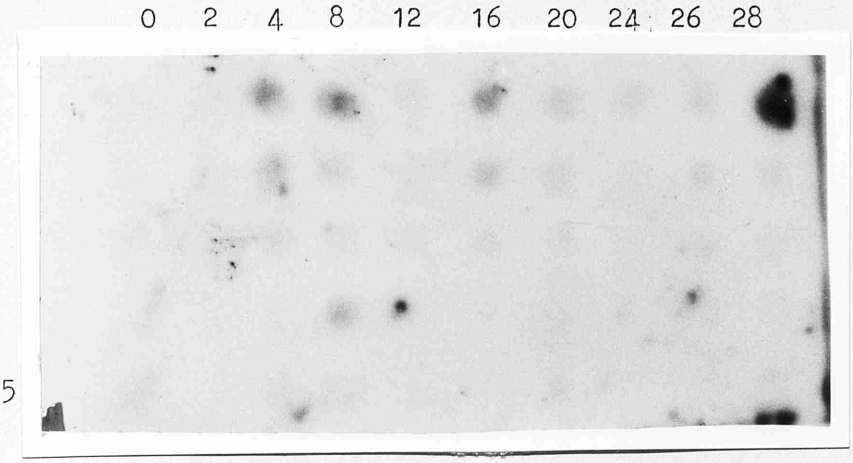
2.5µg of polyA+RNA, obtained as described in materials and methods 3.2.3, 3.3, from the uteri of immature rats given 1µg of oestradiol 4 hours before death, was resolved on a 0.8% agarose/2.2M formaldehyde gel, blotted onto a nitrocellulose filter, and hybridised to plasmid HiHi III labelled with ^{32}P by nick-translation (materials and methods 7.1, 7.3). The filter was then washed and autoradiographed.

The sizes of the bands were estimated using the position of 28S and 18S rRNA markers.

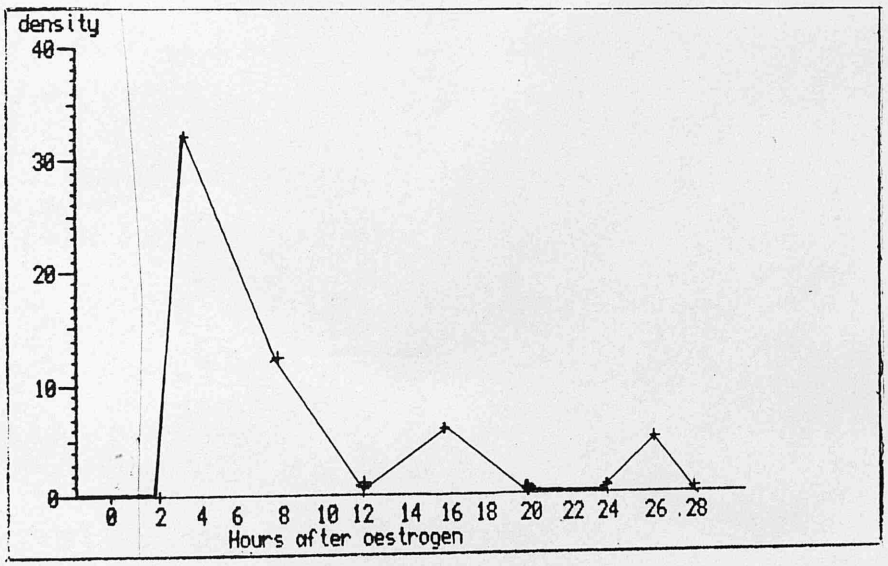

origin
~22Kb
< 5Kb
< 1.2Kb

A.

µg polyA$^+$RNA Hours after oestrogen.



B.



Hours after oestrogen.	0	2	4	8	12	16	20	24	26	28
%S.D.	-	-	13.9	40.6	-	41.6	-	-	37.2	-

Figure 35

The Effect of Oestrogen on the Level of RNA Sequences Complementary to v-Ki-ras in the Immature Rat Uterus

A. Samples of polyA+RNA isolated from the uteri of 21 day old rats weighing 30-35g which had received 1μg of oestradiol in saline, or saline alone, at various times before death by the procedure described in materials and methods 3.2.3, 3.3 and corrected for contamination with rRNA, were denatured and serial dilutions prepared. The RNA was then dotted onto a nitrocellulose filter and hybridised to plasmid HiHi III labelled with ^{32}P by nick-translation (materials and methods 7.2, 7.3). The filter was then washed and autoradiographed.

B. Each row of the autoradiograph shown in A was scanned using a laser densitometer and the mean and percentage standard deviation (% SD) for each polyA+RNA preparation used calculated as described in Results 4.2.1. The mean values were plotted against the duration of treatment.

Figure 36

The Effect of Tamoxifen on the Level of RNA Sequences Complementary to v-Ki-ras in the Immature Rat Uterus

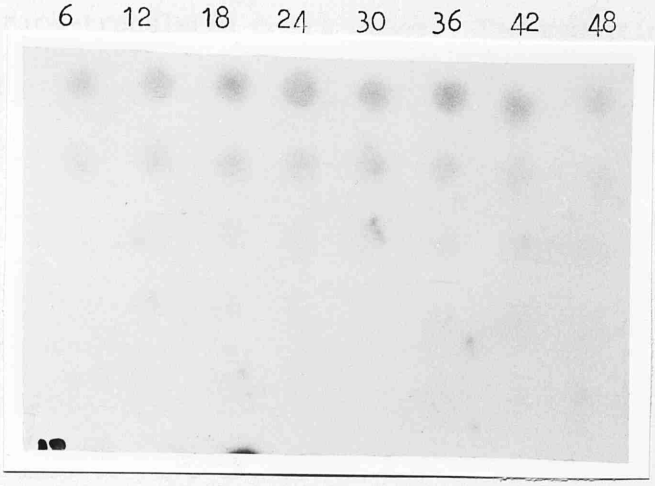
A. Samples of polyA+RNA isolated from the uteri of 21 day old rats weighing 30-35g which had received 30µg tamoxifen in corn oil at various times before death by the procedure described in materials and methods 3.2.3, 3.3 and corrected for contamination with rRNA, were denatured and serial dilutions prepared. The RNA was then dotted onto a nitrocellulose filter and hybridised to plasmid HiHi III labelled with ^{32}P by nick-translation (materials and methods 7.2, 7.3). The filter was then washed and autoradiographed.

B. Each row of the autoradiograph shown in A was scanned using a laser densitometer and the mean and percentage standard deviation (% SD) for each polyA+RNA preparation used calculated as described in Results 4.2.1 The mean values were then plotted against the duration of treatment.

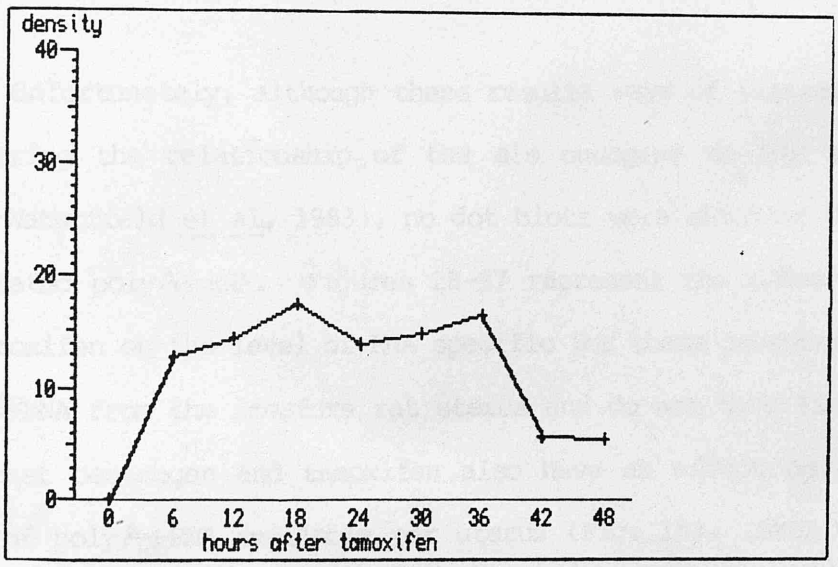
A.

µg polyA$^+$RNA

Hours after tamoxifen.



B.



Hours after tamoxifen.	0	6	12	18	24	30	36	42	48
%S.D.	-	4.6	11.8	5.7	9.7	23.4	19.2	48.9	-

Samples of oestrogen stimulated polyA+RNA were resolved on agarose/formaldehyde gels, blotted onto nitrocellulose filters and hybridised to nick-translated c-sis clone. The resulting autoradiograph, Figure 37A, showed the presence of a c-sis transcript of 4.8Kb in the uterus. Transcripts of 4.2Kb and 5.3Kb have been reported elsewhere (Gazit et al., 1984). Although there is an obvious peak of c-sis RNA at 8-12 hours after oestrogen, the amounts present seemed to increase and decrease rather erratically. Northern blot analysis of tamoxifen-inducecd RNA also demonstrated the presence of one c-sis transcript, again at 4.8Kb, which seemed to have two peaks; one at 12 hours and the other at 36 hours, but neither to the same extent as that observed with oestrogen (Fig. 36B).

Unfortunately, although these results were of interest, especially considering the relationship of the sis oncogene to the growth factor, PDGF (Waterfield et al., 1983), no dot blots were obtained due to lack of quantitated polyA+RNA. Figures 28-37 represent the effect of oestrogen and tamoxifen on the level of RNA specific for these oncogenes per unit of polyA+RNA from the immature rat uterus and do not take into account the fact that oestrogen and tamoxifen also have an effect on the number of units of polyA+RNA available per uterus (Fig. 15). When this is taken into account, by multiplying the amount of RNA specific for each sequence in one unit of polyA+RNA at a particular point by the number of units available per uterus at that point, it can be seen that the effect of oestrogen on c-myc, c-Ha-ras and c-Ki-ras RNAs are very similar, as are the effects of tamoxifen (Fig. 38).

Oestrogen administration resulted in a considerable but short-lived surge in the levels of the RNAs specific for the three oncogenes, myc,

Figure 37

Northern Blot Analysis of the Effect of Oestrogen and Tamoxifen on Sequences Complementary to the c-sis Sequence in the Immature Rat Uterus

2.5µg of polyA+RNA, quantitated as described in Results 4.2.1, from the uteri of 21 day old rats which had received either:

(a) 1µg of oestradiol in saline

(b) 30µg of tamoxifen in corn-oil

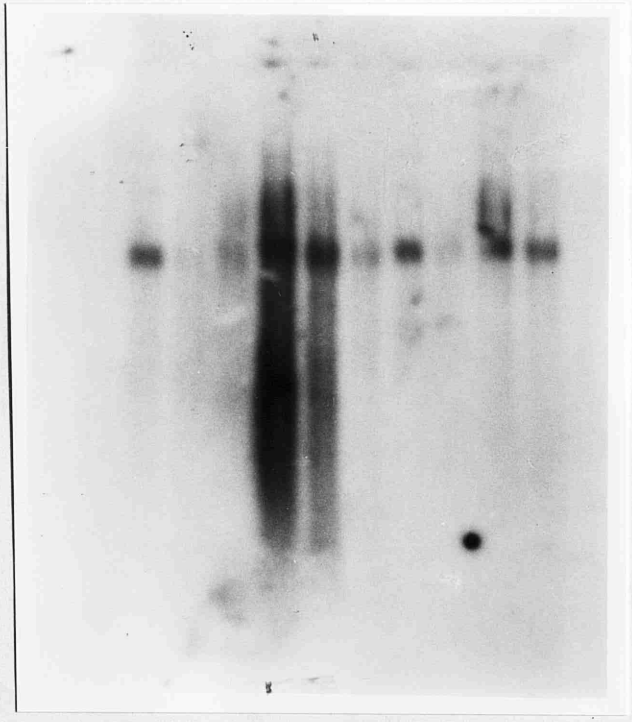
(c) carrier alone

for the times indicated, were resolved on 0.8% agarose/2.2M formaldehyde gels, blotted onto nitrocellulose and hybridised to the nick-translated c-sis plasmid. The filters were then washed and autoradiographed (4.5, 7.1, 7.3).

The sizes of the bands were estimated from the position of the 28S and 18S rRNA markers.

Hours after oestrogen.

0 2 4 8 12 16 20 24 26 28

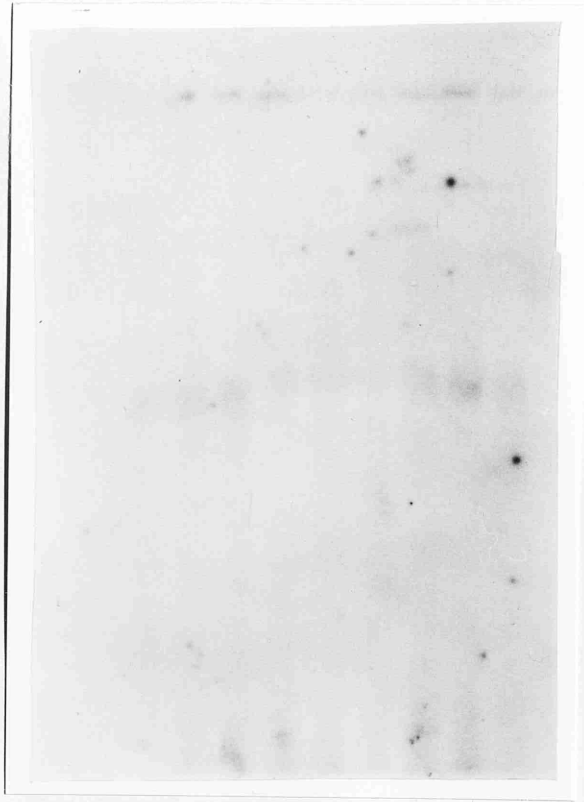


origin

4.8Kb

Hours after tamoxifen.

0 6 12 18 24 30 36 42 48



origin

4.8Kb

Figure 38

The Effect of Oestrogen and Tamoxifen on the Level of c-myc c-Ha-ras, and c-Ki-ras RNA per rat uterus

These figures were derived by multiplying the amount of c-myc, c-Ha-ras and c-Ki-ras RNA per unit of uterine polyA+RNA from oestrogen and tamoxifen treated rats, 21 day old and weighing 30-35g (Figs. 29, 30, 32, 33, 35, 36), by the amount of polyA+RNA available per uterus after each treatment (relative to control uteri) (Fig. 15). The results are expressed as a percentage of the value obtained for the amounts of each RNA per control uterus except for c-Ki-ras, which is expressed in arbitrary units.

A. Effect of oestrogen on the level of c-myc RNA per uterus

B. Effect of tamoxifen on the level of c-myc RNA per uterus

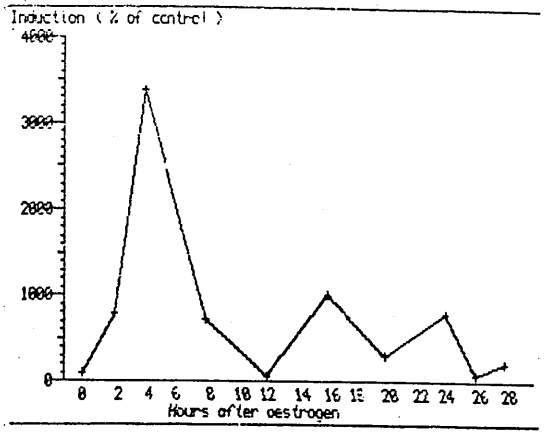
C. Effect of oestrogen on the level of c-Ha-ras RNA per uterus

D. Effect of tamoxifen on the level of c-Ha-ras RNA per uterus

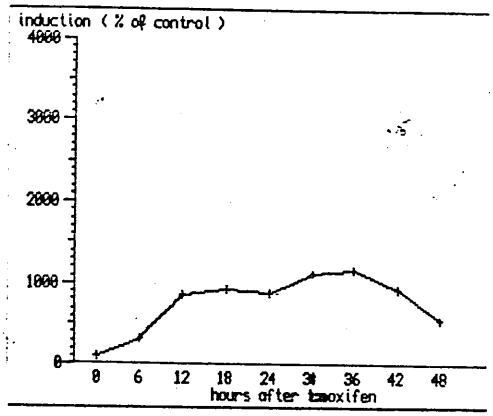
E. Effect of oestrogen on the level of c-Ki-ras RNA per uterus

F. Effect of tamoxifen on the level of c-Ki-ras RNA per uterus

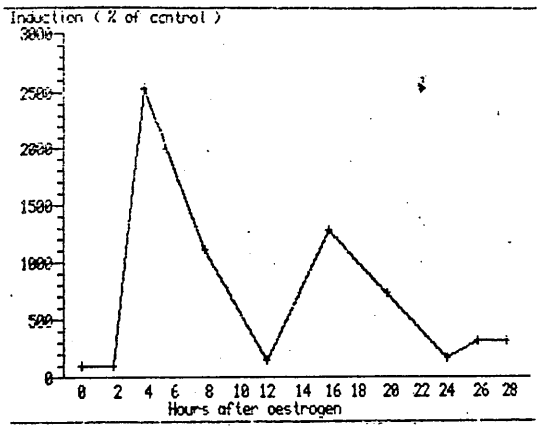
A.


Induction (% of control)
4000
3000
2000
1000
0
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28
Hours after oestrogen

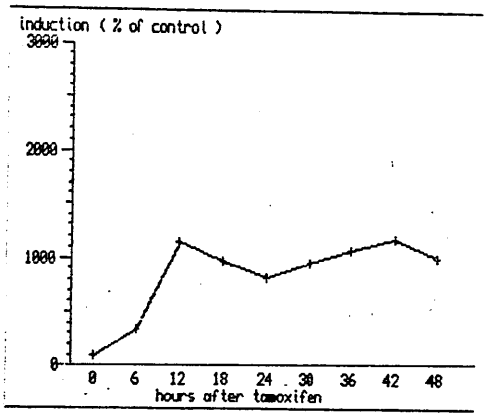
B


induction (% of control)
4000
3000
2000
1000
0
0 6 12 18 24 30 36 42 48
hours after tamoxifen

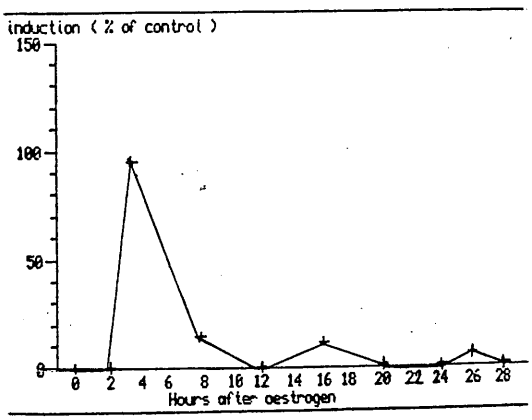
C


Induction (% of control)
3000
2500
2000
1500
1000
500
0
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28
Hours after oestrogen

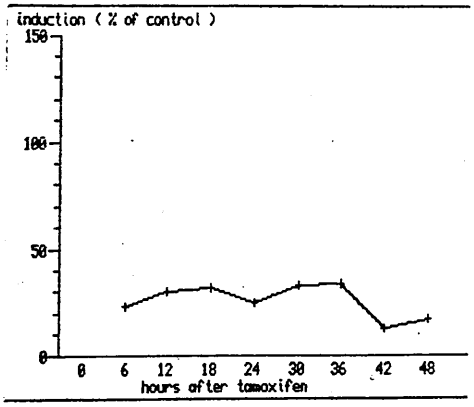
D


induction (% of control)
3000
2000
1000
0
0 6 12 18 24 30 36 42 48
hours after tamoxifen

E


induction (% of control)
150
100
50
0
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28
Hours after oestrogen

F


induction (% of control)
150
100
50
0
0 6 12 18 24 30 36 42 48
hours after tamoxifen

Ha-ras and Ki-ras, with maximum levels being obtained within 4 hours of treatment and control levels being regained by 12 hours. A second, smaller, increase was observed after 16 hours.

Tamoxifen administration resulted, in all three cases, in a smaller more sustained increase than those observed in response to oestrogen, maximum levels being found after 12 hours, and being maintained until 36-42 hours after treatment.

This data suggests a difference in the expression of these three oncogenes in the immature rat uterus in response to oestrogen and tamoxifen. However, taking into account the different rates of uptake, accumulation in the nucleus, the effects on oestrogen receptor replenishment, and the short half-lives of the oncogene mRNAs, a slower induction by tamoxifen would be expected.

Other clones examined were a recombinant plasmid containing a rat skeletal muscle actin cDNA sequence (p749), and an oestrogen-induced cDNA clone isolated from a cDNA library to oestrogen-treated MCF-7 human breast cancer cells (pS2).

5.3 The Effect of Oestrogen and Tamoxifen on Actin and pS2 RNA Expression in the Immature Rat Uterus

5.3.1 Actin

p749 contained a 350bp insert of rat skeletal muscle actin cDNA in plasmid pBR322 and was obtained from Dr D.P. Leader, University of

Glasgow. It was thought that the level of actin mRNA would increase as the cells of the uterus grew in response to oestrogen, however the pattern of this increase was not known.

The effect of oestrogen on actin mRNA levels was examined by northern blotting oestrogen-induced polyA+RNA and hybridisation of this to nick-translated p749. The resulting autoradiograph showed the presence of two actin transcripts at approximately 1.4Kb and 1.5Kb (Fig. 39). Densitometric scans of this film showed that the level of actin mRNA per unit polyA+RNA increased from administration of oestrogen to a peak about twenty-times control values at 12 hours after which it decreased to normal by 16 hours. The effect of tamoxifen was similarly assessed. Tamoxifen also increased the amount of actin mRNA to a peak eight-times control value by 12 hours after administration, and then trailed off until 30 hours (Fig. 40).

Fig. 41 shows the above data, replotted to take account of the increased levels of total mRNA present in the uterus of oestrogen or tamoxifen-treated rats. It can be seen that the response to the hormone and its synthetic counterpart are similar and it is likely that differences in the response are pharmaco-kinetic in origin.

5.3.2 pS2.

The derivation of the pS2 clone will be described in the next section on gene expression in the human breast cancer cell line MCF-7. The uterine polyA+RNA samples; control, oestrogen-induced, and tamoxifen induced, were examined by northern blotting for the presence of pS2 transcripts. No hybridising sequences were detected.

Figure 39

Northern Blot Analysis of the Effect of Oestrogen and Tamoxifen on Sequences Complementary to p749 in the Immature Rat Uterus

2.5µg of polyA+RNA, quantitated as described in Section 4.2.1, from the uteri of 21 day-old rats which had received either:

(a) 1µg of oestradiol in saline

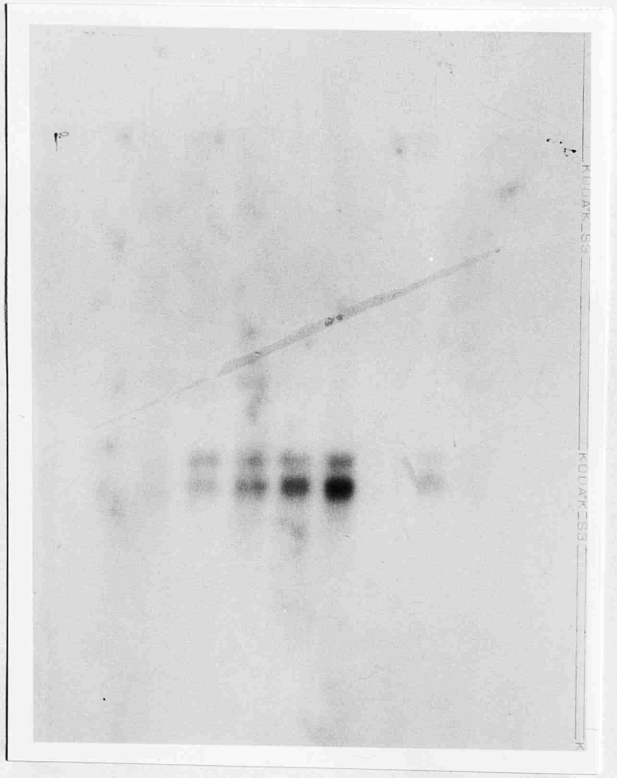
(b) 30µg of tamoxifen in corn-oil

(c) carrier alone

for the times indicated, were resolved on 0.8% agarose/2.2M formaldehyde gels, blotted onto nitrocellulose and hybridised to nick-translated p749 DNA (materials and methods 7.1, 7.3). The filters were then washed and autoradiographed. The sizes of the bands were estimated from the position of the 28S and 18S rRNA markers.

Hours after oestrogen.

0 2 4 8 12 16 24



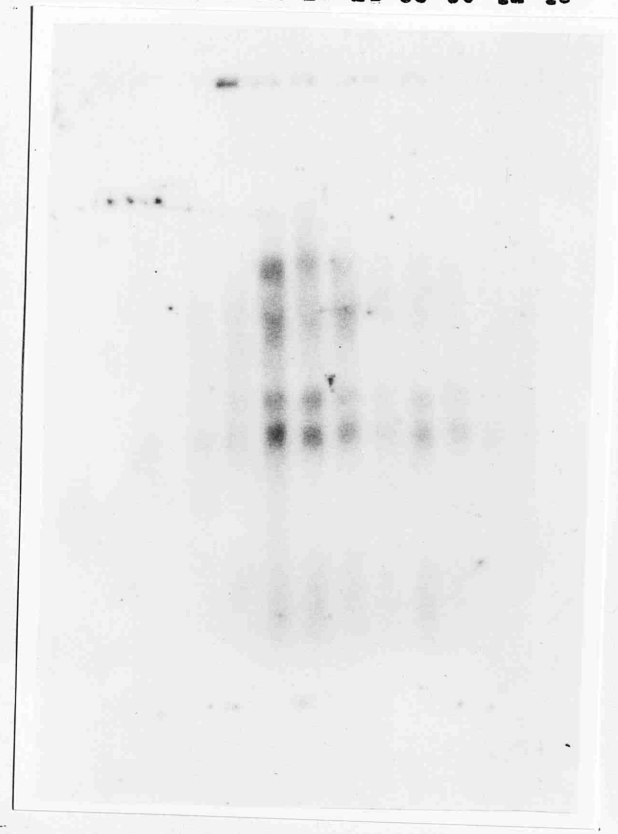
origin

1.5Kb

1.4Kb

Hours after tamoxifen.

0 6 12 18 24 30 36 42 48



origin

1.5Kb

1.4Kb

Figure 40

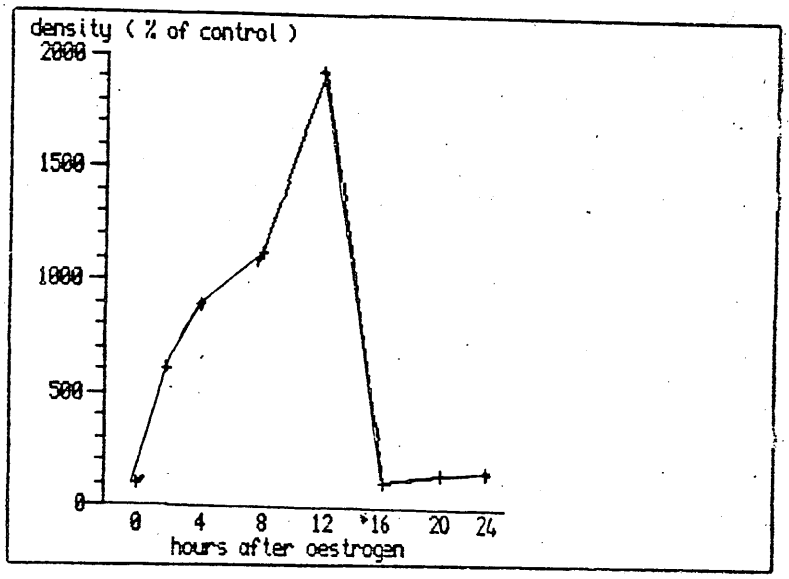
The Effect of Oestrogen and Tamoxifen on the Amount of Actin RNA per Unit of Uterine PolyA+RNA

The autoradiographs in Fig. 39 were scanned using a laser densitometer and the average value, over five scans of each track, plotted against the duration and type of treatment.

A – Oestrogen

B – Tamoxifen

A.


density (% of control)
2000
1500
1000
500
0
0
4
8
12
16
20
24
hours after oestrogen

B.

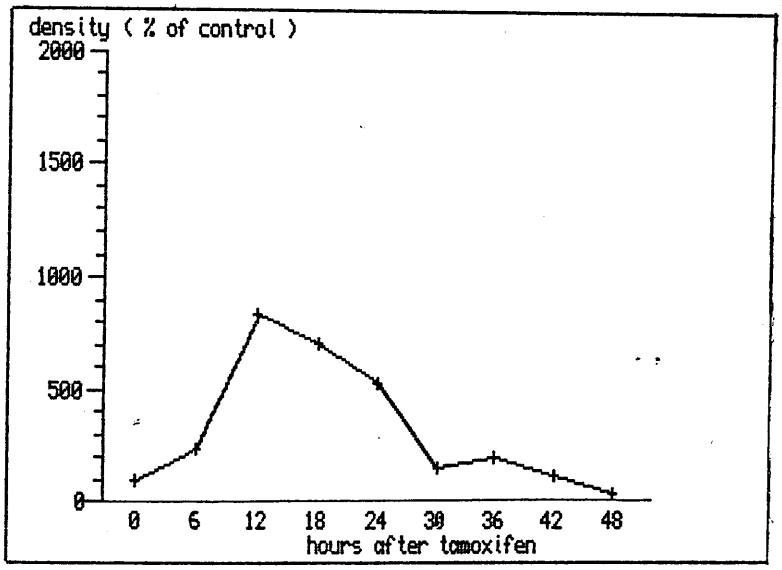

density (% of control)
2000
1500
1000
500
0
0
6
12
18
24
30
36
42
48
hours after tamoxifen

Figure 41

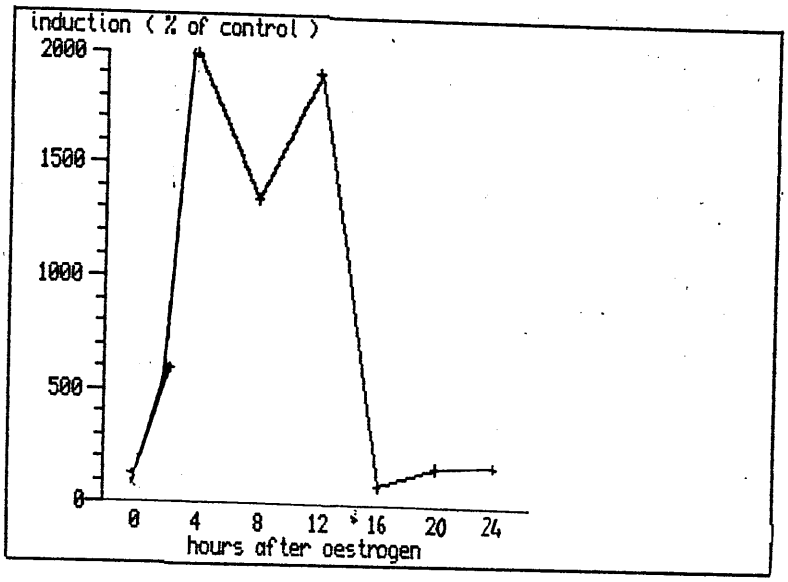
Quantitation of the Effect of Oestrogen and Tamoxifen on the Level of Actin RNA per Rat Uterus

This figure was derived by multiplying the amount of actin RNA per unit of uterine polyA+RNA from oestrogen and tamoxifen treated rats, 21 days old and weighing 30-35g (Fig. 40) by the amount of polyA+RNA available per uterus after each treatment (relative to control uteri) (Fig. 15). The results are expressed as a percentage of the value obtained for the amount of each RNA per control uterus.

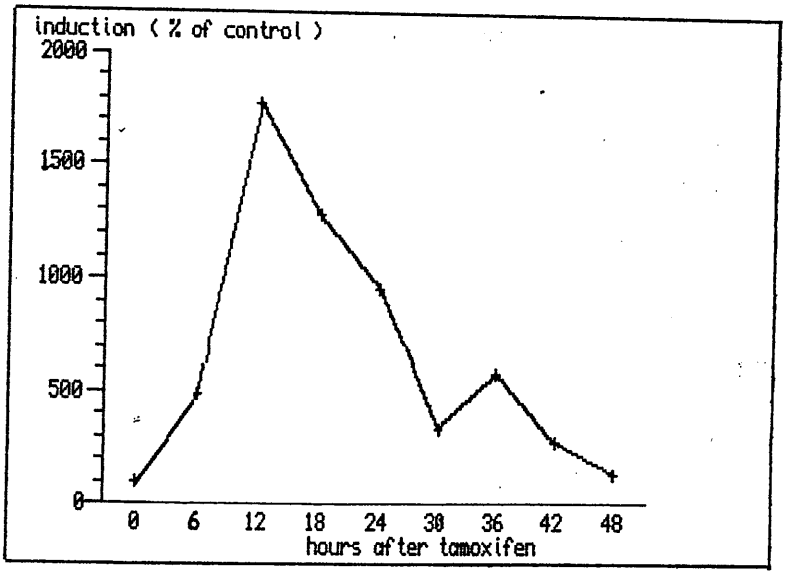
A – Oestrogen

B – Tamoxifen

A.


induction (% of control)
2000
1500
1000
500
0
0
4
8
12
16
20
24
hours after oestrogen

B.


induction (% of control)
2000
1500
1000
500
0
0
6
12
18
24
30
36
42
48
hours after tamoxifen

6.1 The Effects of Oestrogen and Tamoxifen on RNA Levels of MCF-7 Cells

The immature rat uterus has been used as a model to study the effects of oestrogens and antioestrogens. However, as antioestrogens are used to treat breast cancer, a more appropriate tissue to study their action in would be breast carcinoma. This is not easy to obtain in a form suitable for the study of gene expression. However, a number of cell lines derived from breast carcinomas are available. One of these, the MCF-7 cell line, an oestrogen receptor positive cell line derived from a pleural effusion of a metastatic breast carcinoma (Lippman et al., 1976; Brooks et al., 1973), was used to study the effects of oestradiol and tamoxifen, alone and in combination, on total RNA and poly A+RNA levels in these cells. This required, as with the immature rat uterus, the isolation of total RNA from these cells at various times after treatment with oestradiol, tamoxifen, or both, the selection of mRNA from this, and the quantitation of rRNA contamination of these mRNA preparations.

6.1.1 The Extraction of RNA from MCF-7 Cells

MCF-7 cells were grown at ICI pharmaceutical division, Alderley Park, Macclesfield, during the industrial component of this study. The mean doubling-time of these MCF-7 cells, grown under the conditions described in materials and methods 2.3.1, was approximately 36 hours. The time-courses of oestradiol and tamoxifen treatment for these cells were chosen on the basis that:

(i) tamoxifen has been shown to cause accumulation of MCF-7 cells in the G0/G1 phases of the cell cycle and therefore most cells should have been affected by the antioestrogen within one round of this cycle (Sutherland et al., 1983a, b).

(ii) the level of mRNA complementary to the oestrogen-induced MCF-7 clone, pS2, has been shown to increase very soon after oestrogen treatment, that is, within 15 minutes (Brown et al., 1984).

RNA was therefore obtained from cells treated with oestradiol for 0/30 minutes/1 hour/3 hours/8 hours/24 hours and 36 hours, with tamoxifen for the same durations, and with oestradiol and tamoxifen for 3 hours and 24 hours.

This work was carried out at ICI, Alderley Park, and the number of time points of oestradiol and/or tamoxifen treatments obtained was limited by the amount of MCF-7 cells which could be grown, treated, and used to extract RNA, within the period allocated. The time points and treatments described above were therefore a compromise between those desirable and the time available. Additional points at 18, 30 and 72 hours, and at 6 and 12 hours instead of 8 hours would have been of interest.

Oestradiol and tamoxifen were administered to cells in the late log phase of growth, that is when about 70% confluent, the medium having been changed 24 hours previously. This point was chosen so as to maximise the number of available cells while still allowing continued cell growth. As counting the number of cells per flask before and after each treatment in each flask would be very time consuming, five flasks, the number used for each treatment, were seeded with the same batch of cells and grown together until they reached approximately 70% confluence. The cells in these flasks were then counted and the percentage variation over the five flasks found to be 8%.

The cells were not counted after treatment as, within the first 24 hours after treatment, with oestradiol the cells should not divide, and they do not divide at all in response to tamoxifen (McGuire et al., 1981).

The concentrations of oestradiol and tamoxifen used were decided in consultation with Dr A.E. Wakeling of ICI Pharmaceuticals, and were based on data on the dose response of MCF-7 growth stimulation and inhibition by oestradiol and tamoxifen. Oestradiol was used at a final concentration of 10^{-8}M, shown to be the optimal concentration for stimulating cell growth (Lippman et al., 1976). Tamoxifen was used at a final concentration of 10^{-6}M. Use of this concentration avoids the cytotoxic effect of tamoxifen observed in MCF-7 cells when concentrations of 6-10 x 10^{-6}M are used (Green et al., 1981).

RNA was extracted from cells treated with carrier, oestradiol, tamoxifen, or both oestradiol and tamoxifen, for various durations by the same procedure used to extract uterine RNA, that is, the modified Kirby (1956) procedure. This was because this method had been used successfully to prepare uterine RNA, and also, by using this as opposed to one of the other extraction procedures, the results of analysis of the effect of oestradiol and tamoxifen on the level of specific mRNAs in both systems could be compared.

6.1.2 The Effect of Oestrogen and Tamoxifen on Total RNA Levels in MCF-7 Cells

RNA was obtained from cells treated for various times with oestradiol and tamoxifen. This was quantitated and the amount obtained

plotted against the length and type of treatment (Fig. 42). Administration of oestradiol and tamoxifen led to an initial decrease in the amount of RNA obtained from the MCF-7 cells. This decrease and subsequent recovery of RNA levels occurs earlier with tamoxifen than with oestrogen treated cells, however the increase is only maintained until 8 hours of tamoxifen treatment after which it seems to plateau. Conversely, there is a steady increase in the amount of RNA obtained between 3 and 36 hours after oestrogen treatment. This picture is similar to that obtained from the immature rat uterus (Fig. 12) in that tamoxifen causes an increase and levelling off of RNA levels, whereas oestradiol causes a steady increase. The major differences between the effect of these two compounds on MCF-7 cells as opposed to immature rat uterus are:

(i) the large decrease in the amount of RNA obtained within the first few hours of treatment of the cells.

(ii) the much smaller extent of the changes in RNA levels.

Administration of both oestradiol and tamoxifen to MCF-7 cell results, after 3 hours, in much higher recoveries of RNA than obtained when either compound is given alone (Fig. 42). However, after 24 hours the amount of RNA obtained is just slightly less than with oestradiol or tamoxifen alone. This could be due to a slowing down of the effect of oestradiol by having to compete with tamoxifen but more time points would be required to check this.

Figure 42

The Effect of Oestrogen and Tamoxifen on the Amount of RNA per Flask of MCF-7 Cells

MCF-7 cells were grown as described in materials and methods 2.3, until approximately 70% confluent. They were then given:

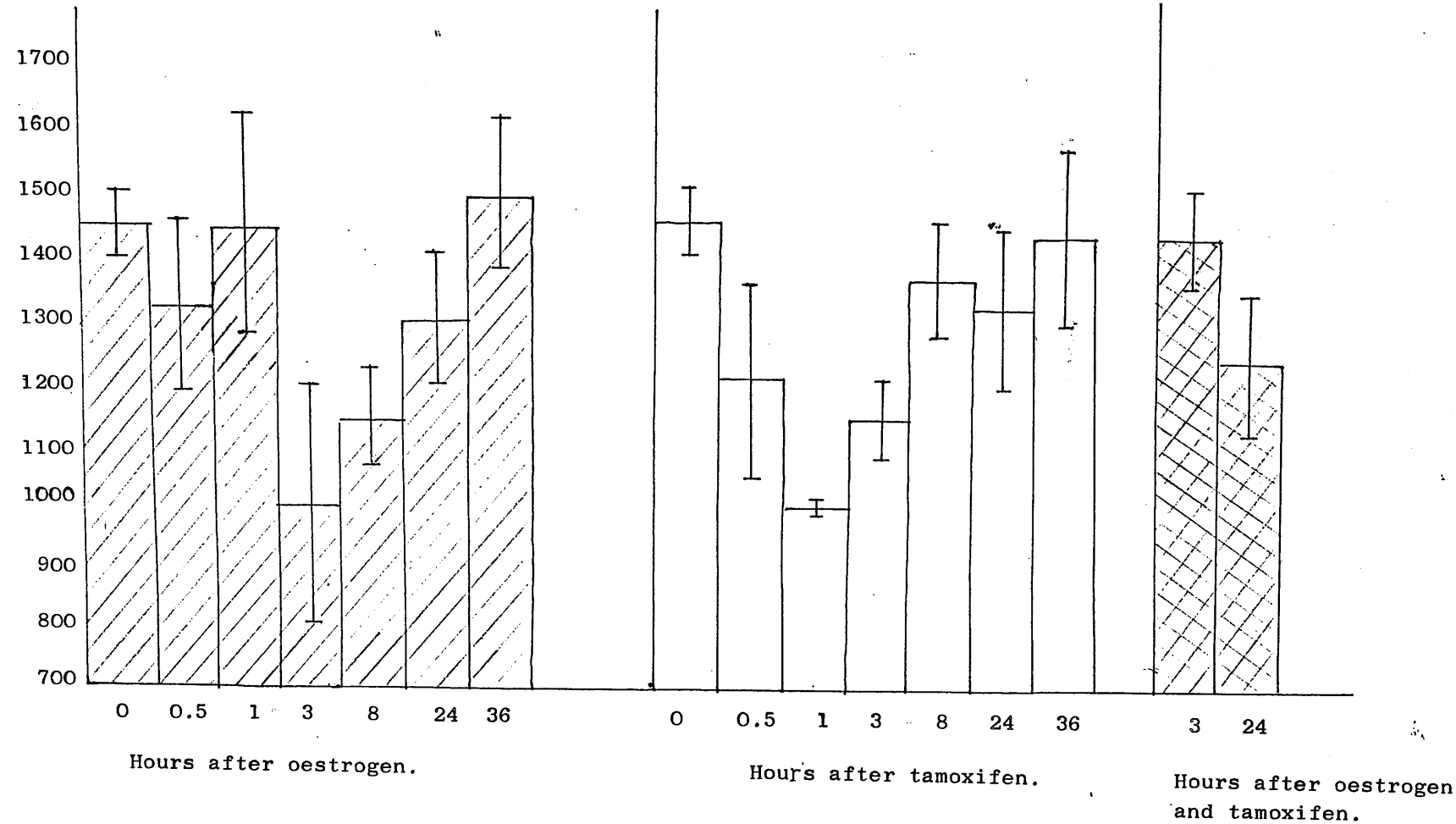
(a) oestradiol to a final concentration of 10^{-8}M

(b) tamoxifen to a final concentration of 10^{-6}M

(c) oestradiol, 10^{-8}M and tamoxifen, 10^{-6}M

Control cells received carrier only.

RNA was then extracted from the cells at various times after treatment, the total amount obtained/flask at each time point calculated and plotted, with error bars, against the duration and type of treatment. The data shows the mean of two separate preparations of five flask each at each time point.


µg RNA/flask
1700
1600
1500
1400
1300
1200
1100
1000
900
800
700
0 0.5 1 3 8 24 36
Hours after oestrogen.
0 0.5 1 3 8 24 36
Hours after tamoxifen.
3 24
Hours after oestrogen and tamoxifen.

6.1.3. The Effect of Oestrogen and Tamoxifen on PolyA+RNA Levels in MCF-7 Cells

PolyA+RNA was selected by Oligo (dT) cellulose chromatography (materials and methods 3.3) from the total RNA preparations from MCF-7 cells and the amount of rRNA contamination, and therefore of pure poly A+RNA, estimated as described in the uterine RNA section (4.2.1). The amount of the total preparation attributable to polyA+RNA was calculated and plotted against the length and type of treatment (Fig. 43).

The level of mRNA falls slightly within 1 hour of oestradiol administration to MCF-7 cells, however by 3 hours it has reached a value 2.2-fold that of control cells which is maintained at the 8 hour time-point. Levels then appear to decline to 1.5 fold control levels at 24 hours. Whether these later fluctuations are significant however must be regarded as doubtful, given that only two analyses were performed. However, this shows a large increase in the amount of mRNA in these cells, at the point when the level of total RNA is decreasing (Fig. 42), that is, between 1-3 hours after oestradiol administration. This is similar to the effect of oestradiol on the immature rat uterus in that while total RNA levels are decreasing between 2-8 hours, the amount of mRNA increases to a maximum at 4 hours.

The effect of tamoxifen is totally different in that, in the immature rat uterus it causes a 2-fold increase in the level of mRNA between 0-18 hours after administration whereas, in MCF-7 cells, no significant change is observed in the first 8 hours, after which there is a steady decline in the level of mRNA.

Figure 43

The Effect of Oestrogen and Tamoxifen on the Amount of PolyA+RNA in MCF-7 Cells

MCF-7 cells were grown under the conditions described in materials and methods 2.3, until approximately 70% confluent. They were then given:

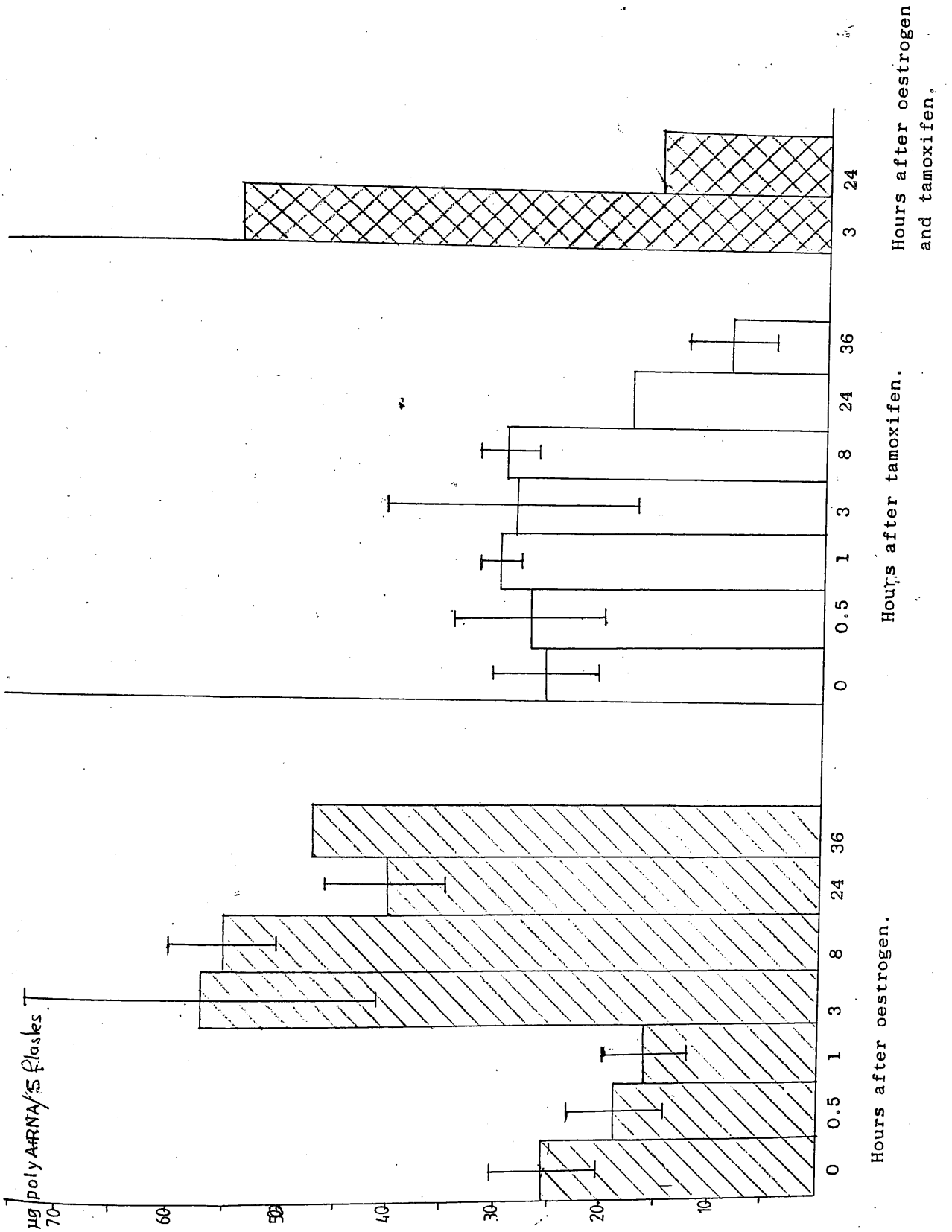
(a) oestradiol to a final concentration of 10^{-8}M

(b) tamoxifen to a final concentration of 10^{-6}M

(c) oestradiol to 10^{-8}M and tamoxifen to 10^{-6}M

Control flasks received carrier only.

RNA was then extracted from the cells after various intervals and polyA+RNA selected by one passage through Oligo (dT) cellulose and the amount of rRNA contamination estimated (materials and methods 3.2.3, 3.3). The actual amount of polyA+RNA available at each time point was plotted against the duration and type of treatment. The data shows the mean value and variation of two separate preparations of five flasks at each time.


µg poly A RNA/5 flasks
70
60
50
40
30
20
10
0
0.5
1
3
8
24
36
Hours after oestrogen.
0
0.5
1
3
8
24
36
Hours after tamoxifen.
3
24
Hours after oestrogen and tamoxifen.

Therefore, although tamoxifen appears to stimulate an increase in total RNA between 3-8 hours this does not result in an increase in mRNA levels during this time but only maintains the existing control level of mRNA.

The cytostatic and cytotoxic effects of tamoxifen at the concentration used (1μM) have been shown, in ZR-75.1 human breast cancer cells, to be completely blocked by the simultaneous administration of 10^{-8}M oestradiol (Allegra et al., 1978) and the effects of prior treatment, at this concentration, on MCF-7 cells have been shown to be reversed by addition of 10^{-8}M oestradiol (Sutherland et al., 1981). The effect on mRNA levels of administering 10^{-6}M tamoxifen and 10^{-8}M oestradiol simultaneously to MCF-7 cells (Fig. 43) shows that, after 3 hours the level of mRNA obtained is almost equal to that obtained with oestradiol alone, whereas at 24 hours it is almost equivalent to that obtained by giving tamoxifen alone. This implies that the effects of tamoxifen on these cells are not completely blocked by administration of this concentration of oestradiol.

7. The Effect of Oestrogen and Tamoxifen on Gene Expression in MCF-7 Human Breast Cancer Cells

The MCF-7 human breast cancer cell line was used to study the effects of oestrogen and the antioestrogen tamoxifen on the level of expression of a number of specific genes previously studied in the immature rat uterus (Results 5).

This study was of interest due to the difference in the degree of agonistic/antagonistic activity demonstrated by tamoxifen in different tissues, for example, tamoxifen is a pure agonist in mouse uterus

(Terenius, 1971), a pure antagonist in chick oviduct (Sutherland et al., 1977), and a partial agonist/partial antagonist in rat uterus (Harper and Walpole, 1967). Therefore, although tamoxifen is, as in the rat uterus, a partial agonist/partial antagonist in MCF-7 cells, the degree of antagonism of oestrogen action varies depending on the parameter being measured. With respect to a particular parameter, for example, expression of a specific gene, the degree of agonistic/antagonistic activity of tamoxifen may also vary between these two systems.

It has been demonstrated that oestrogen stimulates proliferation of MCF-7 cells in culture whereas antioestrogens inhibit their growth (Lippman et al., 1976; Coezy et al., 1982). The antioestrogen tamoxifen is not metabolised in MCF-7 cells (Horowitz, 1972) and at the dose used, 1μM, its growth-inhibitory effects can be completely reversed by oestrogen (Sutherland et al., 1981). At higher doses tamoxifen is cytotoxic.

The possible expression, and effect of oestrogen and tamoxifen on the expression of, some of the rat uterine library clones, oncogene clones, actin and pS2 were examined in MCF-7 cells and the results are shown below.

The MCF-7 cells used in these experiments were grown at ICI Pharmaceuticals, Macclesfield, under the conditions described in materials and methods 2.3.1. The methods used to study the expression of specific genes in MCF-7 cells were the same as those used to study gene-expression in the immature rat uterus, that is both northern and RNA dot blots using the same amount of pure poly A+RNA per time point of treatment.

7.1. The Effect of Oestrogen and Tamoxifen on the Expression of B11, E10 and F4 Specific Sequences in MCF-7 Cells

The B11 plasmid, isolated from the oestrogen-induced uterine cDNA library was hybridised to 8 hour oestrogen-induced mRNA from MCF-7 cells which had been blotted onto a Biodyne A membrane. This showed the presence of three specific complementary sequences at 11.2Kb, 6.4Kb and 4.5Kb (Fig. 44). The 11.2Kb and 4.5Kb sequences were also present in the uterine RNA (Fig. 16). As these autoradiographs were very faint, even after 2-3 weeks exposure, RNA dot blots were used to try and assess the effect of oestrogen and tamoxifen on their expression (in dot blots the RNA is concentrated in a small area and detection of a signal is quicker than on a northern blot).

The results of densitometric scanning of the autoradiographs for this and subsequent experiments with MCF-7 cell RNA were plotted as bar graphs, with the density being calculated as a percentage of the value obtained using control RNA.

The effect of oestrogen and tamoxifen on the level of B11 RNA is shown in Figure 45. Oestrogen treatment of these cells only resulted in a two-fold increase in B11 RNA, this being reached 8 hours after hormone treatment. Complementary sequences had fallen to control level by 24 hours after treatment but had risen to 1.6 times control by 36hours.

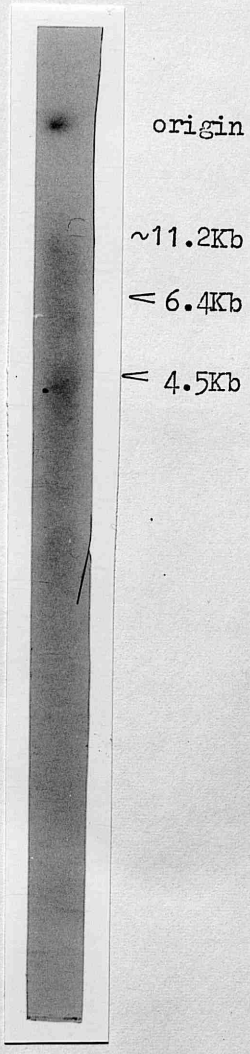
Tamoxifen resulted, after an initial decrease, in very little change in B11 RNA after 3 and 8 hours. These effects are much less dramatic than those observed in the immature rat uterus.

Figure 44

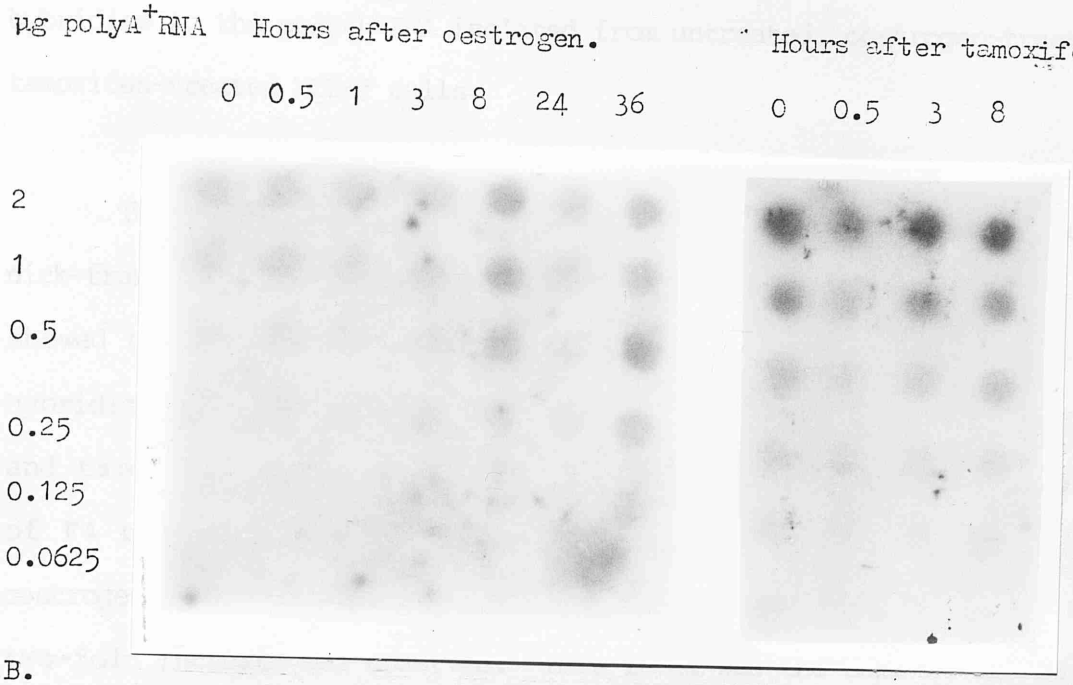
Northern Blot Analysis of Eight hour Oestrogen-Induced PolyA+RNA for Sequences Complementary to Plasmid B11 in MCF-7

A 2.5μg sample of polyA+RNA obtained from MCF-7 cells treated with 10^{-8}M oestradiol for eight hours was resolved on a 0.8% agarose/2.2M formaldehyde gel, blotted onto Biodyne A membrane and hybridised to plasmid B11 labelled with ^{32}P by nick-translation. The membrane was then washed, sealed in a plastic bag, and autoradiographed (materials and methods 7.1, 7.4).

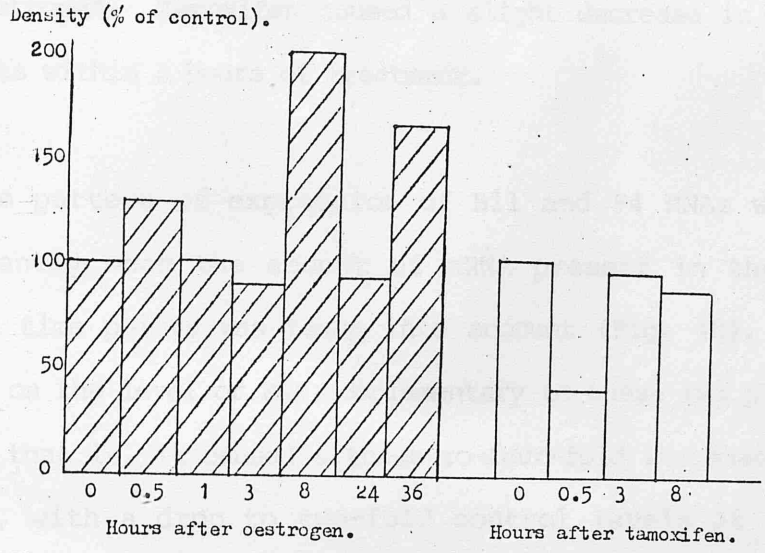
The size of the bands were estimated from the position of 28S and 18S rRNA markers.


origin
~11.2Kb
6.4Kb
4.5Kb

A.



B.



Hours after oestrogen.	0	0.5	1	3	8	24	36
%S.D.	11.3	13.5	7.5	3.9	0.6	12.1	10.5
Hours after tamoxifen.	0	0.5	3	8			
%S.D.	1.5	31.0	13.6	14.9			

Figure 45

The Effect of Oestrogen and Tamoxifen on the Level of Bll Specific RNA Sequences in MCF-7 Cells

A. Samples of polyA+RNA were isolated, by the procedure described in materials and methods Section 3.2.3, 3.3, from MCF-7 cells which had received:

(a) Oestradiol to 10^{-8}M, in alcohol

(b) Tamoxifen to 10^{-6}M, in alcohol

(c) Alcohol alone

for the times indicated. The polyA+RNA was corrected for contamination with rRNA, denatured and serial dilutions dotted onto nitrocellulose filters and then hybridised to plasmid Bll labelled with ^{32}P by nick-translation, washed and autoradiographed (materials and methods 7.2, 7.3).

B. Each row of the autoradiographs shown in A above was scanned using a laser densitometer and the mean and percentage standard deviation (% S.D.) calculated for each mRNA preparation used. The mean values were then plotted as a percentage of the control value and plotted against duration and type of treatment.

Plasmid E10, isolated from the uterine cDNA library, did not hybridise to the polyA+RNA isolated from untreated, oestrogen-treated and tamoxifen-treated MCF-7 cells.

The third plasmid, F4, isolated from the uterine cDNA library was nick-translated and hybridised to polyA+ RNA from untreated cells. This showed the presence of F4 transcripts of 3.6 and 1.2Kb (Fig. 46). When hybridised to dot blots of polyA+RNA from untreated, oestrogen-treated and tamoxifen-treated MCF-7 cells (Fig. 47), it was found that the level of F4 transcripts remained constant within the first 3 hours after oestrogen administration, however by 8 hours post-treatment, an almost two-fold increase was observed. This level was the same 24 and 36 hours after oestrogen. Tamoxifen caused a slight decrease in the level of F4 transcripts within 3 hours of treatment.

The pattern of expression of B11 and F4 RNAs was not altered significantly when the amount of mRNA present in the cells at the different time points was taken into account (Fig. 48). The effect of oestrogen on the level or RNA complementary to these two plasmids was very similar, that is, it caused a three-to-four-fold increase at 8 hours and 36 hours, with a drop to two-fold control levels at 24 hours after treatment. The effect of tamoxifen on these two sequences was also similar within the first 3 hours of treatment, but lack of RNA from later time points of tamoxifen-treated cells prevented further comparison.

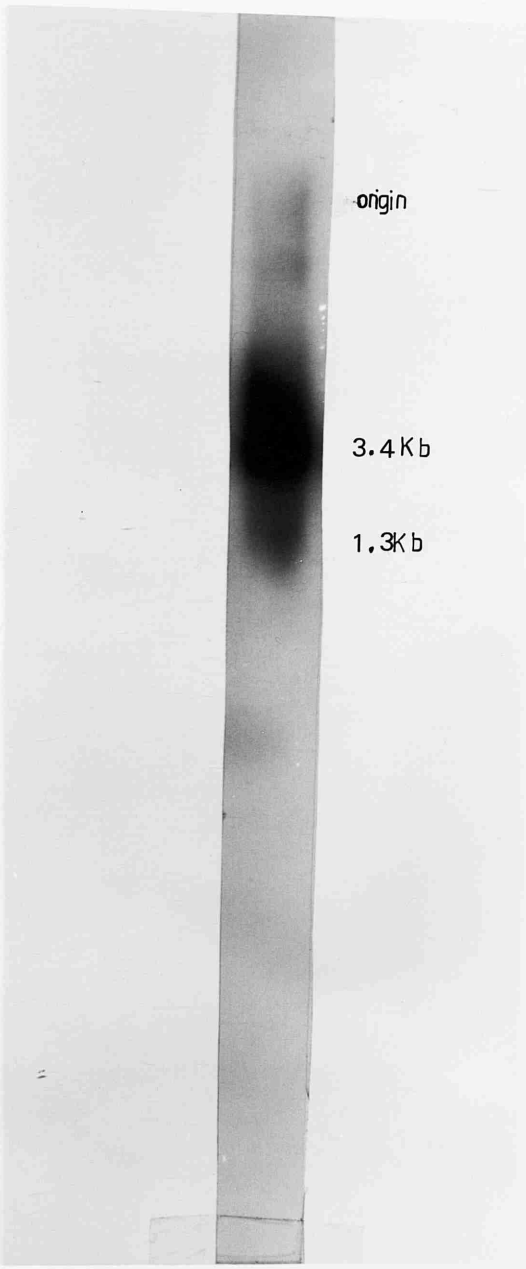
7.2. The Effect of Oestrogen and Tamoxifen on the Expression of c-myc, c-Ha-ras and c-Ki-ras specific RNA in MCF-7 Cells –

Figure 46

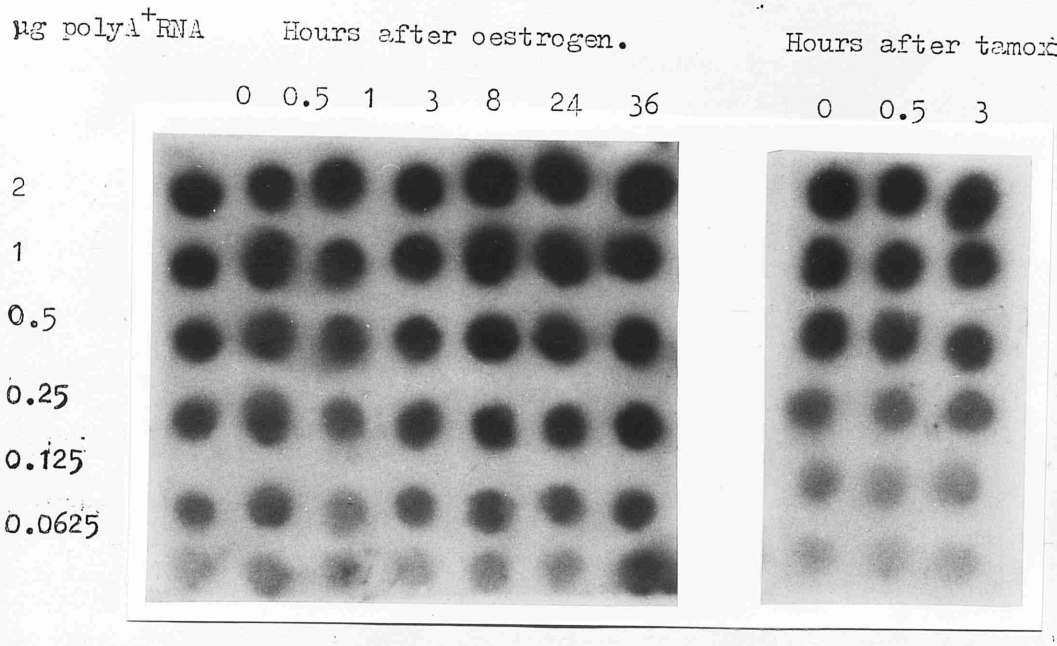
Northern Blot Analysis of PolyA+RNA for Sequences Complementary to Plasmid F4 in MCF-7 Human Breast Cancer Cells

A 2.5µg sample of polyA+RNA, obtained from MCF-7 cells by the procedure described in materials and methods 3.2.3, 3.3 was resolved on a 0.8% agarose/2.2M formaldehyde gel, blotted onto Biodyne A membrane and hybridised to plasmid F4 labelled with ^{32}P by nick-translation. The membrane was then washed and autoradiographed (materials and methods 7.1, 7.4).

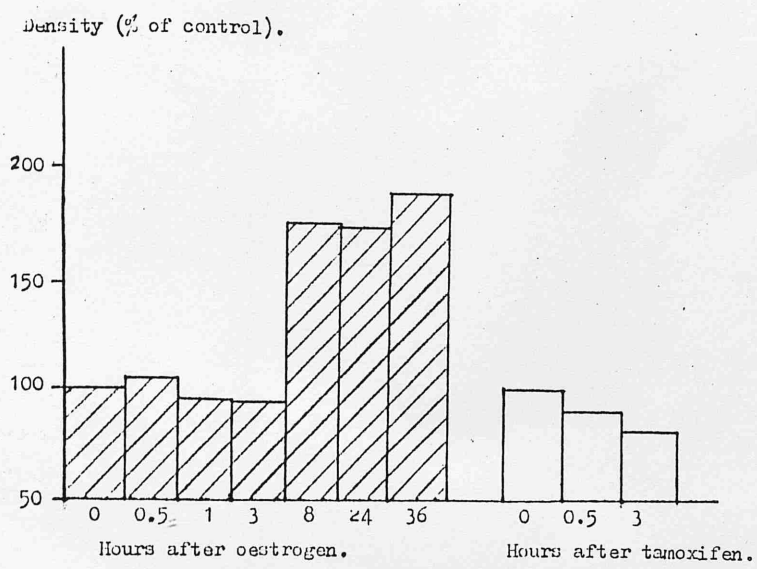
The sizes of the bands were estimated from the position of 28S and 18S rRNA markers.


origin
3.4Kb
1.3Kb

A.



B.



Hours after oestrogen.	0	0.5	1	3	8	24	36
%S.D.	12.5	3.9	3.5	12.9	6.8	14.3	9.1
Hours after tamoxifen.	0	0.5	3				
%S.D.	0.3	1.5	1.3				

Figure 47

The Effect of Oestrogen and Tamoxifen on the Level of F4 Specific RNA Sequences in MCF-7 Cells

A. Samples of polyA+RNA were isolated, by the procedure described in materials and methods 3.2.3, 3.3, from MCF-7 cells which had received:

(a) Oestradiol to 10^{-8}M, in alcohol

(b) Tamoxifen to 10^{-6}M, in alcohol

(c) Alcohol alone

For the times indicated. The PolyA+RNA was corrected for contamination with rRNA, denatured and serial dilutions dotted onto nitrocellulose filters and then hybridised to plasmid F4 labelled with ^{32}P by nick-translation, washed and autoradiographed (materials and methods 7.2, 7.3)

B. Each row of the autoradiographs shown in A above was scanned using a laser densitometer and the mean and percentage standard deviation (% S.D.) calculated for each mRNA preparation used. The mean values were then plotted as a percentage of the control value and plotted against duration and type of treatment.

Figure 48

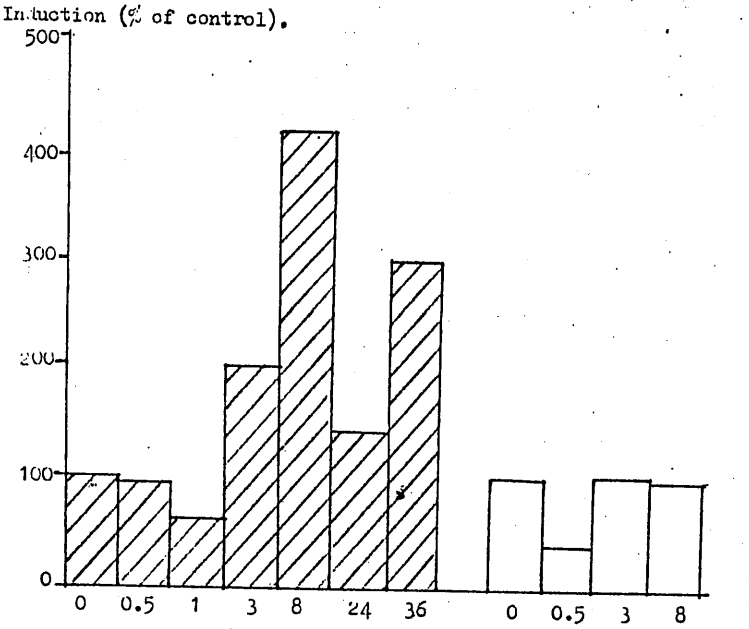
The Effect of Oestrogen and Tamoxifen on the Level of B11 and F4 RNA per Flask of MCF-7 Cells

These figures were derived by multiplying the amount of B11 and F4 RNA per unit of polyA+RNA from control, oestrogen and tamoxifen treated MCF-7 cells (Figs. 45, 47) by the amount of polyA+RNA available after each treatment (relative to control flasks) (Fig. 43). The results are expressed as a percentage of the value obtained for the amount of each RNA per control flask.

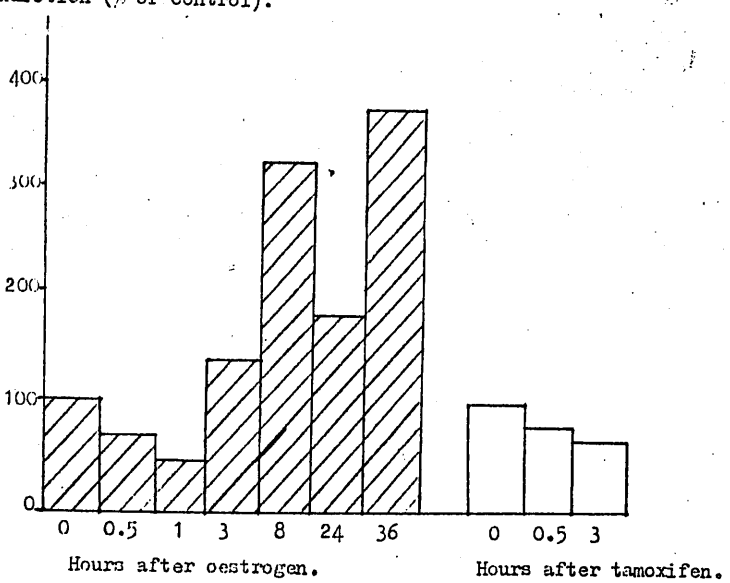
A - B11

B - F4

A Induction (% of control).



B Induction (% of control).



Plasmid p-myc-2, containing a v-myc sequence, was used to detect the presence of c-myc sequences in MCF-7 cell polyA+RNA. Northern blot analysis revealed the presence of three c-myc transcripts in MCF-7 cells at 4.9Kb, 3.6Kb and 2.5Kb (Fig. 49). The levels of c-myc RNA were then quantitated by RNA dot blot analysis. Densitometric scanning of the resulting autoradiographs showed that, after a slight decrease in the first few hours after oestrogen administration, the levels of c-myc RNA rose to a maximum of about two-fold control level after 8 hours. This then fell to about half control levels by 24 hours, and rose slightly at 36 hours after treatment (Fig. 50). Tamoxifen administration resulted in a continual decrease from control cells to an undetectable level in cells treated for 24 hours.

The expression of c-Ha-ras in MCF-7 cells was examined by resolving samples of control, oestrogen and tamoxifen stimulated polyA+RNAs on agarose/formaldehyde gels, blotting them onto Biodyne A membranes and hybridising them to nick-translated BS9 DNA, the v-Ha-ras clone. This demonstrated the presence of two Ha-ras specific sequences, 4.6Kb and 1.8Kb, in MCF-7 cells (Fig. 51). Transcripts of these sizes are also present in the immature rat uterus (Fig. 31).

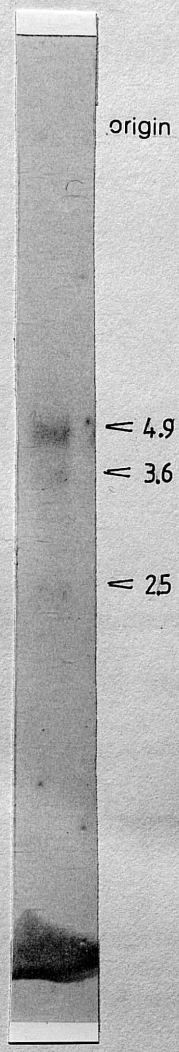
The expression of c-Ki-ras was studied and quantitated using RNA dot blots. This showed that after an initial decrease to half the control value within 3 hours of oestrogen treatment the amount of Ki-ras transcripts increased to twice the control value at 8 hours (Fig. 52). Further time points are required before any statement on the effect of tamoxifen can be made.

Figure 49

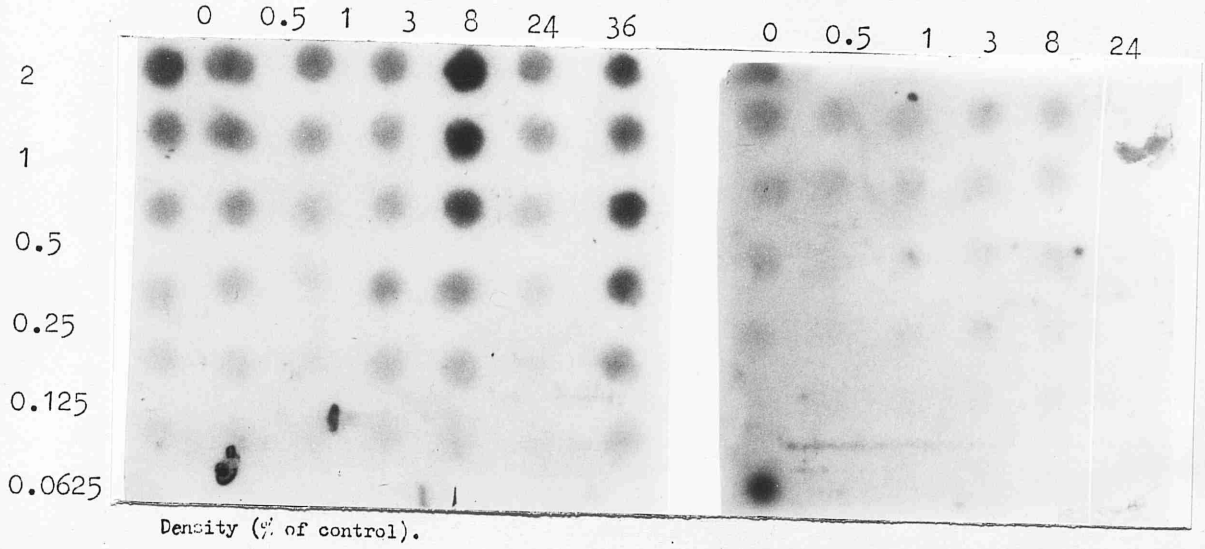
Northern Blot Analysis of PolyA+RNA for Sequences Complementary to v-myc Sequence in MCF-7 Cells

A 2.5µg sample of polyA+RNA, obtained as described in materials and methods 3.2.3, 3.3, from flasks of MCF-7 cells which had received oestradiol (to 10^{-8}M in alcohol) for 8 hours, was resolved on a 0.8% agarose/2.2M formaldehyde gel, blotted onto Biodyne A membrane and hybridised to nick-translated p-myc-2 (materials and methods 7.1, 7.4). The membrane was then washed and autoradiographed.

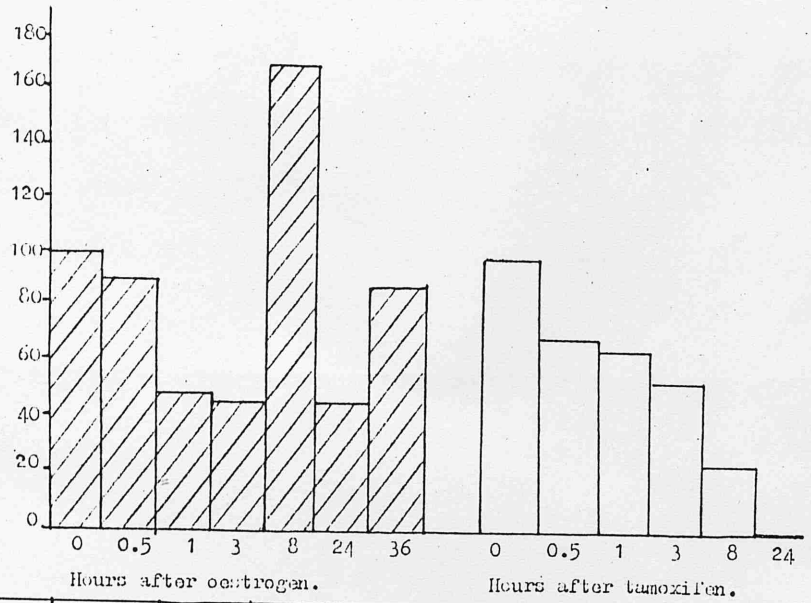
The sizes of the bands were estimated using the position of 28S and 18S rRNA markers.


origin
4.9
3.6
2.5

A.



B.



Hours after oestrogen.	0	0.5	1	3	8	24	36
%S.D.	8.4	11.0	5.1	9.5	2.8	9.6	11.9
Hours after tamoxifen.	0	0.5	1	3	8	24	
%S.D.	20.3	35.7	13.3	9.9	10.7	-	

Figure 50

The Effect of Oestrogen and Tamoxifen on the Level of c-myc Specific RNA Sequences in MCF-7 Cells

A. Samples of polyA+RNA were isolated, by the procedure described in materials and methods 3.2.3, 3.3, from MCF-7 cells which had received:

(a) Oestradiol to 10^{-8}M, in alcohol

(b) Tamoxifen to 10^{-6}M, in alcohol

(c) Alcohol alone

for the times indicated. The polyA+RNA was corrected for contamination with rRNA, denatured and serial dilutions dotted onto nitrocellulose filters and then hybridised to plasmid p-myc-2 labelled with ^{32}P by nick-translation, washed and autoradiographed (materials and methods 7.2, 7.3).

B. Each row of the autoradiographs shown in A above was scanned using a laser densitometer and the mean and percentage standard deviation (% S.D.) calculated for each mRNA preparation used. The mean values were then calculated as a percentage of the control value and plotted against duration and type of treatment.

Figure 51

Northern Blot Analysis of the Effect of Oestrogen and Tamoxifen on c-Ha-ras Specific RNA in MCF-7 Cells

2.5µg samples of polyA+RNA obtained as described in material and methods 3.2.3, 3.3, from flasks of MCF-7 cells which had received:

(a) Oestradiol to 10^{-8}M in alcohol

(b) Tamoxifen to 10^{-6}M in alcohol

(c) Alcohol alone

for the times indicated were resolved on 0.8% agarose/2.2M formaldehyde gels, blotted onto Biodyne A membranes and hybridised to nick-translated BS9 DNA (materials and methods 7.1, 7.4). The membranes were then washed, sealed in plastic bags, and autoradiographed.

The sizes of the bands were estimated using the position of 28S and 18S rRNA markers.

Hours after oestrogen.

0 0.5 1 3 8 24 36

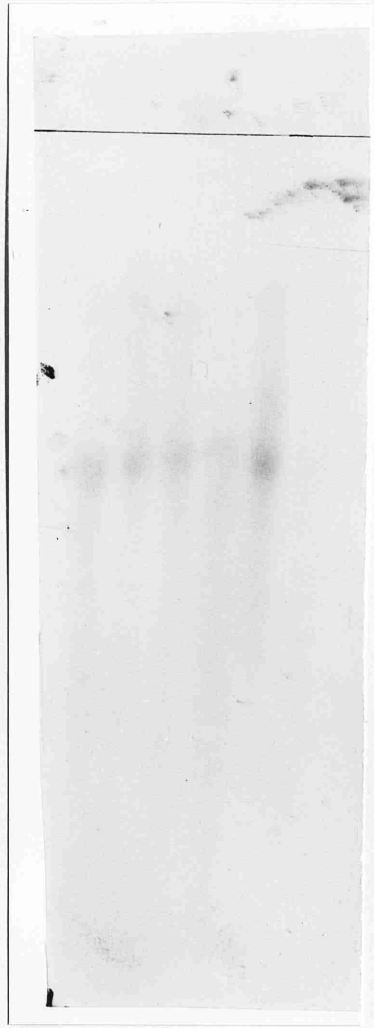
origin

< 4.6Kb

< 1.8Kb

Hours after tamoxifen.

0 0.5 1 3 8 24 36

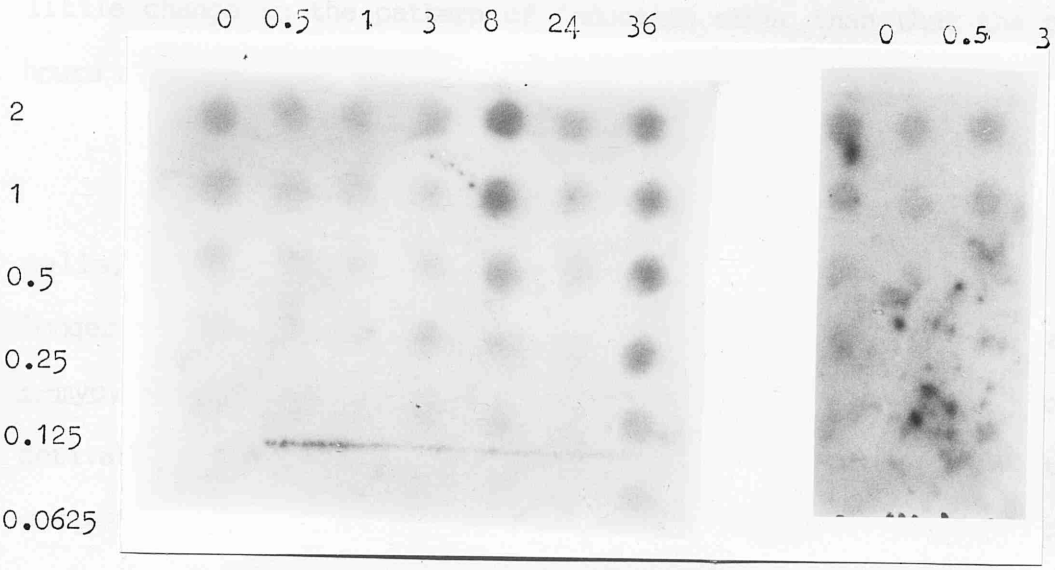


origin

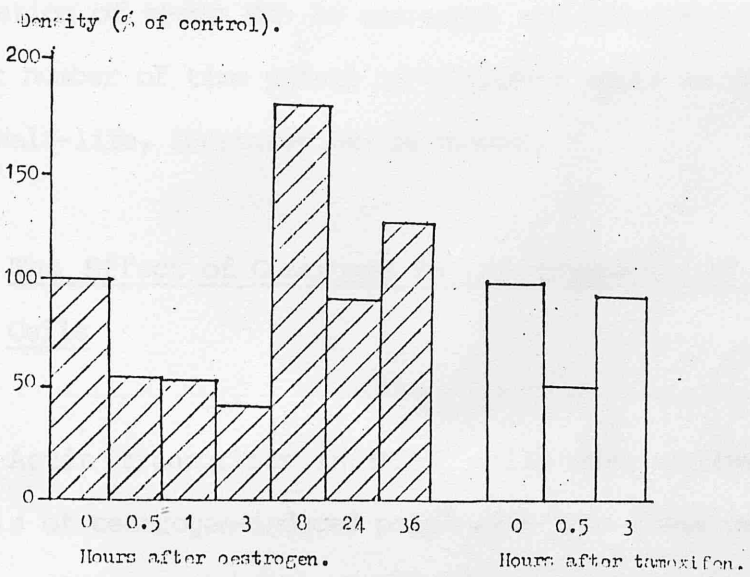
< 4.6Kb

< 1.8Kb

A.



B.



Hours after oestrogen.	0	0.5	1	3	8	24	36
%S.D.	4.8	8.8	2.7	7.5	3.6	7.5	6.2
Hours after tamoxifen.	0	0.5	3				
%S.D.	6.6	13.1	14.7				

Figure 52

The Effect of Oestrogen and Tamoxifen on the Level of c-Ki-ras Specific RNA Sequences in MCF-7 Cells

A. Samples of polyA+RNA were isolated, by the procedure described in materials and methods 3.2.3, 3.3, from MCF-7 cells which had received:

(a) Oestradiol to 10^{-8}M, in alcohol

(b) Tamoxifen to 10^{-6}M, in alcohol

(c) Alcohol alone

for the times indicated. The polyA+RNA was corrected for contamination with rRNA, denatured and serial dilutions dotted onto nitrocellulose filters and then hybridised to plasmid HiHi III labelled with ^{32}P by nick-translation, washed and autoradiographed (materials and methods 7.2, 7.3).

B. Each row of the autoradiographs shown in A was scanned using a laser densitometer and the mean and percentage standard deviation (% S.D.) calculated for each mRNA preparation used. The mean values were then calculated as a percentage of the control value and plotted against duration and type of treatment.

Taking into account the different amounts of poly A+RNA available after the different treatments resulted in Fig. 53. This shows very little change in the pattern of induction other than that the peak at 8 hours after oestrogen is magnified for both c-myc and c-Ki-ras.

Tamoxifen appears to decrease the amount of c-myc RNA in MCF-7 cells, whereas oestrogen seems to stimulate myc expression. However, longer exposures to tamoxifen may result in a stimulation of expression of c-myc, as in these cells tamoxifen is not metabolised to a more active derivative, and therefore, due to the much lower affinity of tamoxifen compared to oestrogen for the oestrogen receptor it may take much longer to exert its effect. Again studies on the rates of synthesis and degradation of c-myc RNA in oestrogen and tamoxifen-treated cells, and a larger number of time points of treatment would be useful as, due to its short half-life, increases may be missed.

7.3. The Effect of Oestrogen on the Expression of Actin mRNA in MCF-7 Cells

Actin transcripts in MCF-7 cells were studied by northern blot analysis of oestrogen-induced poly A+RNA from these cells. Hybridisation of these RNAs to nick-translated p749, an actin clone, demonstrated the presence of two actin transcripts, at 5.8Kb and 2Kb, the levels of which increased steadily to about five-fold control value by 8 hours after administration, fell dramatically at 24 hours, and were approximately eleven-times the control value at 36 hours (Fig. 54). This filter was made using the remainder of the oestrogen induced MCF-7 cell poly A+RNA which was insufficient for any other purpose. There was however no poly

Figure 53

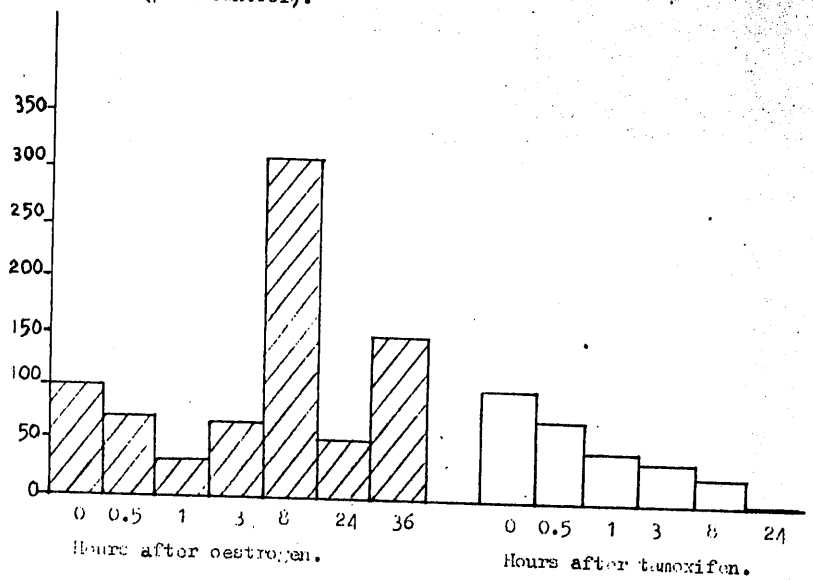
The Effect of Oestrogen and Tamoxifen on the Level of c-myc and c-Ki-ras Specific RNA per Flask of MCF-7 Cells

These figures were derived by multiplying the amount of c-myc and c-Ki-ras RNA per unit of polyA+RNA from control, oestrogen and tamoxifen treated MCF-7 cells (Figs. 50, 52) by the amount of polyA+RNA available after each treatment (Fig. 43). The results are expressed as a percentage of the value obtained for the amount of each RNA per control flask.

A — c-myc

B — c-Ki-ras

A Induction (% of control).



B Induction(% of control).

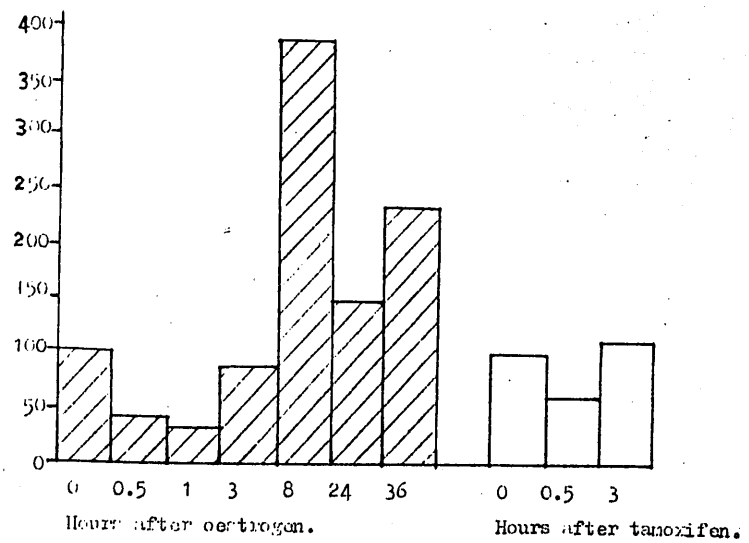


Figure 54

Quantitation of the Effect of Oestrogen on the Expression of Actin RNA in MCF-7 Cells

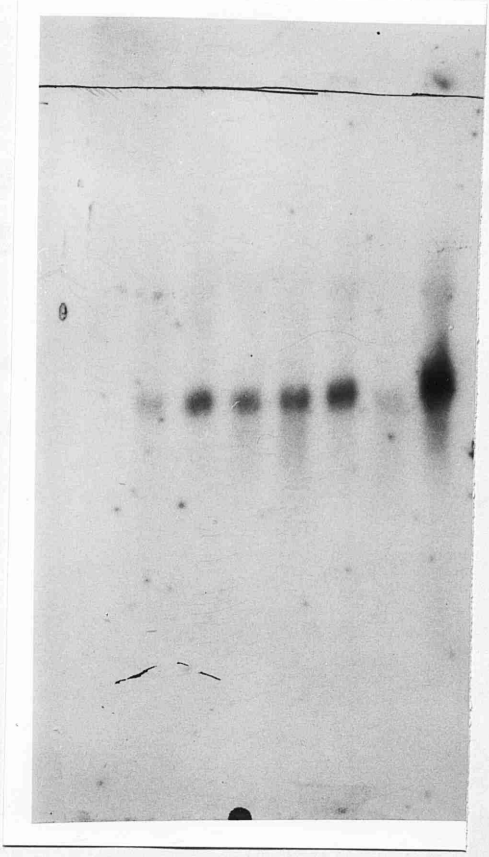
A. 2.5µg samples of polyA+RNA obtained as described in materials and methods 3.2.3, 3.3, from MCF-7 cells which had received oestradiol to 10^{-8}M in alcohol, or alcohol alone, for the times indicated, were resolved on 0.8% agarose/2.2M formaldehyde gel, blotted onto Biodyne A membrane and hybridised to plasmid p749 labelled with ^{32}P by nick-translation, the membrane washed, sealed in a plastic bag, and autoradiographed (materials and methods 7.1, 7.4).

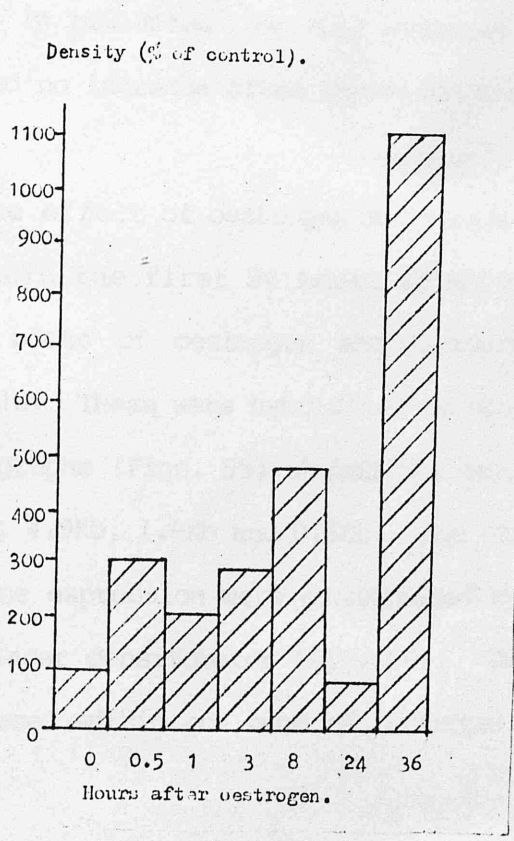
The sizes of the bands were estimated using the position of 28S and 18S rRNA markers.

B. The autoradiograph in A was scanned using a laser densitometer and the average value, over five scans of each track, plotted, as a percentage of the control value, against the duration of treatment.

Hours after oestrogen.

0 0.5 1 3 8 24 36





A+RNA left from some of the tamoxifen treatments and therefore a northern blot of tamoxifen-induced polyA+RNA could not be run for comparison with this.

7.4 The Effect of Oestrogen and Tamoxifen on the Expression of pS2 RNA in MCF-7 Cells

pS2, a cDNA clone of an oestrogen-regulated mRNA of about 600 nucleotides in MCF-7 cells was isolated by the differential screening of a cDNA library prepared from MCF-7 polyA+RNA (Masiakowski et al., 1982). The level of pS2 RNA increases rapidly, within half an hour, of addition of oestrogen to MCF-7 cells, and this increase has been shown to continue to a maximum after 24 hours, which is then maintained until six days (Brown et al., 1984; Westley et al., 1984). Brown (1984) showed that 24 hours of treatment with 10nM oestradiol caused a large increase in pS2 mRNA, whereas treatment with 100nM tamoxifen resulted in a very small increase in pS2 mRNA. He also measured pS2 gene transcription in vitro and showed no increase after tamoxifen treatment.

The effect of oestrogen and tamoxifen on the expression of the pS2 gene within the first 36 hours after treatment was studied by using northern blots of oestrogen and tamoxifen-stimulated polyA+RNAs from MCF-7 cells. These were hybridised to nick-translated pS2. The resulting autoradiographs (Figs. 55) showed the presence of three complementary RNA sequences, 4.9Kb, 1.8Kb and 0.6Kb. The effects of oestrogen and tamoxifen on pS2 gene expression were quantitated by scanning these autoradiographs using a laser densitometer (Fig. 56). This showed that the level of pS2 RNA increased within one hour of oestrogen administration and continued to

Figure 55

Northern Blot Analysis of the Effect of Oestrogen and/or Tamoxifen on pS2 RNA in MCF-7 Cells

2.5µg samples of polyA+RNA obtained as described and methods 3.2.3, 3.3, from flasks of MCF-7 cells which had received:

(a) Oestradiol to 10^{-8}M alcohol

(b) Tamoxifen to 10^{-6}M in alcohol

(c) Oestradiol to 10^{-8}M plus tamoxifen to 10^{-6}M, both in alcohol

(d) Alcohol alone

for the times indicated, were resolved on 0.8% agarose/2.2M formaldehyde gels, blotted onto Biodyne A membranes and hybridised to nick-translated pS2 (materials and methods 7.1, 7.4).

The membranes were then washed, sealed in plastic bags and autoradiographed.

The sizes of the bands were estimated using the position of 28S and 18S rRNA markers.

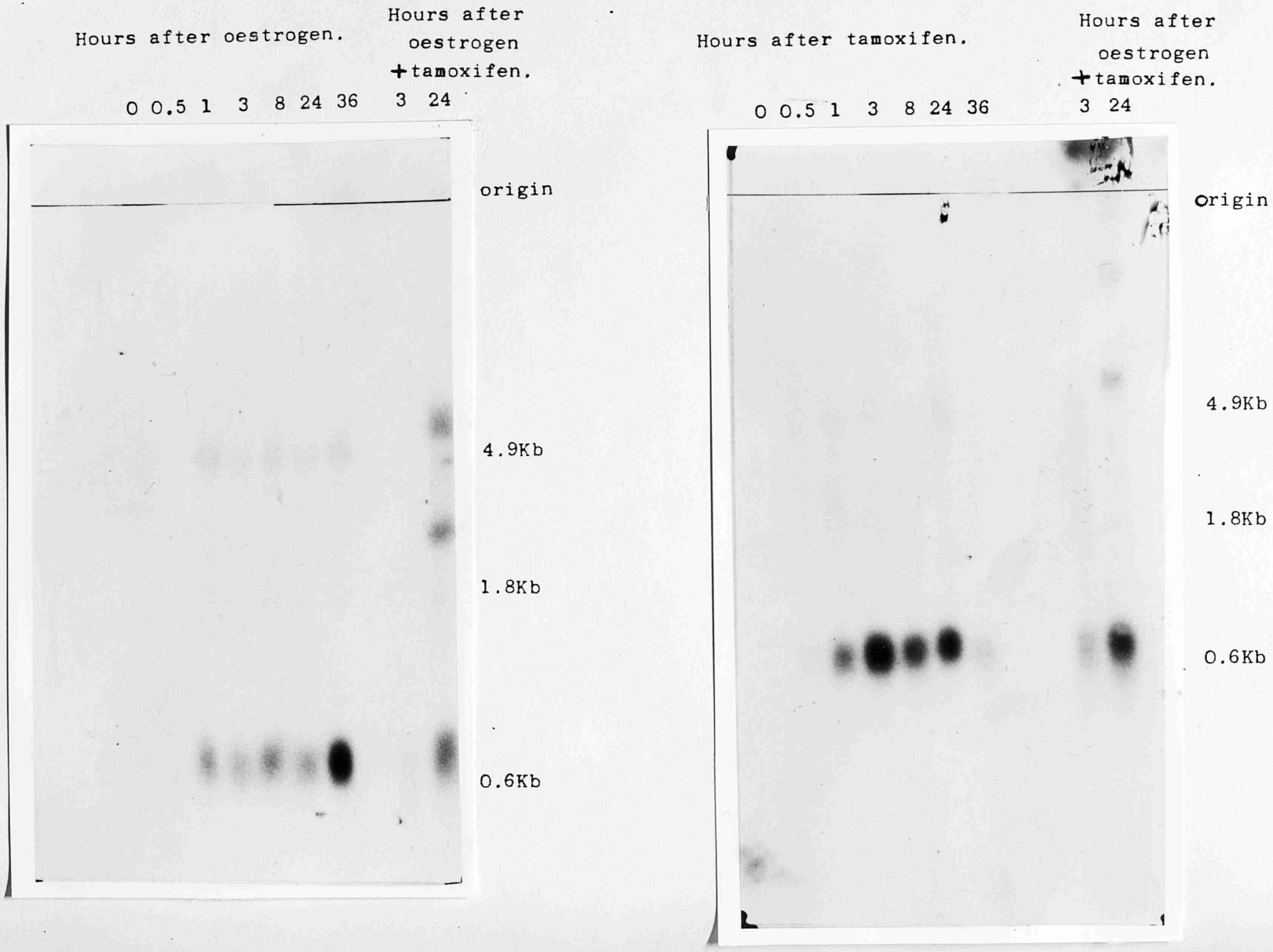

Hours after oestrogen.
Hours after oestrogen +tamoxifen.
0 0.5 1 3 8 24 36 3 24
origin
4.9Kb
1.8Kb
0.6Kb
Hours after tamoxifen.
Hours after oestrogen +tamoxifen.
0 0.5 1 3 8 24 36 3 24
origin
4.9Kb
1.8Kb
0.6Kb

Figure 56

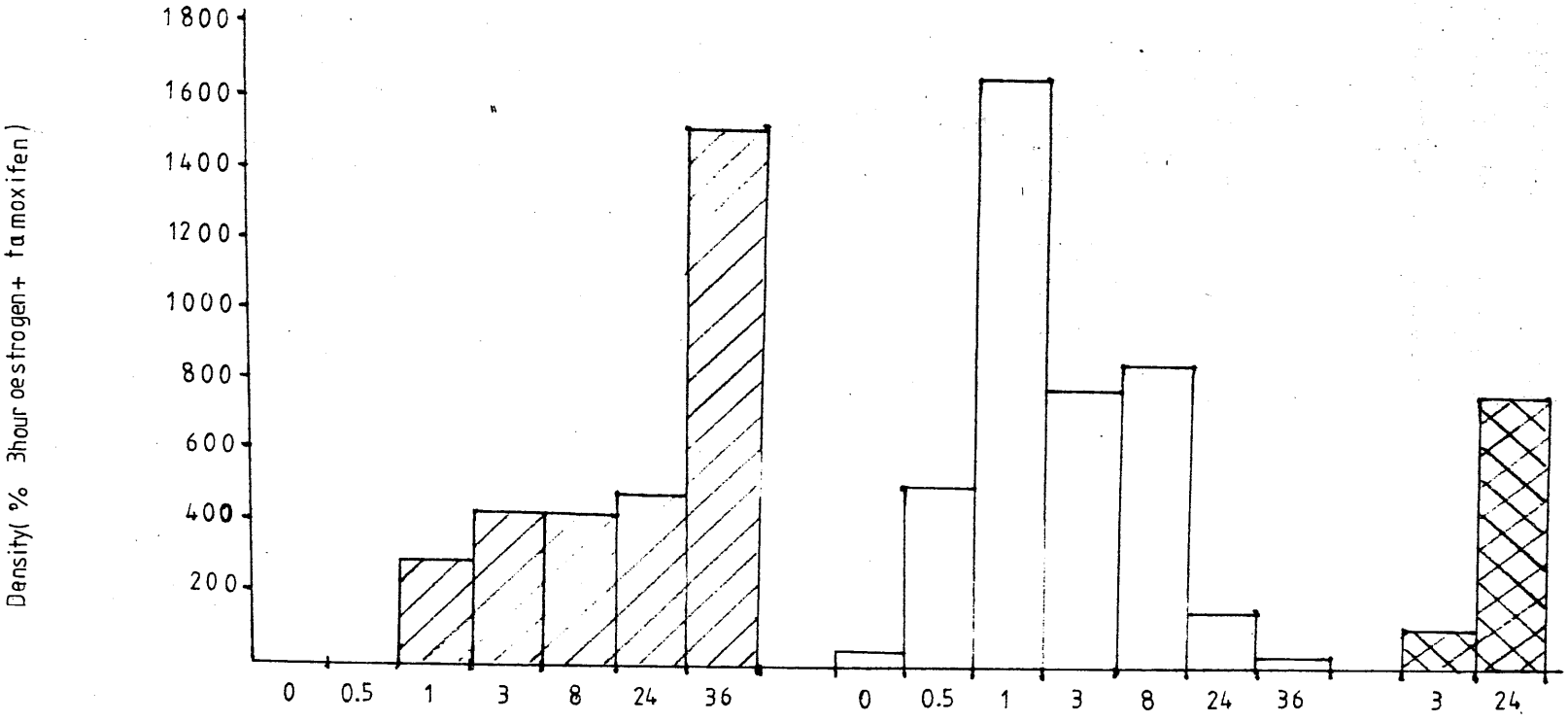
The Effect of Oestrogen and Tamoxifen on the Level of RNA Complementary to pS2 in one Unit of PolyA+RNA from MCF-7 Cells

Each track of the autoradiographs in Fig. 55 was scanned using a laser densitometer and the mean and percentage standard deviation (% S.D.) for each polyA+RNA preparation used calculated as described in Results 4.2.1. The mean values were then calculated as a percentage of the value obtained for 3 hours of oestrogen plus tamoxifen treatment, and plotted against the duration and type of treatment.

A. Oestradiol only

B. Tamoxifen only

C. Oestradiol plus tamoxifen



Hours after oestrogen.	0	0.5	1	3	8	24	36
% S.D.	–	–	13.9	8.9	7.8	57.1	15.4

Hours after tamoxifen.	0	0.5	1	3	8	24	36
% S.D.	–	13.1	8.7	2.6	19.7	34.7	–

Hours after oestrogen plus tamoxifen.	3	24
% S.D.	15.7	6.3

increase throughout the time course studied. Tamoxifen administration resulted in an increase of pS2 RNA to a maximum ten-times control levels within one hour, however levels subsequently fell off throughout the time course. Administration of oestrogen and tamoxifen simultaneously to MCF-7 cells resulted in levels of pS2 RNA, after 3 and 24 hours, almost the same as those obtained due to oestrogen alone.

Fig. 57 illustrates the data of Figs. 55, 56 replotted to take account of the effect of the hormones on the levels of total cellular mRNA as illustrated in Figure 43. This shows that oestrogen induces only a small increase in pS2 RNA over 24 hours, but a large induction by 36 hours. The induction by tamoxifen of pS2 RNA is much-smaller, and levels decrease from the peak at 1 hour after administration to practically nothing after 36 hours. That is, tamoxifen appears to stimulate pS2 gene transcription earlier than oestrogen, but to a lesser, and comparitively short-lived extent.

Figure 57

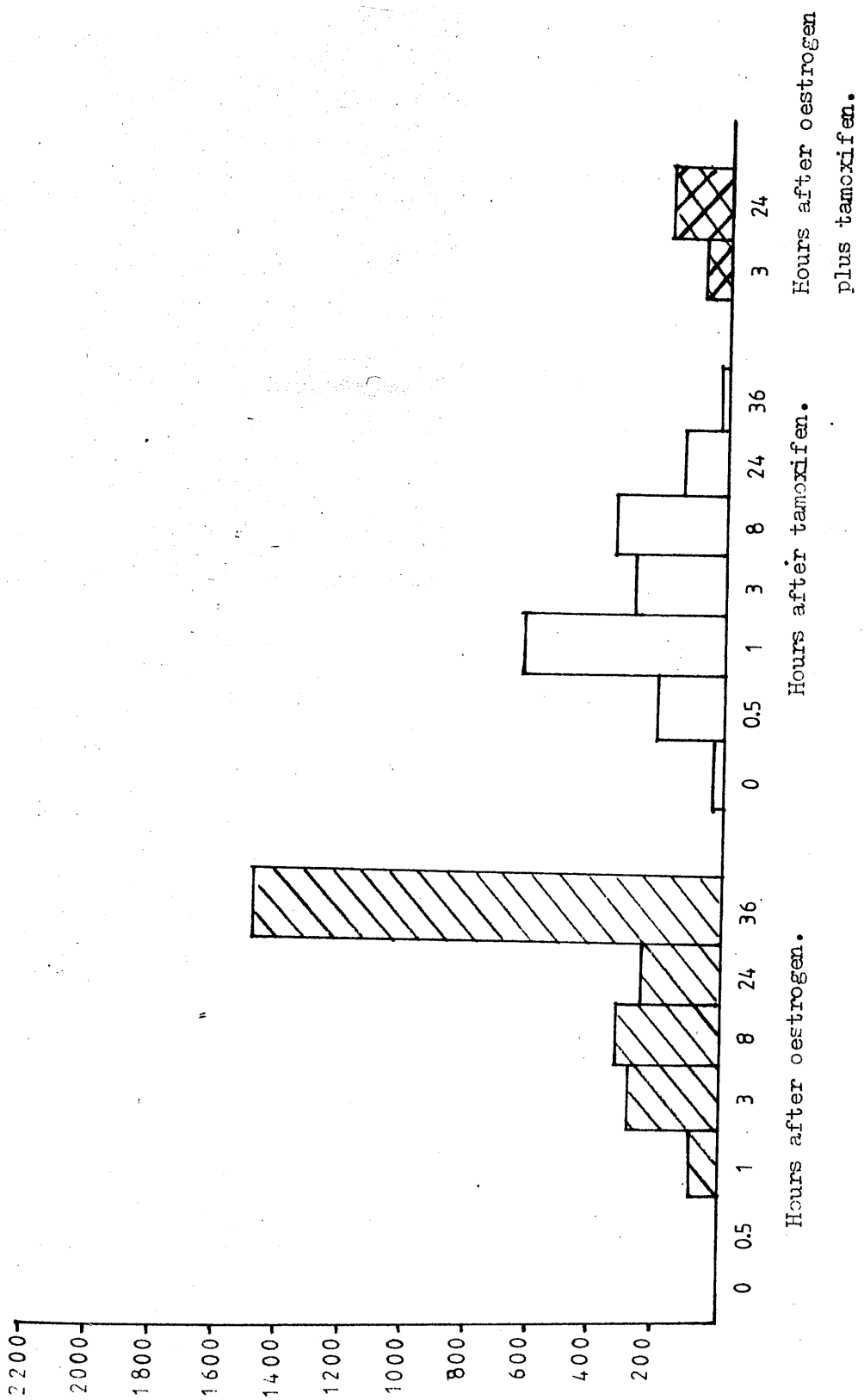
The Effect of Oestrogen and Tamoxifen on the Level of pS2 RNA per Flask of MCF-7 Cells

This figure was derived by multiplying the amount of pS2 RNA per unit polyA+RNA from control, oestrogen, tamoxifen, and oestrogen plus tamoxifen-treated cells (Fig. 56) by the amount of polyA+RNA available after each treatment (Fig. 43). The results are expressed as a percentage of the value obtained for the amount of pS2 RNA per flask of cells treated with oestrogen plus tamoxifen for 3 hours.

A. Oestrogen only

B. Tamoxifen only

C. Oestrogen plus tamoxifen


Induction (% 3 hour oestrogen+tamoxifen)
2200
2000
1800
1600
1400
1200
1000
800
600
400
200
0 0.5 1 3 8 24 36
Hours after oestrogen.
0 0.5 1 3 8 24 36
Hours after tamoxifen.
3 24
Hours after oestrogen plus tamoxifen.

DISCUSSION

The changes in the immature rat uterus, both physiological and biochemical, which are brought about by the administration of exogenous oestrogen to the immature rat, have been extensively studied (Knowler and Beaumont, 1985). The molecular changes which occur have been studied, in this laboratory, by examining both protein (Beaumont and Knowler, 1983) and mRNA populations (Aziz and Knowler, 1979; 1980). Attempts have been made to quantitate these changes, at the level of mRNA populations, by using the technique of Rot hybridisation. This technique, which provides data on the complexity of individual, and differences between, mRNA populations is however subject to a number of inaccuracies (Young et al., 1976; Ryffel et al, 1976; Aziz and Knowler, 1979) and can only provide information on gross changes in mRNA populations.

The next step was therefore to examine and quantitate the effects of oestrogen, and of the antioestrogen tamoxifen, at the level of expression of individual genes. Two approaches to this problem were taken:

(1) The first involved constructing a cDNA library to an mRNA population isolated from the immature rat uterus at a specific point in its response to oestrogen. This was then screened with both homologous and heterologous cDNA probes, to isolate cloned oestrogen responsive messenger sequences. The induction or repression by oestrogen and antioestrogen of the specific mRNAs represented by these clones was then studied using the techniques of northern and RNA dot blotting. The effect of antioestrogen on these mRNAs was examined in order to try and isolate mRNAs differentially affected by oestrogen and antioestrogen.

(2) The second approach was to obtain, from other laboratories, recombinant plasmids with inserts encoding cDNAs to known genes, and to use these to monitor the expression of these genes, and their possible regulation by oestrogen and antioestrogen, in the immature rat uterus. This approach is discussed more fully later.

The mRNA population used to construct the cDNA library was isolated from the uteri of immature rats which had been stimulated with oestrogen for four hours. This particular point in the response of the uterus to oestrogen was chosen for a number of reasons:

(a) This time-point corresponds to the first peak of oestrogen-induced RNA synthesis in the immature rat uterus (Knowler and Smellie, 1971). The subsequent responses of the uterus to oestrogen have been shown, using various inhibitors of protein and RNA synthesis, to be dependent on the initial increase in hnRNA synthesis, its maturation to mRNA, and on its translation into protein (Knowler and Smellie, 1971; Borthwick and Smellie, 1975; Merryweather and Knowler, 1980). This particular mRNA population should therefore contain the messenger sequences whose altered expression in response to oestrogen is responsible for oestrogen-induced cell growth.

(b) This mRNA population has been extensively characterised by Rot hybridisation. This showed that the four hour oestrogen stimulated mRNA population differed markedly from that obtained from either control rats, or rats treated with oestrogen for two hours, especially in the number of sequences of intermediate abundance

(Table 2) (Aziz et al, 1979a, b). The lack of complementarity between these populations implied that by four hours after administration, oestrogen had switched on or increased the transcription of a large number of genes. The four hour oestrogen stimulated mRNA population was also very different from the mRNA population obtained from the mature rat uterus (Table 2).

(c) Heterologous Rot hybridisations, using four-hour oestrogen-stimulated mRNA and mRNA from the peak of tamoxifen stimulated RNA synthesis (12 hours of tamoxifen treatment) have shown that, although all the sequences represented in the four hour oestrogen-induced mRNA population were present in the twelve hour tamoxifen-induced mRNA population, there were quantitative differences in the sequences of intermediate abundance; some being more, or less, abundant in response to oestrogen compared with tamoxifen (Waters et al, 1983a). This could be due to the time-points chosen for comparison but use of this mRNA population for cloning could potentially lead to isolation of messenger sequences differentially regulated by oestrogen and antioestrogen.

12µg of poly A+RNA from the uteri of four hour oestrogen-treated rats was used to construct a cDNA library in plasmid pUC8. This yielded a small library of approximately 2000 recombinants. Because the Rot curve data indicated that the number of oestrogen-regulated sequences was quite large and included some of the abundant mRNA species it was decided that this library should contain some oestrogen-regulated sequences.

Screening of the library resulted in the selection of twelve recombinants whose complementary RNA levels were altered by oestrogen treatment. The time-courses of expression in response to oestrogen and the antioestrogen tamoxifen of three of these plus four oncogene clones, an actin recombinant and the pS2 clone, were studied in the immature rat uterus.

It was of interest to compare the expression of the selected mRNA species in the rat uterus and human breast carcinoma for several reasons. Firstly, if an oestrogen-responsive mRNA species in rat uterus was present and oestrogen-responsive in a cell line derived from a different species and a different tissue then it was likely that the species concerned was of considerable importance in the hormonal response. Such a sequence would have considerable potential value as a marker of oestrogen-responsiveness in the analysis of breast carcinoma and other tumours. Secondly, tamoxifen, itself an anticancer drug, is notoriously variable in its effects on different tissues and species. In some cases its effects are entirely antagonistic, in others agonistic and in still others variable depending on the response measured. Clearly, therefore, it was of interest to compare the effects of tamoxifen on the expression of the chosen mRNAs in the two species.

As oestrogen induces cell proliferation in both of these systems it must induce the transcription of a number of genes for proteins involved in meeting the increased metabolic and structural requirements of growing and dividing cells. These genes are therefore growth responsive and may possibly also be switched on by a number of other mitogens, that is they are not necessarily specifically regulated by oestrogen.

With the above clones we have not tried to determine whether they are specifically induced by oestrogen or are members of a set of proliferation responsive genes. The only method of deducing this would be to stimulate cell proliferation with a number of other mitogens, for example other steroids or growth factors, and to monitor the expression of the genes. Oestrogen-specific genes would not be switched on by other mitogens.

This is extremely difficult in the animal model due to the presence of endogenous growth factors and steroids. In cells in culture the presence of other growth factors and steroids can be controlled by growing the cells in serum-free defined medium. However the number of cells necessary and the cost of such a study was prohibitive.

Whether these genes were specifically regulated by oestrogen or were part of a general proliferation response was not important to their usefulness as probes to study the mechanism of action of tamoxifen. Although this antioestrogen antagonises oestrogen action, possibly by altering the expression of genes specifically regulated by oestrogen, it also, in the systems studied, inhibits cell proliferation. Therefore monitoring its effect on genes involved in proliferation, or in the case of the oncogenes, genes implicated in the regulation of proliferation, may shed light on its mechanism of action.

The effect of oestrogen and tamoxifen on RNA levels in the immature rat uterus and MCF-7 cells.

In order to study the effects of oestrogen and tamoxifen on the levels of expression of specific genes in the immature rat uterus and MCF-7 cells it was necessary to purify polyA+RNA from both systems after various treatments with oestrogen and/or tamoxifen. This allowed the effect of these two compounds on the levels of total and polyA+RNA in both systems to be estimated and compared. In doing this however it was important to try to relate any observed differences to the different pharmacokinetics of oestrogen and tamoxifen in the rat and MCF-7 cells.

In the rat oestrogen has a serum half-life of a few hours whereas tamoxifen has a half-life of approximately four days (Adams et al., 1983). Also tamoxifen is taken up slowly by uterine nuclei, the maximum level of tamoxifen-receptor complexes in the nucleus occurring 24 hours after tamoxifen administration whereas the peak of oestrogen-receptor complexes in the nucleus occurs just two hours after administration and has regained control levels by 24 hours (Clark et al., 1978). Tamoxifen also has an affinity for the oestrogen-receptor of only 2% relative to that of oestradiol, and is only slowly metabolised to the more potent mono-hydroxylated form which has an affinity for the oestrogen-receptor similar to that of oestradiol (Sutherland and Baulieu, 1976). In MCF-7 cells oestrogen is metabolised, but tamoxifen is not (Horowitz et al., 1978). In humans tamoxifen has been shown to be metabolised mainly to N-desmethyl tamoxifen which has a binding affinity for the oestrogen-receptor very similar to that of tamoxifen (Sutherland and Whybourne, 1981).

Both oestrogen and tamoxifen stimulated an increase in the amount of total RNA per uterus (Fig. 12). This was as expected as both oestrogen and tamoxifen stimulate cellular hypertrophy in the immature rat uterus therefore both have to stimulate synthesis of the proteins, and of the respective mRNAs, involved in cell growth.

Oestrogen stimulated an almost steady increase in RNA per uterus over the first 24 hours whereas tamoxifen caused an increase from 12 hours to a maximum at 24 hours, after which it plateaued. The maximum in both cases was 2.5-3 times that obtained per control uterus. These increases were not as dramatic as anticipated from previous studies on uterine RNA synthesis (Waters and Knowler, 1983; Merryweather and Knowler, 1980) which showed a seven-fold increase in RNA synthesis 2 hours after oestrogen and 24 hours after tamoxifen treatment. The reasons for these differences are unknown but may relate in part to differences in the method of RNA extraction.

The slower accumulation of RNA after tamoxifen treatment compared with that induced by oestrogen is probably due to its slower uptake, lower affinity for the oestrogen-receptor and its slow metabolism to a more active form.

The changes in the level of RNA obtained per flask of MCF-7 cells after treatment with oestrogen and tamoxifen were very similar but were less dramatic than those in the rat uterus. Both compounds caused a small initial decrease in the amount of RNA per flask but control levels were recovered by 24 hours (Fig. 42). The total variation however was only from 65-105% of control values. Westley et al. (1984) found that administration of 5×10^{-9}M oestradiol resulted in only a 25% increase in 24 hours in the amount of RNA extracted per T75 flask over control cells and that tamoxifen had no effect. These small changes could be easily masked by error.

Ideally such studies should be related to cell numbers or total cellular protein or DNA. In the present study however these analyses were

not carried out. However it has been shown that tamoxifen administration to MCF-7 cells prevents cell growth (Lippman et al., 1976; Horowitz et al., 1978), and that the administration of oestrogen, although increasing the mitotic index, does not appear to cause significant cell division within the first 36 hours after administration as shown by no increase in cell number (Lippman et al., 1981). Also, to try and negate differences in cell number between individual flasks, five flasks were treated individually at each time point and then pooled before extracting their RNA. The variation in cell number between five flasks plated with the same number of cells at the same time and grown under identical conditions for 5 days was 8%.

When the amount of poly A+ RNA per uterus was estimated after the various treatments it was found that again both oestrogen and tamoxifen stimulated increases, although to different extents. Oestrogen administration resulted in a maximum of three-fold control levels after 4 hours, and a second smaller peak of twice control levels at 16-20 hours after administration. This biphasic pattern of response to oestrogen is in agreement with that found by Merryweather and Knowler, (1980) and Waters and Knowler (1983), except that, as for total RNA the levels of poly A+ RNA are not as high as would be expected. Tamoxifen resulted in a slower, smaller, but sustained increase in poly A+ RNA levels, a maximum twice control level being attained by 12 hours and maintained throughout the time-course. Again these results are similar to those of Waters and Knowler (1983) and are consistent with the fact that tamoxifen, like oestrogen, causes cellular hypertrophy in the immature rat uterus, and therefore has to stimulate the cells to produce the same amount of building materials. The differences in the levels of poly A+ RNA obtained

in response to tamoxifen when compared with oestrogen can be attributed to the different pharmacokinetics of the two compounds; the lower peak in response to tamoxifen possibly resulting from a slower rate of synthesis against an unaffected rate of degradation resulting in a reduced accumulation of transcripts.

When the level of polyA+RNA per flask of MCF-7 cells was estimated it was found that, although the levels of total RNA were not changing very significantly, the levels of polyA+RNA, after an initial decrease, rose from half to twice the control level between 1 and 36 hours after oestrogen. Tamoxifen administration however seemed to have an inhibitory effect on the transcription of mRNA in these cells as the level of mRNA decreased steadily to less than half that of the control level over the 36 hours of treatment. This effect is different from that observed in the immature rat uterus but is consistent with the data of Lippman et al. (1981) who showed that tamoxifen, at concentrations less than 4μM, was capable of inhibiting the growth of MCF-7 cells below that of cells in oestrogen-free medium.

It must be noted that these figures were generated from only two sets of data, however, they do suggest a defect in the tamoxifen-receptor complex compared to the oestrogen-receptor complex in its ability to stimulate transcription in MCF-7 cells, a defect not apparent at this level in the tamoxifen-receptor complex of the immature rat uterus. This emphasises the difference in the degree of oestrogen antagonism displayed by this antioestrogen in different model systems and the necessity therefore of also studying its action in the tissue in which this is employed; human breast cancers.

The effect of oestrogen and tamoxifen on the expression of specific genes in the immature rat uterus and MCF-7 cells.

The effect of oestrogen and tamoxifen on the expression of specific genes in the immature rat uterus and MCF-7 human breast cancer cells was studied by monitoring the level of the RNAs, specific for a particular gene, after various treatments with oestrogen and tamoxifen. This was calculated by measuring the amount of polyA+RNA specific for a particular gene in one unit of polyA+RNA, as a percentage of that present in controls, and then multiplying this by the number of units of polyA+RNA available. This resulted in essentially three patterns of gene expression being observed in the rat uterus.

In the immature rat uterus oestrogen and tamoxifen both stimulate cell growth and therefore increases in total mRNA levels, but only oestrogen can stimulate DNA synthesis and cell division. A number of the genes studied, the library clones B11 and E10, and the oncogene clones myc, Ha-ras and Ki-ras, appeared to be expressed in a pattern very similar to that of the oestrogen and tamoxifen induced changes in total polyA+RNA that is an initial increase to a maximum level at 4 hours, a decline, and a second increase between 16 and 20 hours after oestrogen. They also exhibited similar responses to tamoxifen, namely an increase within 12 hours which was maintained, with a slight fall after 24 hours, until 42 hours after treatment. The differences in the expression of these genes in response to oestrogen and tamoxifen can, like those of total poly A+RNA, probably be explained in terms of the different pharmacokinetics of the two compounds.

The changes were however more dramatic than the two - or three-fold variations observed in total polyA+RNA levels, especially in the case of the oncogenes, for example, Ha-ras specific sequences increased twenty-five fold, and myc specific sequences increased more than thirty-fold in response to oestrogen. These peaks of oestrogen-induced accumulation of oncogene-encoded mRNA were also much greater than those induced by tamoxifen. This is consistent with the fact that the oncogene mRNAs, especially those for c-myc, have very short half-lives (Dani et al., 1984). Their levels therefore decline rapidly after the short oestrogen-induced surge in synthesis. With tamoxifen however the stimulation of transcription is slower due to the slower uptake by uterine cells, and lower affinity for the oestrogen receptor, of this compound compared to oestrogen. A slow, less dramatic but continued induction of transcription of these genes, if accompanied by an unaltered rate of degradation of the messenger sequences, would lead to a smaller accumulation of messenger sequences over a longer period of time.

The polyA+RNAs specific for the library clones B11 and E10 accumulate to about the same extent in the immature rat uterus in response to both oestrogen and tamoxifen, and their increases are not so dramatic. Given the argument presented above, this may be due to:

(1) a greater induction by tamoxifen than oestrogen or

(2) a longer half-life of these messages as opposed to those for the oncogenes or

(3) an alteration in their degradation rate in response to tamoxifen.

These possibilities could be resolved by measuring the rate of synthesis and degradation of these messages in the uterus in response to oestrogen and tamoxifen. A further possibility is that these two message species and those of the three oncogenes studied, may be expressed in different cell populations of the uterus which do not respond equally to tamoxifen (Markaverich et al., 1981). As stated before, separating the cell populations of the uterus in order to obtain RNA for studies using the methods employed here is virtually impossible. However, this could be examined by the technique of in situ hybridisation.

Nevertheless, taking the uterus as a whole, and taking into account the different pharmacokinetics of tamoxifen and oestrogen, tamoxifen appears to be an effective oestrogen agonist with respect to the induction of B11, E10, c-myc, c-Ha-ras and c-Ki-ras gene transcription. And unless a threshold level of one of the oncogenes must be obtained in order to start the cascade of events leading to DNA synthesis and cell division, the effect of tamoxifen on the expression of any of these genes does not appear to be responsible for the antiproliferative effect of this compound in the rat uterus.

The third library clone studied, F4, although exhibiting the same general pattern of oestrogen-induced changes in polyA+RNA levels as were induced in total polyA+RNA, appeared to have either an increased rate of synthesis throughout the time-course studied, with surges at 2-4 hours and 12-20 hours after oestrogen, or to have more stable mRNAs than the other genes studied. The level of polyA+RNA for this particular gene did not decrease to control levels at 8-12 hours, but only from fifteen to six-fold control levels. Expression of this gene also appeared to be

stimulated to a greater extent by tamoxifen than oestrogen and, unlike all the other clones, showed only one peak of synthesis, 0-12 hours after tamoxifen. The slow decline subsequent to 12 hours also suggests that this message has a longer half-life than the others. This gene therefore, even when considering the different pharmacokinetics of oestrogen and tamoxifen, appeared to be differentially regulated by oestrogen and tamoxifen in that tamoxifen failed to induce a second increase at 30-36 hours after treatment.

Actin is an essential structural protein required for cell integrity and it therefore seemed reasonable that transcription of this gene would be increased in cells stimulated to grow. The level of actin mRNA had previously been shown to increase during the exponential growth of cells in culture (Cleveland et al, 1980) and was found to increase in the immature rat uterus in response to both oestrogen and tamoxifen.

The pattern of actin expression in the immature rat uterus in response to oestrogen was somewhat different from that of the other genes studied in that there was one prolonged increase between 0-12 hours after which levels declined rapidly to the control value. Tamoxifen also only stimulated one increase in actin RNA synthesis to a maximum after 12 hours which then declined steadily to control levels by 48 hours.

Taking into account the slower action of tamoxifen compared to oestrogen in the uterus, tamoxifen again would appear to be an effective oestrogen agonist in its stimulation of actin gene expression.

Use of MCF-7 cells as a model in which to study the action of oestrogen and tamoxifen had both advantages and disadvantages. The major

advantages are that the problems of drug accessibility and metabolism are much reduced when compared with the whole animal. Furthermore, only one cell type, epithelial in origin, is present, whereas in the rat uterus data will reflect the combined responses of a number of different cell types.

In MCF-7 cells, as in the immature rat uterus, the antioestrogen tamoxifen is a partial agonist/partial antagonist. However, in the carcinoma cell observed effects are solely due to tamoxifen itself as the compound is not metabolised in these cells (Horowitz et al., 1978). The degree of antagonistic activity is different from that in the immature rat uterus in that tamoxifen has been shown to inhibit the growth of MCF-7 cells below the level observed in control cells. This resulted in smaller amounts of poly A+ RNA being obtained from tamoxifen-treated cells as opposed to control and oestrogen-treated cells and this in turn limited the number of genes whose expression, in response to tamoxifen, could be studied. The effect of tamoxifen in inhibiting cell growth was taken into account when deciding how many flasks of cells to use at each time-point. The cells were grown and their total RNA extracted during the industrial part of this studentship. Yields were not however determined until after a return to Glasgow by which time it was not possible to arrange to obtain more cells.

In response to oestrogen two patterns of expression were observed in MCF-7 cells. In general the pattern of changes in the levels of transcripts for F4, B11, c-myc, c-Ki-ras and actin were the same as those induced in total poly A+RNA (Figs. 48 and 53). An initial small decline within one hour of treatment recovered within 3 hours and rose to a

maximum after 8 hours. The amount present after 24 hours was much lower but increased slightly by 36 hours. The main difference between the total polyA+RNA levels and those for these particular genes was in the 36 hour time-point, but was much lower for all the genes studied, except F4.

The second pattern of expression was observed with the MCF-7 clone, pS2. The levels of this mRNA rose slightly within the first 24 hours after oestrogen addition but was dramatically increased by 36 hours (Fig. 57). This gene showed a slight early induction in response to tamoxifen, but then declined throughout the remainder of the time-course. The only other gene studied throughout the tamoxifen time-course was c-myc: the pattern of c-myc expression in response to tamoxifen appeared to follow that of total polyA+RNA, that is a steady decline in levels from control value to about zero by 24 hours after treatment. It should be noted that, as previously stated, the changes occurring within 36 hours of administration of the two compounds, oestrogen and tamoxifen, are due to changes per cell and not to changes in cell numbers (Lippman et al., 1981).

The main problems in trying to interpret these results are the lack of time-points and the short half-lives of some of the mRNAs being studied. For instance, the peak of expression of c-myc cannot be said to be 8 hours after tamoxifen administration, but can only be placed between 3 and 24 hours after administration. If the peak of expression of such a short-lived mRNA has been missed by several hours, this could account for the much less dramatic increases over the control levels observed in MCF-7 cells as opposed to the immature rat uterus. Similarly the lack of stimulation of pS2 and c-myc expression in response to tamoxifen may be due to the time-points chosen. Conversely, the observed low stimulations

might be genuine and result from the lower affinity of tamoxifen for the oestrogen receptor, and the lack of metabolism of tamoxifen to a more active derivative.

The most that can be said therefore is that the genes B11, F4, c-myc, c-Ki-ras, c-Ha-ras, actin and pS2 are transcribed in MCF-7 cells and that their transcription is increased in response to oestrogen. More time-points are required in order to ascertain exactly when, and to what extent, the transcription of each gene occurs.

The fact that the majority of the genes studied responded in a similar way to oestrogen in the immature rat uterus and MCF-7 cells, and that the pattern of response was similar to that found for total poly A+RNA in both cases, suggested that they were involved in the growth response and were not specifically oestrogen responsive. This conclusion was supported to some extent by the fact that they were expressed similarly in response to tamoxifen which also causes cell growth in the uterus. It was unfortunate that more tamoxifen-induced poly A+RNA from MCF-7 cells was not obtained as tamoxifen inhibits MCF-7 growth and therefore possibly the expression of these genes, for example, within the limits already discussed, c-myc expression.

In a continuation of this study, it is hoped to determine whether these genes are specifically oestrogen-regulated or are part of a more general growth response. MCF-7 cells will be stimulated with various mitogens, for example, EGF and PDGF, and gene expression monitored. It is also hoped that more data can be generated on the effects of tamoxifen. Monitoring the expression of the library clone, F4, is of special interest

in that it appears to be differentially regulated by oestrogen and tamoxifen in the uterus and, if this is also the case in MCF-7 cells, its further characterisation will be undertaken.

The expression of the oncogenes, c-myc, c-Ha-ras and c-Ki-ras, in the immature rat uterus in response to oestrogen again emphasizes the role of their encoded proteins in normal cell function.

The product of the c-myc oncogene has been implicated in inducing growth competence, that is, in recruiting cells from the quiescent state (Go) into the actively growing state (G1), and has been found to be expressed in response to a variety of mitogens in a variety of cell types (Robertson, 1984). The majority of cells in the immature rat uterus are quiescent, that is not cycling (Sutherland et al., 1983) and administration of oestrogen or tamoxifen results in recruitment of cells into the actively growing state and an early increase in c-myc expression. This is consistent with the postulated role of the c-myc product. If, as the limited data obtained suggests, tamoxifen inhibits c-myc expression and cell growth in MCF-7 cells, this obviously fits in with the postulated role of the c-myc product. Nevertheless it also raises questions about the different degrees of antagonism of oestrogen action exhibited in different species and cell populations by this compound. Do the differences relate to the oestrogen-receptor and therefore the antioestrogen-receptor complex and its ability to bind to the correct chromatin sites? Alternatively are the differences in the DNA or chromatin binding sites themselves?

The recent cloning and sequencing of the oestrogen-receptor from MCF-7 cells (Green et al., 1986) should now allow comparison of the

sequences of the oestrogen receptors from other cell types to be made. Also, studies into the binding sequences for the receptor on the chromatin, and their structure, are continuing and may shed some light on this area.

Another possibility is that the antioestrogen-specific binding sites (AEBS) may be playing either an active or passive role in the degree of oestrogen antagonism demonstrated by tamoxifen in different cell types. These sites are present in nearly all tissue types and their concentration does not parallel that of the oestrogen receptor (Murphy et al., 1981). It has been suggested that the different affinities of the various antioestrogens and their metabolites for these sites may alter their apparent distribution volume and therefore their biological potency in different tissues. Also, the fact that about 50% of oestrogen receptor positive breast tumours fail to respond to tamoxifen therapy, whereas 10-15% of oestrogen receptor negative tumours do respond suggests that tamoxifen has an effect on its own; dissociated from the oestrogen receptor pathway (Murphy et al., 1981). This may or may not be related to the affinity of tamoxifen and its metabolites for the AEBS, or the concentration ratio of AEBS to oestrogen receptor, but it does point to the possibility that the differential regulation of genes by oestrogen and antioestrogen may be the result of two different receptor pathways.

The expression of the ras oncogenes has been linked to cell growth and proliferation in that they were found to be expressed before the wave of DNA synthesis in regenerating rat liver (Makino et al., 1984). Campisi et al., (1984) proposed that the ras gene product was involved in

regulating events in the G1 phase of the cell-cycle which prepared the cell for DNA synthesis.

In the immature rat uterus the *ras* genes are expressed early and in response to both oestrogen and tamoxifen, even though tamoxifen does not stimulate DNA synthesis in most uterine cells. It would appear therefore that if the *ras* genes are involved in regulating DNA synthesis, tamoxifen must either affect the expression of a gene further along the regulatory pathway, or affect the ras gene product directly. This is possible in that the ras gene product is a GTPase which is membrane bound and thought to transduce signals generated by ligands binding to cell surface receptors (Beckner *et al.*, 1985). Therefore, the triphenylethylene derivative could, at the concentrations used, affect the cell membrane and therefore the ras gene products function. An alternative suggestion could be that the level of the ras gene product has to reach a threshold value which cannot be attained due to the slower pharmacokinetics of tamoxifen. Therefore subsequent events, for example, DNA synthesis cannot occur. A third possibility is that although tamoxifen inhibits DNA synthesis in most uterine cells, it does stimulate it in stromal cells. Whether this induction would be enough to account for the ras gene expression could be determined by *in situ* hybridisation.

In summary, both oestrogen and tamoxifen induce uterine cellular hypertrophy and the majority of genes studied appeared to be induced by both compounds in this tissue. The differences observed could be explained in terms of the different pharmacokinetics of the two compounds.

Only one gene, isolated from the cDNA library to 4 hour oestrogen-stimulated rat uterine mRNA, appeared to be differentially regulated by oestrogen and tamoxifen in the uterus, and it is hoped to be able to further investigate the expression of this gene, and possibly some of the others isolated from the library, in a number of other oestrogen responsive and unresponsive systems.

Most of the genes studied were also induced by oestrogen in the MCF-7 human breast cancer cell line, but the effect of tamoxifen was difficult to estimate due to its inhibitory effects on total poly A+RNA synthesis. It is therefore hoped to obtain more RNA from this system in order to complete this study.

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