GENE EXPRESSION

IN

HUMAN BREAST CANCER

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DECLARATION

In accordance with the regulations of the University of Edinburgh, I declare that this thesis has been composed by me. The work reported in this thesis is my own with all contributions from other workers clearly indicated in the text or in the acknowledgements section.

Alastair Mark Thompson.

ABSTRACT

Evidence exists which suggests that the expression of certain genes is associated with the development of human malignancy.

The aims of the work reported in this thesis were:
(i) to quantitate accurately the expression (ie transcription) of selected important genes in human breast cancer, to relate this to clinical and pathological factors and to other genetic changes in the tumours and
(ii) to develop a xenograft model in which to study the effect of an anticancer agent on breast tumour gene expression in vivo and thus to establish whether these approaches might have therapeutic implications.

The particular approach used to achieve these objectives was the development of molecular techniques to measure gene expression and loss of genetic material at the nucleic acid level.

Gene expression was measured in primary breast cancers from 80 female patients and compared to that in normal control tissues from other patients who had surgical removal of normal breast tissue. Ribonucleic acid (RNA) was extracted from the tumours and probed with radiolabelled gene probes. Gene expression (messenger RNA) was then quantified using laser densitometry on the resultant Northern blots. Increased expression of three

oncogenes (p53, c-myc, c-erbB-2), a growth factor gene (TGF-ß) and an oestrogen regulated mRNA (pS2) was detected in varying proportions of the tumours. Overexpression of individual genes was found to correlate significantly with different clinical and pathological parameters.

Since the loss of specific regions of deoxyribonucleic acid (DNA) is associated with oncogenesis, DNA was also extracted from the paired venous blood and tumour tissue of each patient. Allele loss specific to the tumour DNA was then determined using Southern blots probed with radiolabelled DNA. The p53 gene is located near the tip of the short arm of chromosome 17 at 17p13 and so this region was examined for loss of genetic material. Specific DNA loss in this region was demonstrated in 64% of tumours. Loss close to, but not including, the p53 locus correlated with p53 overexpression. Using the polymerase chain reaction, exons 5 and 6 of the p53 gene were selectively amplified and subsequent sequencing suggested the overexpressed p53 may be mutant and oncogenic in at least some tumours. These results suggest that chromosome 17p is important in breast cancer. Lack of normal p53 tumour suppressor gene expression or deletion of a nearby regulatory locus resulting in overexpression of mutant p53 are proposed as key events in the pathogenesis of breast cancer.

A mouse xenograft model, based upon the oestrogen dependent human breast cancer cell line MCF-7, was established. Changes in specific gene expression measured by mRNA production were demonstrated following oestrogen stimulated growth of the xenograft tumours. Treatment with the endocrine agent tamoxifen resulted in a high level of TGF-ß expression and a reduction in tumour size. To examine the clinical relevance of these findings, the level of TGF-ß expression was determined in a subgroup of 11 patients treated with tamoxifen prior to surgery. Tumours which increased in size despite tamoxifen treatment were found to have high levels of TGF-ß mRNA. This suggests that such tumours have escaped the growth inhibitory effect of TGF-ß that may be demonstrated in vitro and in the MCF-7 xenograft model system.

It is concluded that the use of molecular approaches, such as those employed in this work, will help not only to elucidate the pathogenesis of breast cancer, but also to clarify the mechanisms by which some forms of anticancer therapy are mediated and hence lead to new therapeutic approaches.

INTRODUCTION

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General considerations

Breast cancer is common. In the United Kingdom, one in twelve women are affected (Fentiman, 1989), with breast cancer cited as the cause of death in 15,073 women in 1985 (Forrest, 1986). World-wide, there were an estimated 572,100 new cases of breast cancer in 1980 (Parkin et al., 1988), the disease accounting for 15-20% of cancer deaths and 2-5% of all deaths (Boyle, 1989).

Due to the complex, dynamic nature of cancer and the interactions between tumour and host, many factors have been implicated in the dynamic process of carcinogenesis. A number of epidemiological factors have been suggested as risk factors for breast cancer. The incidence of breast cancer is much higher in women than men, increases with age (Doll, 1975), is higher in social classes I and II (Kelsey, 1979) in urban than rural populations (Boyle, 1989) and in western compared to third world women (Waterhouse et al., 1976) although migrating populations acquire the cancer pattern of the new home in time. Dietary factors (Kalsouyanni et al., 1987, Holm et al., 1989) including alcohol (Longnecker et al., 1988), vitamin D intake and high body mass (Rose, 1986) have also been implicated, but little progress has been made recently in our understanding of the epidemiology of breast cancer and its relationship to the biology of the disease (Boyle and Leake, 1988).

In recent years considerable attention has been paid to

hormonal influences on the breast. Identified environmental risk factors appear to relate mainly to duration and periodicity of oestrogen exposure (Aldercreutz, 1989). These influences can be considered under three main headings: ovarian factors, external hormonal influences and previous breast disease.

The role of ovarian function on the development of human breast cancer relates to the menstrual cycle and child bearing, with risk factors for breast cancer highest in early reproductive life (Pike, 1987). Early menarche and late menopause increase the risk of developing breast cancer (Kelsey and Hildreth, 1983, Miller and Bulbrook, 1986), suggesting the cumulative number of cycles may be important (Olsson et al., 1983) particularly as oophorectomy is protective, with a greater reduction in risk the earlier it is performed (Feinlib, 1969).

Late age at first full-term pregnancy or nulliparity increases the risk of developing breast cancer (Miller and Bulbrook, 1986); the protective effect of parity, once thought to be explained by early maternal age at first birth (MacMahon et al., 1970), is in fact independent of maternal age at first birth (Ewertz and Lund, 1989). Lactation may cause hormonal changes (via modulation of pituitary and/or ovarian activity), resulting in a negative relationship between lactation and breast cancer (Byers et al., 1985). This is independent of parity (Byers et al., 1985) with 5 or more

children actually increasing the risk (Duffy et al., 1983). The effect of other reproductive factors (early terminated pregnancies, spacing of childbirths and age at last pregnancy) have not yet been defined.

A substantial proportion of British women are exposed to external hormonal influences, particularly the oral contraceptive pill and hormone replacement therapy. Early suggestions of increased risk from oral contraceptive pill use, particularly the early use of an oral contraceptive pill (Pike et al., 1983) and increased duration of total use have not been confirmed in large studies with a high proportion of young women (Stadel et al., 1985, Paul et al., 1986).

The situation remains uncertain (Chilvers & Deacon, 1990) but even if one assumes one additional breast cancer before the age of 35 years per 7,000 years of oral contraceptive pill use , this effect is outweighed by the reduction in risk of ovarian and endometrial cancer (Kay and Hannaford, 1989).

Similarly, although studies of hormone replacement therapy have shown a minimal association with the development of breast cancer (Kelsey & Berkowitz, 1988), evidence from prospective studies suggests an association with long-term hormone replacement therapy is likely (Hunt et al., 1987).

Nevertheless, there is no clear evidence that an excessive oestrogenic stimulus is a determinant in the

initiation and promotion of breast cancer (Bulbrook et al., 1989). However, both oestrogen and progesterone may be involved in breast carcinogenesis as evidenced by the antitumour effects of the oestrogen antagonist tamoxifen (Stewart et al., 1989) and the use of progestins in the treatment of breast cancer (Chamness, 1989).

Some women with a past history of benign breast disease have an increased risk of breast cancer (Roberts et al., 1984) probably confined to epithelial proliferation lesions (Page et al., 1987, Tavassoli & Norris, 1990), with benign breast disease, hyperplasia and hyperplasia with cellular atypia progressively increasing the relative risk of breast cancer by factors of 1.5, 1.9 and 3.0 respectively (Carter et al., 1988).

The only direct cause and effect relationship for breast cancer is with ionising radiation. Studies of radiogenic breast cancer in female survivors of the Japanese A-bomb (Takunaga et al., 1982), breast cancer mortality in tuberculosis patients who had multiple fluoroscopies (Howe, 1984), patients who received radiotherapy for benign breast conditions (Baral et al., 1977) or for intrathoracic lymphoma (Anderson & Lokich, 1990) demonstrate an increased risk with increasing dose, decreased risk with increasing age at exposure and latency of at least 10 years (Hindreth et al., 1989, Miller et al., 1989). Ionising radiation damages cells by disrupting, by whatever means, the genetic material of a

cell.

In addition, evidence for a genetic (familial) predisposition to breast cancer has been recognised since Roman times (reviewed in Lynch et al., 1984).

It is claimed that at least 5% of breast cancers have a hereditary basis, rising to 11.5% of cancers diagnosed before the age of 50 (Lynch et al., 1984). Such patients, according to Lynch, characteristically have an early age of onset, excess of bilaterality and multiple primaries, vertical transmission (mother to daughter) within the family and impaired survival compared to sporadic breast cancer (Lynch et al., 1984). The issue of early age of onset in familial cases is still contentious and the findings may be influenced by biased ascertainment of cancer families (Tulinius, 1989). However, there is certainly an increase in incidence of breast cancer in the close female relatives of breast cancer patients compared to the general population (Kelsey, 1979) rising to a 9 fold increase in age-specific risk in the first degree female relatives of a premenopausal breast cancer patient with bilateral disease (Anderson, 1971).

Although little recent progress has been made in our understanding of the epidemiology of breast cancer (Boyle and Leake, 1988), the cumulative body of evidence from the epidemiology, biology, particularly the radiobiology, and family studies of breast cancer suggests that the

fundamental abnormalities in breast tumorigenesis lie at the molecular level of nucleic acids which comprise the genetic material of the cell.

To understand the molecular mechanisms which underlie breast cancer it is helpful first to review the multistep theory of carcinogenesis which encompasses the concepts of tumour suppressor genes and oncogenes. This leads to consideration of nucleic acid studies (DNA and mRNA) in relation to specific genes.

Multistep theory of carcinogenesis

The evidence for a clonal origin of tumours, even of those in which the progeny of a single cell have diversified to heterogeneous cell phenotypes, is compelling (Bishop, 1987). Tumour cells therefore become increasingly different from the host with derangement of functions controlling growth and differentiation (Vile, 1989), metabolism of precarcinogens to carcinogens, impaired DNA repair mechanisms, or altered immune surveillance (Friend et al., 1988).

The thesis that carcinogensis is the cumulative effect of a series of discrete genetic lesions (Friend et al., 1988) was first proposed by Nordling (1953). The epidemiology of the age specific incidence of several common cancers suggested 6 or 7 events were required (Armitage & Doll, 1954). The requirement for cumulative events in the evolution of a single tumour to explain the

limited penetrance of any one cancer gene was subsequently modified to suggest as few as 2 mutations were required for carcinogenesis where an external factor (such as hormonal influences in the breast) might confer a slight growth advantage on the altered cells (Armitage & Doll, 1957). While an autonomously oncogenic single mutation in the germ line would probably be lethal (any target cell would be malignant), a non-lethal constitutional abnormality is conceivable. It would necessarily be less severe and probably require another event to produce cancer, one step perhaps involving immortalisation and the other involving transformation.

Further evidence for at least 2 hits (Knudson, 1971) came from observations on 48 cases of the ocular childhood tumour, retinoblastoma and subsequently from a few other rare heritable tumours with high penetrance. Precancerous lesions in the hereditary form of a cancer are multicellular in origin (resulting from local phenomena that affect numerous cells) but malignant tumours arising from these are clonal in origin resulting from a rare event that affects a single cell (Knudson, 1985).

Molecular analysis of common tumours such as colorectal cancer (Vogelstein et al., 1989) points increasingly to the presence of multiple abnormalities at the DNA level; however malignant cells tend to accumulate multiple aberrations in their DNA including gene deletions (Povey

et al., 1980, Sondberg, 1980) and it is unlikely that all of these contribute to the malignant state.

Genetic loci may be important in either the initiation or progression of tumours (Bishop, 1987, Vile, 1989), with cells already capable of abnormal proliferation becoming more aggressive (Bishop, 1987). There may be genetic variation in the pathways of tumour progression (Hall et al., 1989), with sequential selection of subclones, on the basis of accumulated oncogenic events that alter sensitivity to external signals and hence confer a proliferative advantage (Cline et al., 1987, Steel, 1989). Such events may involve oncogene activation (c-k-ras), loss of oncogene expression (v-abl) or no discernable change in oncogenes (reviewed in Nicholson, 1987), loss of tumour suppressor function, the emergence of drug resistance or loss of intimate intercellular contacts (Steel, 1989). The propensity for metastasis may be linked to disordered function of oncogenes encoding cytoskeletal elements (reviewed in Waxman and Sikora, 1988). Indeed, oncogene expression (for example of the protein p21 - Ohuchi et al., 1986) may be a feature of earlier rather than later stages of mammary carcinogenesis, so oncogene expression may contribute to carcinogenesis rather than determine metastatic progression (Nicholson, 1987). Conversely, in breast cancer families, it has been suggested that oncogenes may be involved in tumour progression rather than as primary

alterations leading to breast cancer (Hall et al., 1989). The hypothesis that somatic mutations lead to cancer implies that there are critical genetic loci the mutation of which leads to cancer (Knudson, 1985). The isolation of candidate genes involved in the aetiology of human cancer has resulted from 3 main approaches: transfection studies using DNA from human tumours and immortalised cell lines, the study of tumour-producing viruses and molecular comparisons of malignant and non-malignant tissues. This has led to the identification of 2 groups of important genes: tumour suppressor genes and oncogenes.

Tumour Suppressor Genes

Tumour suppressor genes (also variously termed oncosuppressor genes, anti-oncogenes, recessive oncogenes, orthogenes, emergenes or plato-genes) are defined as genes the deletion, reduced expression or inactivation of which contributes to carcinogenesis. Tumour suppressor genes were originally recognised when cancer cells, fused with normal cells resulted in non-tumorigenic hybrids (Harris et al., 1969, Harris, 1988) although expression of the transforming gene was not down-regulated. Some cells subsequently shed specific chromosonal material resulting in reversion to the tumorgenic phenotype of transformed morphology, low serum requirements and lack of contact inhibition

(Harris, 1988, Stanbridge, 1988). As a refinement of this early work, specific DNA fragments introduced into a cell were found to suppress the tumorigenic phenotype (reviewed in Hansen and Cavenee, 1988) the clearest example of which remains the effect of the normal retinoblastoma gene introduced into a retinoblastoma derived cell line (Huang et al., 1988).

Tumour suppressor genes may also play a role in terminating embryogenesis where genes active during embryogenesis (transforming genes) are suppressed at differentation by dominant suppressor genes.

In Drosophila, 25 or more recessive genes have been implicated in developmental tumours. Reintroduction of the missing gene fragment into the germline (mutant lava) prevents tumorigenesis and unblocks differentiation arrest (reviewed in Anderson and Spandidos, 1988).

In mammalian cells, loss of growth suppressor genes from a cell removes the cell from normal growth constraints (Weinberg, 1988). Such genes are regarded as recessive since both copies of the gene must show loss of function to allow neoplastic development (Vile, 1989). Moreover, it has long been held (Knudson, 1985), that if genes are important in the origin of common tumours, it is improbable that only one dominantly expressed genetic locus is involved.

The concept of tumour suppressor genes was shown to be directly relevant to human malignancy following studies

of retinoblastoma, a paradigm for hereditary cancer in humans (reviewed in Murphree & Benedict, 1984). Knudson (1971) first proposed an explanation for the familial and sporadic forms of retinoblastoma, a childhood tumour of the retina of the eye, based on mathematical analysis of the disease.

In the familial form, one retinoblastoma locus or gene on 13q14 (Yunis, 1978) is inherited in a defective, nonfunctional form as a germ line event. Only a single further somatic lesion of the retinoblastoma gene is required to cause a tumour and this may be a submicroscopic mutation, deletion, genetic recombination - with or without duplication - or chromosome loss (Cavenee et al., 1983, Knudson, 1985). Thus multiple tumours can occur. In the sporadic form, both retinoblastoma loci are intact and so 2 "hits" or somatic mutations/deletions in the same cell are required to inactivate the tumour suppressor function of the retinoblastoma protein and thus sporadic tumours are invariably single.

This may be explained on biochemical grounds. The retinoblastoma gene encodes a protein that binds to other proteins in the nucleus, possibly forming a complex that regulates cellular differentiation (Friend et al., 1986). The retinoblastoma protein also binds to DNA tumour virus proteins (Whyte et al., 1988, De Capiro et al., 1988) suggesting that some oncogenic viruses may act by

sequestering tumour suppressor gene products (Windle et al., 1990) thereby blocking cell differentiation (Vile,1989). Functional inactivation of the retinoblastoma tumour suppressor genes (by whatever means) therefore removes a barrier to cell proliferation, allowing inappropriate expansion of a cell clone (Friend et al., 1988). Conversely, reintroduction of the normal retinoblastoma gene renders a retinoblastoma-derived cell line non-tumorigenic (Huang et al., 1988).

Similar strong evidence for tumour suppressor genes in familial tumours has emerged in particular from studies of Wilm's tumour (the gene for which is located on 11p13, Franke et al., 1990) and familial adenomatous polyposis (FAP; Bodmer et al., 1987). Familial tumours in general seem to be associated with inactivated or deleted loci, compatible with loss of tumour suppressor gene function. Tumour suppressor genes may act pleiotropically in the differentiation programs of several cell types (Vile, 1989): for example, such functions have been suggested for the retinoblastoma gene in small cell lung cancer (Harbour et al., 1988) and in breast cancer (T'Ang et al., 1988). Overall, the case for tumour suppressor gene malfunction in human cancer is strong.

Oncogenes

Oncogenes can be defined as any gene, the activation overexpression or amplification of which contributes to

tumorigenesis and disordered proliferation or differentiation.

The evidence for oncogenes came initially from viral and gene transfection studies. Acutely transforming oncogenic viruses contain not only the "gag" (core antigen) "pol" (reverse transcriptase) and "env" (envelope protein) found in slowly transforming viruses but also an "onc" gene specific to each virus and responsible for the induction of malignancy in infected cells (Bister and Duesberg, 1982). These oncogenes are homologous to human DNA sequences "proto-oncogenes", (Stehelin et al., 1976, review in Slamon et al., 1987) and probably arose from the DNA of higher organisms including man (Stehelin et al., 1976, Bishop, 1981). In their new setting of retrovirus DNA, these cellular genes may be oncogenic (Bishop, 1987), particulary when viral DNA is reintegrated into chromosomal DNA (Bishop, 1987). Although over 20 oncogenes have been identified among DNA and RNA tumour viruses (Bishop, 1983), and the central role of certain oncogenes in tumorigenesis due to chronic transforming retroviruses such as avian leukosis virus has been established (Hayward et al., 1981) there remain few examples in humans. Burkitt's lymphoma (Epstein-Barr virus), hepatocellular carcinoma (hepatitis B), cervical and laryngeal carcinoma (human papilloma virus) are generally attributed to DNA viruses and AIDS related malignancies to the Human immunodeficiency RNA

virus. However, a viral "onc" gene has not been unequivocally implicated in any of these instances and other, less direct mechanisms may be at work.

Gene transfection, that is the experimental uptake of DNA from human tumours or tumour cell lines into cells (such as the immortal NIH3T3 or C127 mouse fibroblasts) in vitro, can transform the cells (Shih and Weinberg, 1982). These transformed cells are tumorigenic in immunosuppressed mice, suggesting that the DNA responsible for the original tumour has been integrated into the transformed cell DNA (Krontiris and Cooper, 1981, Porteous et al., 1986).

However, the mere process of DNA uptake may itself induce genetic instability (Kerbel et al., 1987) and normal cells (as opposed to the immortal NIH3T3 or C127 cells) may require co-transfection with at least two different oncogenes (Land et al., 1983) for transformation to occur. Out of some 50,000 human genes, fewer than 100 may be oncogenes (Weber and McClure, 1987). Proto-oncogenes are normal componants of cellular DNA, their scheduled expression being necessary for the proper growth and differentiation of cells (Nicholson, 1987, Anderson and Spandidos, 1988) as supported by the homology between the proto-oncogene c-sis and platelet derived growth factor (Heldin and Westermark, 1984) with adjacent sequences serving to prevent unscheduled activation (Anderson and Spandidos, 1988). Proto-oncogenes behave as oncogenes

when activated, giving cells a continuous signal to proliferate and thus blocking specific steps in cellular differentiation (Vile, 1989).

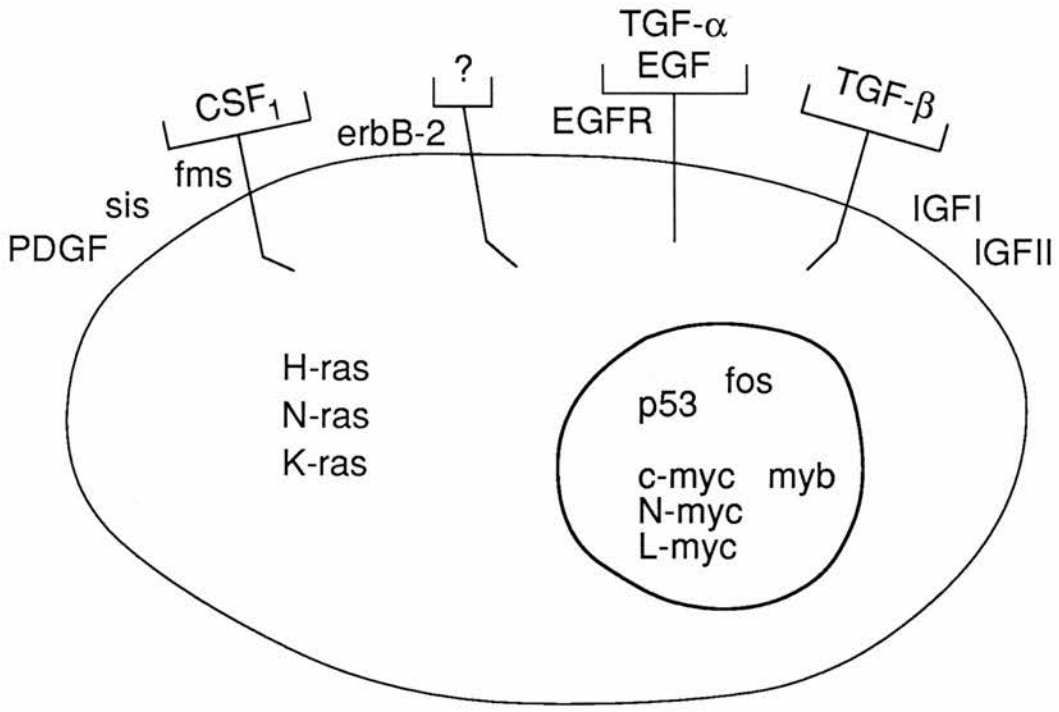
Oncogenes may be classified according to the mechanism by which they arise from the normal proto-oncogene DNA. The transition from normal proto-oncogene DNA to oncogene may be due to mutation for example ras point mutation at codons 12 or 61; (Reddy et al., 1982, Goldfarb et al., 1982) resulting in the production of an abnormal protein product, truncation (src, fms) or fusion (abl/bcr). Translocation of a gene may affect the expression or biochemical function of that gene (Bishop, 1987) - for example chromosome 8 to 14 translocation of c-myc in Burkitt's lymphoma results in inappropriate production of c-myc as a consequence of disruption of exon 1 of the gene (Klein et al., 1981, Taub et al., 1984, Leder et al., 1986, Bishop, 1987) and failure to terminate c-myc expression with B-cell differentiation (Curran et al., 1984, Gonda et al., 1984).

While normal levels of proto-oncogenes may be adequate for cell division, supra-normal expression may be of significance (James et al., 1988). Overexpression may result from multiple gene copies (amplification) (Semba et al., 1985), a feature which frequently affects proto-oncogenes that regulate cell proliferation and may persist in dividing cells if it provides a selective advantage (reviewed in Bishop, 1987). Thus,

overexpression of a gene product such as c-erbB-2 (Slamon et al., 1989) or normal H-ras protein p21 (Pulciani et al., 1985) may be an important pathogenic event in some tumours. In addition, rearrangements or deletions may influence the activity of such genes.

Alternatively, oncogenes can be grouped according to the type of their product action rather than how they arise (James et al., 1988) (figure 1). For example, growth factors (platelet derived growth factor, transforming growth factors), growth factor receptors (epidermal growth factor receptor, c-erbB-2), G proteins (Ras), intracellular transducers (abl) or nuclear acting oncogenes affecting gene expression (myc).

Figure 1 : A classification of oncogenes according to the site of action.



A diagramatic representation of genes potentially involved in carcinogenesis. Examples include growth factors such as platelet derived growth factor (PDGF), transforming growth factors (TGF-a and TGF-ß), epidermal growth factor (EGF), colony stimulating factor (CSF1) and insulin-like growth factors I and II (IGF I and IGF II); growth factor receptors including epidermal growth factor receptor (EGFR), growth factor receptor-like genes c-erbB-2, sis and fms; G proteins (H-ras, N-ras, K-ras) and nuclear acting genes (c-myc, N-myc, L-myc, myb, fos and p53).

In addition, while oncogenes may be involved in the pathogenesis of cancer, in certain situations they may play a role in tumour progression (N-myc in neuroblastoma; Brodeur et al., 1984) or the organisation of a different phenotype for example v-H-ras transfection of hormone dependent MCF-7 cells can confer hormone independence (Kasid et al., 1987).

The distinction between dominant oncogene expression and recessive tumour suppressor genes expression is essentially functional. Indeed, one gene may act as a tumour suppressor gene or an oncogene under different circumstances; for example the oncogenic effect of mutant ras is enhanced by deletion of the residual normal gene (Anderson and Spandidos, 1988). Oncogenes appear to play a major role in cell proliferation during histogenesis (Knudson, 1985). The normal proto-oncogene and tumour suppressor genes are thus involved in growth and differentiation signalling (Bishop, 1988) with the inactivation of tumour suppressor genes as important as the activation of oncogenes (Friend et al., 1988). It is not surprising therefore that the activation of one or more oncogenes occurs as cumulative steps on the route to colonic cancer (Vogelstein et al., 1988) and this model may be valid for other human malignancies.

A third key factor in tumorigenesis, with tumour suppressor genes and oncogenes, may be the influence of growth factors in the promotion of carcinogenesis.

Growth Factors

Transformed cells have relaxed cell cycle control and may traverse the cell cycle in the absence of exogenous growth factors (Heldin & Westermark, 1984). Growth factor independence and autonomous growth of transformed cells may be due to autocrine growth factor production, enhanced receptor expression or subversion of the intracellular signal system that ultimately leads to the initiation of DNA synthesis (Heldin & Westermark, 1984). Alternatively, it may be due to loss of responsiveness to growth inhibitory signals, or to any combination of these mechanisms.

The ability of many oncogenes to confer growth factor independence on cancer cells usually seems to be related to their alteration of a receptor or of a post receptor signalling pathway (making activation of the receptor independent of the effector), rather than to a primary alteration in the synthesis and release of a specific growth factor (Sporn & Roberts, 1985). Enhanced cell response to a growth factor may result form a change in the number or affinity of receptors for the growth factor (Sporn & Roberts, 1985) or to a change in the sensitivity of the post-receptor signal pathway.

Many types of tumour cells release polypeptide growth factors (eg transforming growth factor-alpha, platelet derived growth factor, transforming growth factor-ß) into

their conditioned medium when grown in cell culture and these same tumour cells often possess functional receptors for the released peptide (Sporn & Roberts, 1985).

Just as homology between the oncogene c-sis and platelet derived growth factor suggest that an oncogene may code for a growth factor, expression of a viral oncogene may turn on the production of a cellular growth factor that results in the transformation of a cell (reviewed in Heldin & Westermark, 1984).

Another feature of the relationship between growth and oncogenes is illustrated by the functional similarities between some oncogene proteins and growth factor receptors (for example epidermal growth factor and c-erbB-2; Semba et al., 1985). This is consistent with the observation that the inner aspect of the cell membrane is the major site of action of the tyrosine kinases associated both with oncogene products and with growth factor receptors (reviewed in Heldin & Westermark, 1984).

Growth factors often function in a localised manner, acting either on the cells which themselves have produced the growth factor (autocrine action) or on neighbouring cells (paracrine action). Stimulation of cells by an abnormal growth factor could result in a polyclonal proliferation of pre-neoplastic cells, mutation within which would lead to an apparently monoclonal tumour

(Editorial, Lancet, 1986). In addition, growth factors may have important roles in tumour promotion (James et al 1988), with the response of a cell to a given growth factor changing from enhanced proliferation at one stage to growth arrest later on (Steel, 1989). The tumour promoting effects of growth factors may sometimes be secondary to an inability of the cells to progress to an advanced state of differentiation (Steel, 1989).

Negative autocrine growth factors may themselves control aberrant cell growth and malignant transformation or promotion may be due to failure of cells to synthesis, express or respond to negative growth factors (Sporn & Roberts, 1985). Thus loss of normal inhibitory signals may be at least as important as growth promoting factors in tumorigenesis (Editorial, Lancet, 1986).

Modifications of the extracellular matrix and stroma can lead to alterations in gene expression and cell phenotype (reviewed in Nicholson, 1987). The final growth rate is the net result of the joint effect of positive and negative factors and separate mechanisms account for these individual effects.

In the MCF-7 breast cancer cell line, studies of growth associated polypeptides show that insulin like growth factor-I, transforming growth factor-alpha and platelet derived growth factor may be induced by oestrogen (Kasid & Lippman, 1987). Thus, aberrant production of growth factors, triggered either by activated oncogenes and

oestrogen stimulation in hormone dependent cells, or by increased constitutive production in hormone-independent cells may be associated with the neoplastic growth of breast cancer in an autocrine, paracrine or endocrine manner (Kasid & Lippman, 1987).

Cell to cell interactions are also be important as highly metastatic mammary epithelial cells often fail to communicate via cell junctions, while counterpart benign or normal epithelial cells are tightly coupled (Nicholson, 1987).

The Molecular biology of breast cancer

Evidence from familial, epidemiological, cytogenetic and molecular genetic studies support the notion that cancer is a profoundly genetic disease (Hansen and Cavenee, 1987). The molecular approach to cancer based on these concepts of multistep carcinogenesis involving tumour suppressor genes, oncogenes and growth factors has been applied to breast cancer in cytogenetic, deoxyribonucleic acid (DNA) and messenger ribonuceic acid (mRNA) studies.

Cytogenetics

Despite the technical difficulties of cytogenetic analysis of solid tumours, studies of direct tumour preparations using Giemsa-banding techniques have demonstrated aberrations of chromosomes 1, 3, 5, 6, 9,

10, 11, 12, 15, 16, 17, 18, 21 (Pathak and Goodacre, 1985, Ferti-Passantonopoulou and Panani, 1987) with chromosone 1q most frequently involved. Such cytogenetic studies alone are relatively crude but do suggest possible loci for further, more detailed examination.

DNA Studies

Cancer family studies

Cancer families may be defined as families in which 2 or more relatives, including the proband, are affected within the modified nuclear pedigree - that is including grandparents, aunts and uncles (Lynch et al., 1984). Three important concepts of familial cancers have been established (Knudson, 1989).

1. In familial cases, an inactive/physically deleted gene is inherited.
2. The inherited defect alone is insufficient to cause the cancer, one or more further somatic events are required.
3. Genetic loci of familial cancer may also be the sites of somatic mutation/deletion or chromosonal translocation in sporadic tumours of the same histological type. Hence, analysis of molecular lesions in sporadic tumours may identify candidate loci for cancer susceptibility genes in cancer families.

In familial breast cancer (reviewed in Schneider et al.,

1986), analysis of such families suggests that breast cancer may be transmitted as an autosomal dominant trait with high penetrance (Newman et al., 1988). A disease allele frequency of 0.7% and penetrance varying with age has been suggested (Williams and Anderson, 1984).

A candidate locus, identified by the glutamate-pyruvate transaminase (GPT) gene on chromosome 16 was suggested (King et al., 1980) but not subsequently confirmed (King et al., 1983). More recently, exclusion of genetic linkage to oncogenes (including H-ras, myc and int 2) in breast cancer families has been demonstrated (Hall et al., 1990). A recent review of segregation analysis concludes that the existence of a single major gene is not sufficient to explain familial distribution of breast cancer, suggesting the genetic basis is heterogeneous and complex (Andrieu et al., 1989). Thus, the genes involved in familial breast cancer remain obscure. However, the concept that the same region of the genome is involved in familial and sporadic cancers is now well established, for example in retinoblastoma (Cavenee et al., 1983) and for familial polyposis coli (FAP) (which invariably progresses to colon cancer) and sporadic colorectal cancer (Solomon et al., 1987, Ashton-Rickart et al., 1989). These studies suggest that advancing knowledge of sporadic breast cancer may yet yield dividends for breast cancer families.

DNA changes in sporadic breast cancer.

The possibility of developing a blood lymphocyte DNA marker for breast cancer, in the form of rare alleles at the Harvey ras locus on chromosome 11p (Krontiris et al., 1985, Lidereau et al., 1986, Saglio et al., 1988) no longer seems viable (Corell and Zoll, 1988, Sheng et al., 1988, White et al., 1988, Mackay et al., 1988a) particularly as a test of the familial susceptibility to breast cancer (Hall et al., 1990). However, somatic changes in the tumour DNA fingerprint were identified using mini-satellite probes in 2 out of 4 breast cancers studied (Thein et al., 1988). Further studies of allele losses from specific chromosomal loci (consistent with possible tumour suppressor gene deletion or loss of function) and other genetic abnormalities in breast cancer implicate loci on chromosones 1, 11, 13 and 17 in sporadic breast cancer.

A common region of deletion of 26 centimorgans on 1q 23-32 has been demonstrated in a quarter of 48 breast cancers with allele loss (Chen et al., 1989) compatible with cytogenetic studies (Pathak and Goodacre, 1985, Ferti-Passantonopoulou and Panani, 1987).

Allele loss for H-ras (11p1.4) has been demonstrated in 8.6% to 27% of breast cancers (Theillet et al., 1986, Yokota et al., 1986a, Mackay et al., 1988a, Biunno et al., 1988, Lidereau, 1988), correlating with various poor

prognostic tumour features. Several loci on 11p may be involved (Ali et al., 1987) and 39% (19/49) tumours show allele loss of informative loci on chromosone 11 (Mackay et al., 1988a) but no consistent region of deletion. Turning to the long arm of chromosome 11, heterozygous female carriers for the ataxia-telangiectasia gene on chromosone 11q22-23 (Gatti et al., 1988) have increased risk of breast cancer (Swift et al., 1987).

On chromosome 13, allele loss from 13q was found in human ductal breast cancers (Lundberg et al., 1987). Specific studies of the retinoblastoma locus on 13q demonstrated rearrangements in 19% of primary breast tumours (Varley et al., 1989).

Allele loss from the tip of 17p (at 17p 13.1) has recently been demonstrated (Mackay et al., 1988b, Devilee et al., 1989a, 1989b) in about 2/3 of breast tumours which bears comparison with 17p allele loss in 80% of colorectal cancers (Fearon et al.,1987). Loci already yielding significant frequencies of allele loss in other tumours such as chromosomes 5, 6, 8 and 18 (Vogelstein et al., 1989, Ashton-Rickart et al., 1989, Ehlen & Dubeau, 1990) may also merit further study.

Just as allele loss or gene mutation may represent loss of tumour suppressor gene function, the contribution of oncogenes to breast carcinogenesis may be deduced from the observed amplification of myc and erbB-2 oncogenes in breast cancers, discussed in a later section. Similarly,

a combination of allele deletions and oncogene amplification was demonstrated in 58% of 53 primary breast cancers (Cline et al., 1987). However, doubt continues to be expressed as to whether, in the germline, loss of a single allele at any of the loci listed above will lead to an increased risk of developing cancer (Vile, 1989) or whether the altered sequences observed in primary breast cancer are merely epiphenomena (Hall et al., 1989). One further consideration is that epigenetic mechanisms such as DNA methylation, may play a role in phenotypic expression (Nicholson, 1987).

Indeed, the function of cancer cells may be more important than structural modifications. For example, transcriptional activation of the erbB-2 gene may precede amplification of erbB-2 in early tumours (Borg et al., 1989, Slamon et al., 1989) and some genes amplified in breast cancer are not expressed (for example erbA, van de Vijver et al.,1987). Hence, the expression of genes merits considerable attention. While measurements of gene protein products can be informative, some tumours produce mRNA but no protein can be detected (Yokota et al., 1988). Moreover, messenger RNA transcription (synthesis) is the most common and immediate focal point of genetic regulation in eukaryotic cells (Johnson and McKnight, 1989). Therefore, detection and quantitation of mRNA will give direct evidence for functional genetic changes which may be important in carcinogenesis.

Messenger RNA

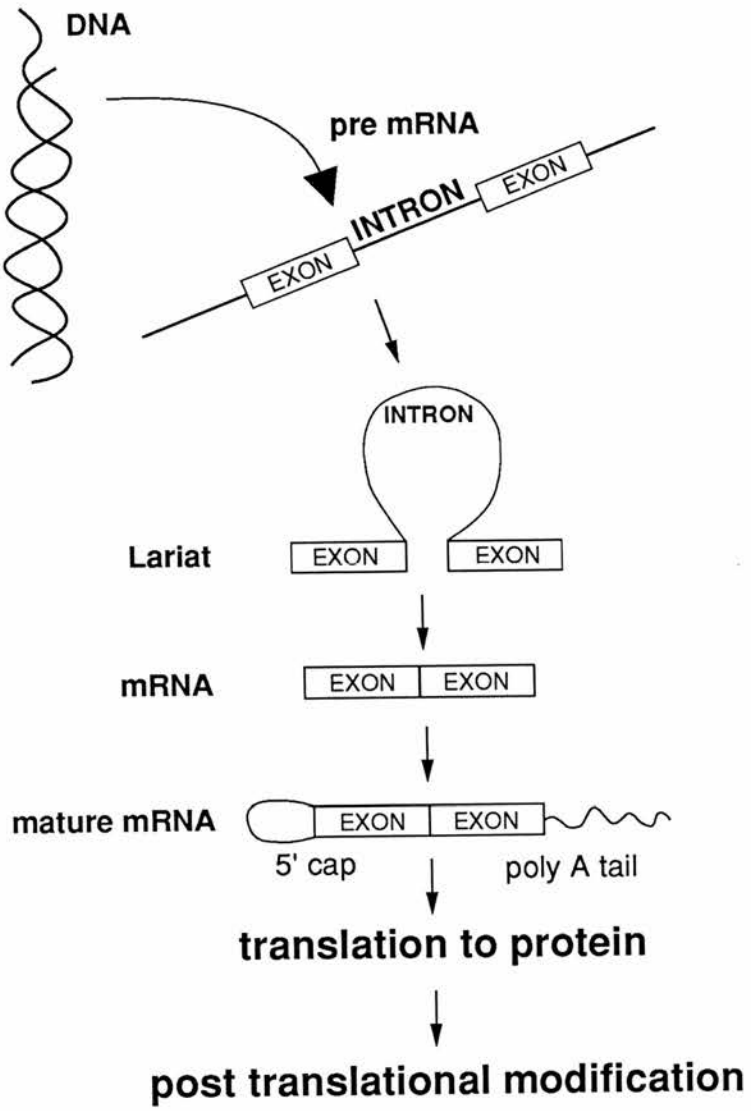
Messenger RNA (single stranded) is faithfully transcribed from double stranded DNA (figure 2), with the possibility of alternative sites of transcription initiation, to form precursor mRNA comprising exons (coding sequences) and intervening introns (non-coding).

Introns are removed from mammalian RNA precursors (pre-mRNA's) by two successive cleavage and splicing reactions; this involves formation of a "lariat" with subsequent release of the intron mediated by spliceosomes, which are ribonucleoprotein particles (snRNPs) (reviewed in Robertson, 1988). Indeed, an intron has recently been identified within a gene that encodes a spliceosomal RNA (Tani & Ohshima, 1989). Transcripts can be differentially spliced (and in Drosophila this may determine the organism's sex; Robertson, 1988).

Thereafter there is addition of a 5'cap and variable polyadenylation at the 3' end to form a 3'tail. Subsequent translation of the nucleotide sequence occurs, with AUG acting as a signal to start protein synthesis (translation) from the mRNA; three triplets (UAA,UAG,UGA) act as termination signals (reviewed in Kingston, 1989). The resultant protein may subsequently undergo considerable post translational modification.

From a given gene, different mRNA products may be formed in the same tissue, in different tissues and at different stages of development.

Figure 2 : The pathway for messenger RNA formation



A diagramatic representation of messenger RNA formation and function. From double stranded DNA, precursor single stranded mRNA is transcribed, introns are removed by formation of a lariat and release of the exons which are spliced together. Following addition of a 5' cap and 3' tail, the mRNA is translated to the protein product.

Formation of the precursor mRNA by transcription may be regulated by non-coding regions in the DNA - in the 5' flanking region the TATA box and the CAT box - by other enhancer sequences and by variation in the extent of cytosine methylation. Transcription may also be influenced by more distant regulatory elements on the same or on other chromosomes. One aspect of this is the concept of imprinting (Monk, 1987), the selective inactivation of a locus, without loss, dependent on passage through male or female gametogenesis. In Wilms' tumour, there is evidence that the paternal chromosome 11 allele is retained but inactive due to imprinting (Wilkins, 1989). Allele losses in a few cases of Wilms tumour show a bias towards retention of the paternally derived chromosome in the tumour (Schroder et al., 1987) supporting this concept of epigenetic effects caused by imprinting (Ponder, 1988). Gene expression can be differentially regulated by use of distinct promotors and mRNA alternatively spliced to create numerous isoforms (Feener et al., 1989). Interaction of cis acting negative regulatory elements (on the same chromosome) with proteins may be important for control of gene expression (Anderson & Spandidos, 1988).

Interactions between sequences within each mRNA can contribute to the stability of the mRNA (Kabnick & Housman, 1988). One example of this is the way in which disruption of the non protein coding exon 1 of the c-myc

gene increases the mRNA stability (Klein et al., 1981, Taub et al., 1984, Leder et al., 1986, Bishop., 1987) allowing post-transcriptional regulation of c-myc gene expression through control of mRNA degradation. Alternatively, a protein such as c-fos may regulate its own synthesis by binding to its own mRNA (reviewed in Anderson & Spandidos, 1988).

Thus, transcription of genes from DNA to mRNA and the subsequent fate of the genetic message is a complex matter. It is, however, amenable to investigation in malignancy. Initial studies of human tumours suggested that several oncogenes were overexpressed in some breast cancers (for example myc, ras, fes, fms, Slamon et al., 1984, Whittaker et al., 1986). The oncogenes implicated in breast tumorigenesis include c-myc, c-erbB-2, H-ras, int 1 and int 2, c-fos, and c-mos. Each one of these oncogenes merits some individual consideration.

c-myc

c-myc is the cellular human homologue of the principle oncogenic sequence carried by the avian myelocytomatosis acute retrovirus MC29 (Morse et al., 1988). Located on chromosone 8q24 (Dalla-Favera et al., 1982) and expressed in a wide variety of mammalian cell types (Marcu, 1987), the c-myc gene comprises 3 exons (the first non-coding) and 2 introns (Watt et al., 1983, Croce, 1986), encodes a 2.2 to 2.5 kb mRNA (Ar-Rushdi et

al., 1983, Alitalo et al., 1983, Erisman et al., 1985, Guerin et al., 1985, Escot et al., 1986, Dubik et al., 1987) that is translated to a 62 kd nuclear phosphoprotein (Stone et al., 1987). c-myc is involved in cell cycle regulation promoting G0/G1 transition (Parkin & Sonenberg, 1989), shortening G1 phase (Karn et al., 1989) and inhibiting terminal differentiation (Dmitrovsky et al., 1986). It has recently been suggested that c-myc may promote cellular DNA replication by binding to the origin of DNA replication (Iguchi-Ariga et al., 1987).

Activation of c-myc has been demonstrated in a wide range of human malignancies (reviewed in Escot et al., 1986) and expression of the myc gene in transgenic mice results in mammary cancers (Stewart et al., 1984)

However, recent evidence suggests that aberrant expression rather than mutation is the method of oncogenic conversion (Lang et al., 1988).

Amplification of c-myc by 2-15 fold has been identified in up to 35% of breast tumours (Guerin et al., 1985, Escot et al., 1986, Yokota et al., 1986a, Varley et al., 1987b, Adnane et al., 1989, Nikiforova, 1988, Sheshadri et al., 1989, Tavassoli et al., 1989). Although amplification is more frequently found in recurrent and metastatic tumours (Lidereau et al., 1988) it has been identified in benign lesions (Varley et al., 1987a, Bonilla et al., 1988).

Rearrangements of the c-myc gene have been demonstrated

in a small proportion of breast tumours (Escot et al., 1986) may result in increased disease aggression (Varley et al., 1987a) and in one study 27/48 (56%) of breast tumours showed c-myc amplification and/or rearrangement (Bonilla et al., 1988). c-myc amplification has been correlated with large tumour size (Seshadri et al., 1989), tumour aggression (Yokota et al., 1986a), poor short term prognosis (Varley et al., 1987a), age over 50 years and tumour type (Escot et al., 1986).

However, overexpression of c-myc may occur in the absence of c-myc amplification (Escot et al., 1986) and early studies suggested myc expression could be detected in a high proportion of breast cancers (Whittaker et al., 1986). Furthermore, increased c-myc expression was associated with a high degree of malignancy in breast tumours (Guerin et al., 1985) and the antiproliferative effects of transforming growth factors have been associated in some target cells with selective reduction in the expression of c-myc (Kimchi et al., 1988).

c-erbB-2

The erbB-2 oncogene was initially identified in a series of ethylnitrosourea induced rat neuroblastomas (Shih et al., 1981). c-erbB-2 has also been termed neu (Shih et al., 1981), HER-2 (Coussens et al., 1985), MAC 117 (King et al., 1985) and NGL (Kokai et al., 1988), although sequence homology of c-erbB-2, neu and HER-2 has been

confirmed (Coussens et al., 1985, Schecter et al., 1985). Located on 17q 12-22 (Coussens et al., 1985, Schecter et al., 1985, Fukushiga et al., 1986, Popescu et al., 1989) the gene encodes a 4.4 to 4.8 kb mRNA (King et al., 1985, Semba et al., 1985, Kraus et al., 1987, Slamon et al., 1987, van de Vijver et al., 1987) that is translated into a 185kd protein (Schecter et al., 1985) with 74% to 78% homology to, but distinct from, the transmembrane and cytoplasmic domains of the epidermal growth factor receptor (Semba et al., 1985, Coussens et al., 1985). The ligand that binds to this putative receptor (the extra cellular domain) remains obscure but presumably activates the intracellular domain with tyrosine kinase activity (Yamamoto et al., 1986).

c-erbB-2 can induce transformation (Shih et al., 1981, Di Fiore et al., 1987, Hudziak et al.,1987) in NIH 3T3 cells. Mutant rat erbB-2 in transgenic mice resulted in all the mice expressing the transgene developing breast tumours (Muller et al., 1988). However, over expression of the normal erbB-2 rather than gene amplification may be an important pathogenic event in some tumours (Slamon et al., 1989), since 26% to 48% of breast tumours had moderately increased erbB-2 protein levels in the absence of gene amplification (Berger et al., 1988, Slamon et al., 1989). Tumours that do show c-erbB-2 amplification can express the c-erbB-2 gene product at high levels (Di Fiore et al., 1987). Up to 30 fold amplification of

c-erbB-2 has been demonstrated in as many as 33% of breast tumours (Yokota et al., 1986b, Venter et al., 1987, Slamon et al., 1987 and 1989, Zhou et al., 1987, Berger et al., 1988, Zeillinger et al., 1989, Zhou et al., 1989, Lacroix et al., 1989, Slamon et al., 1989). Expression of the c-erbB-2 gene does appear to delineate a poor prognostic group as an independent predictive factor (Varley et al., 1987a, Slamon et al., 1987, van de Vijver et al., 1988, Berger et al., 1988, Wright et al., 1989, Slamon et al., 1989, Zeillinger et al., 1989) and this suggests it may have a role in the biological behaviour of breast cancer. Patients with c-erbB-2 amplification (especially with greater than 5 copies) and lymph node involvement with tumour have a shorter time to disease relapse and worse overall survival (Slamon et al., 1989, Wright et al., 1989). Amplification is more common in tumours of advanced stage (Zhou et al., 1987, Berger et al., 1988, Seshadri et al., 1989).
Conversely, tumours which do not express erbB-2 but do express oestrogen receptors may be particularly favourable for patient survival (Hynes et al., 1988).

H-ras

The Harvery-ras (H-ras) oncogene first identified as a transforming sequence in bladder carcinoma (Shih and Weinberg, 1982, Goldfarb et al., 1982) and named from its homology to the Harvey murine sarcoma virus is located on

11p (McBride et al., 1982). The H-ras gene encodes a 1.2kb to 1.4kb mRNA (Spandidos and Agnatis, 1984, Theillet et al., 1986, Biunno et al., 1988, Redmond et al., 1988) for a 21kd 189 amino acid protein, p21 (Capon et al., 1983, Taperowsky et al., 1983). Ras proteins bind Guanosine tiphosphate (GTP), regulating signal transduction from the cell membrane, with GTPase activating protein the likely biological target for regulation by p21 (Cales et al., 1988, Sigal 1988).

Activation of ras, particularly by mutation of codons 12 and 61 (Reddy et al., 1982) has been demonstrated in up to 80% of chemically induced rat mammary tumours (Sukamar et al., 1988) but occurs infrequently in human breast cancer (Biunno et al., 1988, Rochlitz et al., 1989). Similarly, although ras gene amplification may serve as an alternative mechanism to ras mutation in the development of neoplasia (Pulciani et al., 1985), few tumours exhibit amplification (1/65 Biunno et al., 1988, 4/22 Nikiforova, 1988).

Early studies suggested that ras mRNA was expressed in 16/22 invasive ductal breast carcinomas (Theillet et al., 1986) and at higher levels in tumour than in normal tissue (Spandidos and Agnatis, 1984), although H-ras transcripts were detected in neither normal nor cancer tissue in 2 later studies (Whittaker et al., 1986, Biunno et al., 1988). H-ras may provide a competence function (Kelekar and Cole, 1987) requiring activation of a second

oncogene before the cell becomes strongly tumorigenic (Clark et al., 1988). In the MCF-7 cell lines viral ras induces oestrogen independence (Kasid et al., 1985) by secreting mitogens which may function in an endocrine manner (Kasid et al., 1987).

Studies of ras p21 protein show higher levels of expression in malignant than in normal or pre-malignant breast tissue (Horan-Hand et al., 1984, Ohuchi et al., 1986, Whittaker et al., 1986). It has variously been suggested that p21 expression is important in the early stages (Ohuchi et al., 1986) or progression of breast cancer (Lundy et al., 1986) and p21 may be a useful prognostic indicator (Clair et al., 1987).

Int 1 and int 2

From evidence of mouse mammary tumour virus integration into the host genome initiating a tumour by specific gene activation (Dickson et al., 1984), human homologues of int 1 (van-t-Veer et al., 1984) and int 2 (Casey et al.,1986) have been mapped with the int 2 gene located on the same chromosomal band (11q23) as the progesterone gene (Law et al., 1987).

While int 1 and int 2 (Nusse et al., 1984, Dickson et al., 1984) may act co-operatively in the genesis of 50% of mouse mammary carcinomas (Peters et al., 1986), their role in human breast cancer is uncertain. Int gene amplification has been described in a small proportion of

breast cancers (Lidereau et al., 1988, Zhou et al., 1989) with co-amplification and mRNA expression of int 2 and another fibroblast growth factor like gene hst identified in 41/238 (17%) of breast cancers (Theillet et al., 1990). Clinically, co-amplification of int 2 and hst 1 has been correlated with aggressive disease (Zhou et al., 1988). Recently, it has been suggested that a genetic element between Hst/int 2 and a nearby gene BCL-1 could be important in the development of a subset of mammary tumours (Theillet et al., 1990).

Other oncogenes

Other oncogenes implicated in breast cancer include c-mos, a rare polymorphism of which results from a point mutation and was found more frequently in breast cancer patients than in the normal population (Lidereau et al., 1985, Lidereau et al., 1987) although this finding does not appear to be important in breast cancer families (Hall et al., 1989) and no evidence of c-mos amplification was found in one other study of 121 breast cancers (Escot et al., 1986).

c-fos has been implicated in the control of cell proliferation and differentiation and is induced by growth factors but not by oestrogen in MCF-7 breast cancer cells (Wilding et al., 1988). Studies of c-fos mRNA suggest higher levels of mRNA relate to increased metastatic ability of rat mammary tumour clones (Yuhki et

al., 1986), but there is little evidence for a role for c-fos in human breast cancer.

No alteration of N-myc or N-ras oncogenes was identified in 41 breast cancers, and only 2 fold amplification of L-myc in the same series (Varley et al., 1987a). No significant expression of the sis oncogene was found in either malignant or normal breast tissue (Spandidos and Agnatis, 1984).

Similarly, although other oncogenes such as Kirsten-ras closely related to H-ras (Yee et al., 1989), c-myb (Masuda et al., 1987a) and the dbl/mcf-2 gene isolated from the MCF-7 cell line (Noguchi et al., 1988) have been suggested as factors in breast carcinogenesis, there is as yet no strong evidence for the involvement of these genes in human breast cancer.

Tumour suppressor genes

p53

p53, initially considered to be an oncogene has subsequently emerged as a candidate tumour suppressor gene and spans the oncogene/tumour suppressor gene divide. First identified in 1979 (Lane and Crawford, 1979, Linzer and Levine, 1979) the p53 gene is located at 17p13 (Isobe et al., 1986, Miller et al., 1986, McBride et al., 1986). The gene encodes a 2.8 kb mRNA (Harlow et al., 1985) expressed in most adult tissues (Oren, 1988)

that is translated into a short lived (Reich and Levine, 1984) 53kd nuclear phosphoprotein regulated by phosphorylation Mihara et al., 1989). The p53 gene comprises 20kb with 11 exons (Lamb and Crawford, 1986), has structural features of a "housekeeping gene" including absence of a TATA box (Reynolds et al., 1984, Bienz-Tadmor et al., 1985) and DNAase hypersensitive sites (Shimada and Neinhuis, 1985) indicative of a consistently expressed gene. In addition, the open chromatin structure in the promotor region of p53 may allow the binding of mitogenic transcriptional regulators (Lubbert et al., 1989).

Although the normal function of p53 is not known, it may be involved in G1 to S transition of the cell cycle (Mercer et al., 1982, Mercer et al., 1984) with p53 essential for continuous cell proliferation (Milner, 1981, Shohat et al., 1987). It may modulate transcriptional activation by binding to DNA as does myc protein (Donner et al., 1982) or bypass the need for platelet derived growth factor in the induction of competence (Oren, 1988).

p53 is often found in high amounts in transformed cells (De Leo et al., 1979, Linzer and Levine, 1979, Benchimol et al., 1982, Crawford et al., 1981, Rotter et al., 1983, Thomas et al., 1983). Studies up to 1987 suggested p53 was an oncogene (Jenkins et al., 1985, Eliyahu et al., 1984, Eliyahu et al., 1985; Parada et al., 1984, Rovinski

and Benchimol, 1988, Editorial, Oncogene, 1988, Oren 1988), largely on the basis of co-transformation of rat fibroblasts with mutant H-ras. Subsequent studies (see discussion) suggest that the normal gene "wild type" p53 acts as a tumour suppressor (Green, 1989, Wang et al., 1989) in contrast to the mutant, oncogenic p53.

DNA rearrangements can alter the expression of the p53 gene leading to complete gene inactivation (Mowat et al., 1985). Rearrangements were identified in 2 osteosarcomas, but not breast tumours (Masuda et al., 1987b). p53 activation by mutation to oncogene may increase stability of the short lived protein (Jenkins et al., 1985) and thus compete with wild type, tumour suppressor gene p53 product interfering with its normal function. A similar inactivation may result from the formation of a complex observed between p53 and SV40 large T Antigen (Lane and Crawford, 1979, Linzer and Levine, 1979) adenovirus Elb (Sarnow et al., 1982) and heat shock proteins (Pinhasi-Kimhi et al., 1986, Hinds et al., 1987).

Thus the p53 story is complex and during the course of the work outlined in this thesis has continued to evolve.

The retinoblastoma gene

The retinoblastoma gene, the first tumour suppressor gene characterised, has recently been examined in breast cancer. Structural aberrations of retinoblastoma

resulting in absence or truncation of the retinoblastoma transcript have been observed in 25% of breast tumour cell lines and 7% of primary tumours (T'Ang et al., 1988). Rearrangements of the retinoblastoma gene have also recently been thoroughly documented (Varley et al., 1989). A substantial proportion of the retinoblastoma gene mutations are partial or complete deletions that extend an unknown distance beyond one or both ends of the gene (Bookstein et al., 1989).

Despite the undoubted interest of these findings, it must be acknowledged that no obvious excess of breast cancer has been recorded in survivors of familial retinoblastoma and linkage of breast cancer susceptibility to the retinoblastoma gene has recently been excluded (Bowcock et al., 1990). Thus the nature of the retinoblastoma lesions in breast cancer may be different in some way from those occurring in retinoblastoma itself.

Growth factors

While a number of growth factors may play a role in breast cancer, transforming growth factor alpha, transforming growth factor ß, epidermal growth factor and the epidermal growth factor receptor and the insulin-like growth factors I and II merit particular consideration. However, other growth factors such as platelet derived growth factor may also be implicated in the control of mammary epithelial cell proliferation (Yee et al., 1989).

Transforming growth factor alpha

Transforming growth factor alpha (TGF-alpha, Derynck et al., 1984) is a potential autocrine growth factor in human breast cancer cell lines. A 4.8kb transforming growth factor alpha mRNA has been detected in the MCF-7 cell line (Imanishi et al., 1989) and as a biologically active 30kd transforming growth factor alpha in MCF-7 and MDA-MB-231 (Lupu et al., 1988) although regulation of transforming growth factor alpha expression was unrelated to the proliferative behaviour of T47-D cells (Murphy and Dotzlow., 1989).

Transforming growth factor alpha mRNA was detected in 9/18 breast tumours (Ciardiello et al., 1989) and using high performance liquid chromatography, biologically active transforming growth factor alpha sufficient to promote cell division was found in the majority of breast tumour extracts (Gregory et al., 1989). In addition, oestrogen withdrawl and the anti-tumour agent tamoxifen cause a significant reduction in tumour transforming growth factor alpha (Gregory et al., 1989, Murphy and Dotzlaw, 1989).

Transforming growth factor alpha is a ligand which, like epidermal growth factor (EGF), binds to the epidermal growth factor receptor (Todaro et al., 1980, Ro et al., 1988). The EGF gene maps to 4q (Brissanden et al., 1984) and produces a 53 amino acid, 6kD peptide which binds to

specific high affinity EGF receptors (Nicholson et al., 1988, Waterfield, 1989).

Epidermal growth factor receptor

The EGF receptor (EGFR) gene or c-erbB-1 which encodes 3 of the 4 EGFR domains (7q 13-11.2, Meera Khan & Smith, 1984, Coussens et al., 1985), encodes mRNA variously reported as being of 1.8kb (Lebeau & Goubin, 1987), 2.9kb (Semba et al., 1985, Lebeau & Goubin, 1987), 2.7 kb (King et al., 1985), 5 kb (Murphy et al., 1988), 5.8kb and 10 kb (Ullrich et al., 1984, King et al.,1985) which produces a 170 kD receptor protein.

The EGF receptor comprises a ligand binding domain transmembrane domain and cytoplasmic domain with tyrosine kinase activity (reviewed in Nicholson et al., 1988a).

EGFR amplification was detected in 2-4% of tumours (Slamon et al., 1984, Lacroix et al., 1989); gene rearrangement (Slamon et al., 1987) and occasional overexpression have also been reported (Ro et al., 1988). It has been suggested that, although present in normal and cancerous breast tissue, EGFR expression is associated with dedifferentiation of breast cancer (Pekonen et al., 1988). In keeping with this, EGF appears to down regulate the oestrogen receptor by 50%, a possible mechanism of inducing oestrogen independence in oestrogen receptor positive cells (Cormier et al., 1989). Certainly, EGF receptor protein correlated inversely with

oestrogen receptor (Sainsbury et al., 1985, Nicholson et al., 1988b, Pekonen et al., 1988) and using 10 fmol mg^{-1} protein as a cut off point, EGFR positive tumours are associated with failure to respond to endocrine therapy in primary breast cancer (Nicholson et al., 1988b) and have a positive correlation with early disease recurrence and early death (Nicholson et al., 1988a).

Insulin-like growth factors

The somatomedins, insulin-like growth factor I (IGF-I, which maps to chromosome 12, Brissenden et al., 1984) and insulin-like growth factor II (IGF-II, which maps to the short arm of chromosome 11, Brissenden et al., 1984), are single chain proteins of 70 and 67 amino acids respectively. These two growth factors act on specific receptors and may behave as autocrine regulators in tumours (reviewed in Bell et al., 1984, Tricoli et al., 1984 and Slack, 1989). Although it has been suggested that the insulin-like growth factors may be significant autocrine growth factors in human hormone dependent breast cancer cells (Watson and Sikora, 1988), insulin-like growth factor II was not induced by oestrogen treatment in an oestrogen sensitive line and was not detected in an oestrogen independent cell line suggesting insulin-like growth factor II may be produced predominantly by stromal rather than cancer cells (Yee et al., 1988).

Transforming growth factor ß

By contrast with transforming growth factor alpha, epidermal growth factor and the somatomedins, transforming growth factor ß has an inhibitory effect on breast epithelial cells (Sporn et al., 1987) either in an autocrine (Sporn and Roberts, 1985) or paracrine (Knabbe et al., 1987) fashion.

There are three major forms of transforming growth factor ß: transforming growth factor ß1, transforming growth factor ß2 and transforming growth factor ß1.2 which result from the homodimeric and heterodemeric combination of 12.5kd ß1 and ß2 subunits (Cheifetz et al., 1987). The transforming growth factor ß originally described consists of two subunits of ß1 (Derynck et al., 1985) which is more abundant than ß2 (Roberts et al., 1988).

The transforming growth factor ß mRNA of 2.5 kb (Derynck et al., 1986, Knabbe et al., 1987) which predominates over minor species of 1.9 and 4.8 kb (Akhurst et al., 1988, Derynck et al., 1986) is expressed in all tumour tissues and cell lines (Derynck et al., 1987) in higher amounts than in adjacent normal breast tissue (Derynck et al., 1987).

Transforming growth factor ß is secreted from cells in a precursor form and 98% remains biologically inactive, unable to bind to the transforming growth factor ß receptors unless activated by proteolysis, or in the

experimental setting by transient acidification disrupting a noncovalent complex (Sporn et al., 1987). To further complicate the autocrine loop, not only are there 3 forms of transforming growth factor ß, and an activation step but 3 glycoprotein transforming growth factor ß receptors of 65kd, 85kd and 280kd. The 2 smaller receptors preferentially bind transforming growth factor ß1 (Massague, 1987). Thus there is potentially great flexibility in regulation of tissue growth by the transforming growth factor ß system (Chiefetz et al., 1987).

The functions of transforming growth factor ß encompass embryogenesis, inflammation, tissue repair and carcinogenesis (Sporn et al., 1986, Heine et al., 1987, Massague 1987, Sporn et al., 1987).

Increased secretion of transforming growth factor ß accompanies viral transformation of cells with down regulation of the transforming growth factor ß receptor (Anzano et al., 1985). Conversely, transforming growth factor ß could be oncogenic by defect, releasing cells from their proliferative constraints (Sporn and Roberts, 1985, Roberts et al., 1985). The action of transforming growth factor ß, as inhibitor or enhancer may depend not only on the cell type but also other growth factors present (Roberts et al., 1985, Fernandez-pol et al., 1987) and enhancing effects on stromal tissues and cells

(Wrann et al., 1987, Sporn et al., 1987, Massague et al., 1988).

Transforming growth factor ß is a negative growth factor for mammary epithelial cells (Sporn et al., 1987) and secretion of transforming growth factor ß is induced from MCF-7 cells treated with growth inhibiting concentrations of antioestrogens (Knabbe et al., 1987); the transforming growth factor ß thus produced also inhibiting oestrogen insensitive MDA MB 231 cells. Thus, transforming growth factor ß may play an important role in breast carcinogenesis and in vitro studies have suggested there may be some interaction with the hormonal aspects of the disease.

Hormone related genes

Oestrogen receptor gene

The oestrogen receptor gene, which maps to chromosone 6, produces a Mr 65000 oestrogen receptor protein homologous to the erbA protein of oncogenic avian erythroblastosis virus and the human glucocorticoid receptor (Green et al., 1986). Since cDNA for the oestrogen receptor gene was cloned and sequenced (Walter et al., 1985, Green et al., 1986) much of the molecular work has studied the mRNA, although at the DNA level absence of an RFLP allele for oestrogen receptor was found in breast cancer cells which fail to express the oestrogen receptor (Hill et al., 1989).

The oestrogen receptor gene generates mRNA species variously described as 3.7kb to 6.3kb (Green et al., 1986, Rio et al., 1987, Barrett-Lee et al., 1987, Parl et al., 1987, Henry et al., 1988) with all oestrogen receptor positive tumours having detectable oestrogen receptor mRNA (Henry et al., 1988) and 15 of 21 tumours with no detectable oestrogen receptor protein expressing mRNA for the oestrogen receptor (Henry et al., 1988). Therefore, many tumours may not be able to translate the mRNA to receptor protein (Barrett-Lee et al., 1987).

Hormone receptor complexes are presumed to interact with specific DNA sequences (Brown et al., 1984). Supporting evidence for this comes from the identification of functionless defective oestrogen receptor hormone complexes which are unable to bind to nuclear material (Raam et al., 1988) and thus cannot activate transcription. Although the oestrogen receptor protein is of considerable clinical importance, neither oestrogen dependence of the tumour nor clinical response to hormonal therapy is guaranteed by the presence of oestrogen receptors (Raam et al., 1988). Interest has therefore grown in the pS2 gene, the mRNA for which is oestrogen regulated (Masiakowski et al., 1982).

pS2

Expression of the pS2 gene in mRNA increases in response to oestrogenic stimulus (Masiakowski et al., 1982, Brown

et al., 1984) but is not influenced by progestins, glucocorticoids or androgens (Brown et al., 1984). pS2 transcription coincides with the appearance of highly associated oestrogen-receptor complexes in the nucleus (Brown et al., 1984).

The 600 bp mRNA (Masiakowski et al., 1982) encodes for an 84 amino acid protein which is cystene rich (Rio et al., 1987) and has structural similarities to IGF-I (Rio et al., 1987) but does not stimulate DNA synthesis (Kida et al., 1989). Thus, although the significance of oestrogen receptor protein and the oestrogen inducible pS2 requires to be more closely defined, subclassification of breast tumours on the basis of molecular studies of oestrogen receptor and pS2 may define patients with tumours more likely to respond to endocrine therapy (Raam et al., 1988, Henry et al., 1988) and those likely to have a short time to disease relapse (May et al., 1989).

Multiple drug resistance gene

Among the other genes considered for study was the multiple drug resistance gene, MDR1, responsible for multiple drug resistance, which maps to 7q 21.1 (Fairchild et al., 1987). This gene encodes a 4.8 kb mRNA and 170 kd membrane glycoprotein that exports drugs, subverting their intracellular cytotoxic activity. The degree of MCF-7 resistance to adriamycin correlated with overexpression of MDR1 mRNA (Fairchild et al., 1987) and

MDR1 overexpression may precede MDR1 gene amplification (Shen et al., 1986). While the study of MDR1 in breast tumours has been limited, 15% of breast tumours show low levels of MDR1 and 2/2 post chemotherapy resistant breast tumours expressed MDR1 (Goldstein et al., 1989).

It is evident that in order to seek evidence for the multistep theory of carcinogenesis and the involvement of tumour suppressor genes and oncogenes in human breast cancer, one must select key genes to study. On the basis of the work published up to the commencement of the work encompassed in this thesis (October 1987) it was elected to pursue the lines of enquiry outlined below.

AIMS

The first aim of the work reported here was to quantitate accurately the expression of the oncogenes c-myc, erbB2, H-ras, the tumour suppressor gene/oncogene p53, the growth factor gene transforming growth factor ß and the oestrogen regulated gene pS2 in human breast tumour tissue. These genes were selected on the basis of the published evidence outlined above. To quantitate expression it was elected to study gene transcription (mRNA), the most sensitive measure of gene activity (Johnson and McKnight, 1989).

The expression of the genes under study would then be related to clinical and pathological factors of significance in patient management, and one gene would be chosen for further molecular studies at the genetic (DNA) level.

While it is desirable to examine tumours from patients, there remained a need to examine the dynamic changes in gene expression occurring within a breast tumour in response to oestrogen stimulation or a therapeutic agent. Such studies are not generally possible in human subjects, therefore the second aim of this thesis was to develop a xenograft model in which to observe the changes in gene expression (mRNA) occuring in vivo, particularly in response to the commonly used anti-tumour agent, tamoxifen.

At the conclusion of this molecular approach to breast cancer, the intention was to integrate the findings from human material and the xenograft system to contribute towards greater understanding of the pathogenesis of breast cancer, clarify the mechanisms by which the effects of anticancer therapy are mediated and look to future prospects.

MATERIALS AND METHODS

Materials

Methods

Cell lines

- -culture
- -contamination

Xenografts

- -mice
- -oestrogen supplementation
- -serum oestrogen
- -tumour growth
- -tumour transplantation
- -tumour treatment
- -pathology
- -DNA synthesis

Human material

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- -tumour collection
- -patient groups
- -patient data
- -patient followup
- -normal tissue
- -pathology
- -oestrogen receptor
- -DNA ploidy and antibody studies

Nucleic acid studies

- -RNA extraction
- -selection of polyA+ RNA
- -RNA electrophoresis
- -Northern blotting
- -hybridisation and washing conditions
- -DNA extraction from blood and tumours
- -DNA digestion
- -DNA electrophoresis
- -DNA blotting
- -hybridisation and washing conditions
- -probe growing
- -probes

DNA sequencing

- -polymerase chain reaction
- -DNA purification
- -DNA sequencing

Spectrophotometry

Photography

Autoradiography

Densitometry

Statistical analysis

Materials

(By supplier)

Chemicals

Abbott Laboratories

Enzyme immunosorbent assay (EIA) for oestrogen receptor protein

Amersham

^{32}P alpha CTP

^{32}P gamma ATP

^{35}S dATP

^{3}H Thymidine

Hybond N Nylon membrane

Baxter Health Care

Radioimmunoassay for oestradiol

BDH Analar

Acetic acid

Ammonium acetate

40% Bis/acrylamide

Butan-1-ol

Calcium chloride

Chloroform

Ethanol

Ethylene diamine tetra acetate (EDTA)

Formaldehyde

Glucose

Glycerol

Hydrochloric acid

Isoamyl alcohol

Isopropyl alcohol

Liquid paraffin

Methanol

Potassium acetate

Proteinase K

Sodium acetate

Sodium chloride

Sodium citrate

Sodium dodecyl sulphate (SDS)

Sodium hydroxide

Disodium hydrogen phosphate

Sucrose

Trichloro acetic acid

Tris hydrochloride

Triton X

Bio-Rad

Mixed bed resin AG 501-X8

Boehringer Mannheim

Enzymes: BamHI (buffer B)

EcoRI (buffer H)

HindIII (buffer B)

MspI (buffer L)

TaqI (buffer B)

Enzyme buffer B : 10mM Tris HCl, 5 mM $MgCl_2$, 100mM NaCl, 1mM 2-mercaptoethanol, pH 8.0.

Enzyme buffer H : 50mM Tris HCl, 10 mM $MgCl_2$, 100mM NaCl,

1mM 1-dithiothreitol, pH 7.5.

Enzyme buffer L : 10mM Tris HCl, 10 mM $MgCl_2$,

1mM 1-dithiothreitol, pH 7.5.

Proteinase K

Randomprime DNA labelling kit

BOC

Liquid nitrogen

Boots

Arachis oil

Bethesda Research Laboratories

High purity agar

Low melting point agar

Cetus Corporation

Amplitaq kit

Difco

Trypsin

Fluka

Formamide

Gibco

Dulbecco modified eagle medium (DMEM).

Foetal calf serum (FCS).

Imperial Chemical Industries

Tamoxifen (Nolvadex, ICI 46,474)

Innovative Research

1.25mg slow release pellet of 17ß oestradiol benzoate

Intervet

17ß oestradiol benzoate in arachis oil

Kodak

Kodak professional film 4 inch x 5 inch.

XAR film

May & Baker

Ether

National Diagnostics

Sequagel sequencing system

Oswell

Oligonucleotides : 5'TTCCTCTTCCTGCAGTACTC (5' primer)

5'AGTTGCAAACCAGACCTCAG (3' primer)

Oxoid

Phosphate buffered saline

Pharmacia

Dextran sulphate

Ficoll

5% - 30% Ficoll 400 gradient

Opti-Scint

Pfizer

Cytosine arabinoside

Rathburn Chemicals

Glass distilled grade phenol

Sigma

Ammonium persulphate

Ampicillin

Bovine serum albumen (Fraction V BSA)

BR9

5-Bromodeoxyuridine

Bromophenol blue

Chloramphenicol

Diethyl pyrocarbonate (DEPC)

Dimehylsulphoxide (DMSO)

Dispase (neutral protease)

Dithiothreitol (DTT)

Ethidium bromide

Fluroscein isothiocyanate-labelled goat anti mouse conjugate.

Lithium chloride

Lysozyme

Morpholinopropanesulphonic acid

Mouse IgG

Oligo(dT) cellulose

Orange G

Phycoerythrin

Polyvinylpyrrolidene

RNAase-A

Salmon sperm DNA

Sodium heparin

Sodium pyrophosphate

Spermine tetrahydrochloride

Tetramethylethylenediamine (TEMED)

Tetracycline

Urea

Xylene cyanol

Stratech Scientific

Geneclean kit

US Biomedical Corporation

Sequenase DNA sequencing kit

Solutions

Denaturing solution : 5g NaOH (0.5M), 219.15g NaCl (1.5M) in 2.5 litres.

Denhart's solution : 5g Ficoll, 5g polyvinyl pyrrolidine, 5mg bovine serum albumin.

Lysis buffer : 0.1M Tris HCl, 20mM NaCl, 1mM EDTA.

Neutralising solution : 292.9g NaCl (2M), 394g Tris base (1M) in 2.5 litres. Add HCl to pH 5.5.

Phosphate buffer : 23g KH_2PO_4, 125g K_2HPO_4 in 1 litre.

Resuspension buffer : 10mM Tris HCl, 150mM NaCl, 10mM EDTA.

Running buffer (DNA) : 30% sucrose, 0.4% bromophenol blue.

20x SSC : 876.6g NaCl, 441.2g Na_3citrate in 5 litres.

20x TAE gel buffer : 484g Tris base, 114.2ml acetic acid, 200ml 0.5M EDTA in 5 litres.

10x TBE : 1M tris borate 20mM EDTA pH 8.3

TE Buffer : 10mM Tris HCl pH7.5, 0.5mM EDTA.

T-broth : 6g tryptone, 5g yeast extract, 5g NaCl, 1ml 1N NaOH in 1 litre. Add 50ml phosphate buffer before use

Wash buffer 0.1x SSC : 50ml 10% sodium pyrophosphate, 25ml 20% SDS, 25ml 20x SSC in 5 litres.

Wash buffer 0.5x SSC : 50ml 10% sodium pyrophosphate, 25ml 20% SDS, 125ml 20x SSC in 5 litres.

Hardware

Alpha Lab

Plastic disposable 'S' transfer pipette

Becton Dickinson

50ml Falcon tubes

96 well Falcon 3072 microtitre plate

FACScan flow cytometer

Bio-Rad

Gel drier model 583.

Sequi-Gen Nucleic Acid Sequencing Cell

Braun

Mikrodismembrator II

Bethesda Research Laboratories

20cm x 30cm model H-1 gel tank

Calhene

Nude mouse incubators

Camlab

KWB Swiss graduated calipers

Costar

Spinex tubes

Crouzet

Pump type 82344

E-C Apparatus Corporation

EC 105 power source

Flow Laboratories

IR 1500 Automatic CO_2 incubator.

Fuji

RGII X-ray film processor

Gallenkamp

Orbital shaker

Gilson

Eppendorf tips

Pipettes

Grant

Grant SU6 waterbath

Grant SS40-2 shaking waterbath

Jencons

Vortex mixer

Luckham

Shaker R100

Mettler

PM4600 Balance

MSE Scientific Instruments

Microprobe part No. 38121-114

Microcentaur 13000 eppendorf microcentrifuge

Soniprep 150 ultrasonic disintegrator

Nuclear Enterprises

Farmer dosemeter 2570 probe

Nunc

75cm^2 Nunclon tissue culture flasks

Packard

Packard 1600CA analyser

Pharmacia

Nick column sephadex G-50.

Polaroid

Land camera

Sarstedt

13ml polypropylene tubes

Scot

Scotfresh regular handtowels

Scotlab

Eppendorf tubes

1.5ml freezing tubes

Siemens

X ray source - Stabilpan II TH250 therapy tube housing.

Sigma

3MK Centrifuge with Nr. 12056 rotor

Sorval

Centrifuge 3MK

Sterilin

Sterile universal containers

Techne

PHC-1 Dri-block

Hot block

Ultraviolet Products

UV Transilluminator

Unicam

SP1750 UV Spectrophotometer

Union Carbide

LR 40 liquid nitrogen refrigeration tank

Whatman

3mm filter paper

17mm filter paper

Wifug

500E Benchtop centrifuge

Methods

Cell Culture

Human breast tumour cell lines MCF-7 (Soule et al., 1973) T-47D (Keydar et al., 1979), MDA-MB-231 (Cailleau et al., 1974) were obtained from Dr. W.R. Miller, Department of Surgery, Royal Infirmary, Edinburgh.

All lines were cultured in 75 cm^2 Nunclon flasks, fed regularly with Dulbecco Modified Eagle Medium (DMEM) (Dulbecco et al.,1959) supplemented with 12% foetal calf serum (FCS) and maintained in an incubator in an atmosphere containing 5% CO_2 at 37^0C. Medium was changed twice each week or more frequently if the phenol red indicator in the DMEM became yellow. The cells were harvested in the logarithmic (80% confluent) phase of growth (Kelekar & Cole, 1987), by pouring off the DMEM and washing the cells gently with warm phosphate buffered saline (PBS) to remove the residual medium. The cells were bathed in 10ml of a cold 50:50 mixture of 0.04% EDTA and 0.2% trypsin for 5 minutes to remove the cells from the flask wall. The cell suspensions were transferred to 50ml Falcon tubes and pelleted in a swingout rotor in a Wifug 500E benchtop centrifuge centrifuge at 2500 rpm (1000g) for 5 minutes, resuspended in PBS and pelleted in the same way. The resultant pellet was either taken up in 1ml freezing mix (95%FCS with 5% Dimethyl sulphoxide, DMSO) in a 1.5ml polypropylene tube and stored frozen in liquid nitrogen tanks or used to reseed further flasks,

used for nucleic acid extraction or injected into mice.

Culture Contamination

Any culture flask where yeast or bacterial contamination was suspected or evident was destroyed.

All cultures were tested for mycoplasma using conventional immunofluoresence techniques which frequently provides the first evidence of contamination. The supernatant was tested according to standard methods (Barile, 1973) each month by Mr. W. Christie, MRC Human Genetics Unit.

Xenografts

Mice

A long established breeding colony of CBA/Ca strain mice (Festing, 1979) held at the Institute of Animal Technology, Western General Hospital, Edinburgh were used to establish a human breast tumour model in thymectomised, irradiated mice (Steel et al., 1978) under Home Office Licence CMS313. Maintained as described by Hay and collegues (1985), they undergo normal growth and development. Immediately after weaning at age 21 days, each mouse was anaesthetised with ether in a glass dessicator and fixed in the supine position using elasticated clips. Following skin preparation with 70% ethanol, a standard procedure of thoracotomy and removal by suction of the bilobed thymus, with one stage closure of the skin using michel clips was used. The mice recovered in a warmed cage. Three weeks following

thymectomy, the mice were irradiated to a total body dose of 7.50 Gy recorded using a dosememter probe placed in the centre of a perspex jig within which the animals were contained but could move freely. Radiation was delivered from a stabilpan II TH250 X ray source (250v : 0.3-0.4 Gy/min) with a Thoreus II filter over approximately 20 minutes. The mice were protected from this otherwise lethal dose of irradiation by 200mg kg^{-1} cytosine arabinoside injected by the intraperitoneal route 48 hours prior to irradiation.

Twenty homozygous nu/nu mice (Fogh & Hajdu, 1978) of mixed background from the Imperial Cancer Research Fund, London, which are congenitally athymic, were also used in these studies. The nu/nu mice were maintained in sterile incubators by skilled technical staff who performed all injections and tumour cell or tumour fragment implants in these nu/nu mice.

Oestrogen Supplementation

Oestrogen supplementation was administered in the form of 17ß oestradiol benzoate dissolved in and diluted with arachis oil and administered by subcutaneous injection into the nape of the neck at dose regimes 1ug/day, 1ug/2days, 2ug/day, 5ug/day, 5ug/week, 10ug/week, 30ug/2 weeks, 50ug/3 weeks.

The regime used for serum oestradiol measurement and tumour studies for the CBA strain mice was 50ug 17ß oestradiol in 50ug arachis oil administered every 3

weeks. This regime was chosen since it did not kill the mice but did allow tumour growth.

Twenty homozygous nu/nu mice were also used for tumour studies with oestrogen supplement of 1.25mg slow release subcutaneously placed pellet replaced every 6 weeks.

Serum Oestradiol

Oestradiol was measured in 50ul of serum from individual samples of 0.3 to 0.8 ml blood extracted by cardiac puncture from each of 30 mice at specified times following injection of 50ug 17ß oestradiol benzoate in 50ul arachis oil. The radioimmunoassay used oestradiol-6-carboxymethyloxime^{125}-1-iodohistamine as radiolabel and rabbit antiserum against oestradiol-6-carboxymethyloxime-bovine serum albumin. Separation of free from bound label was achieved by means of a solid phase second antibody. Organic solvent extraction of samples prior to assay was not necessary; oestradiol is displaced from its serum binding protein by an excess of blocking agent. Within assay CV was 5% and between assay CV was 6%. The serum oestradiol measurements were performed by S. Barnes and V. Sweeting, Department of Clinical Chemistry, Royal Infirmary, Edinburgh.

Tumour Growth

In vivo growth of breast cancer cell lines was studied in groups of 20 CBA mice which had been thymectomised and irradiated in the same batch. A known number of MCF-7 cells (10^5 up to 10^8 cells checked for viability by dye

exclusion) were suspended in 50 - 100 ul PBS and were injected into the right 4th mammary fat pad of healthy mice one to 3 weeks after irradiation. Some mice received oestrogen supplementation which commenced at the same time, by subcutaneous injection into the nape of the neck. This injection was repeated every three weeks to the same site.

Mice were checked daily and the animals examined for signs of disease or debility, including the cell line injection site and the oestrogen injection site. Tumours were measured in 2 dimensions (maximum and minimum diameters) by the author twice each week using 0.1mm graduated calipers. The volume of the tumour was calculated using the formula pi/12 x (mean diameter)^3.

Tumour bearing mice were killed at specific times following injection of oestrogen; 6, 12, 18, 24, 36, 48, or 72 hours or 3, 7, 10, 14, or 21 days after oestrogen injection. The excised tumours were frozen immediately in liquid nitrogen or fragments fixed for pathologial examination or analysed for oestrogen recptor protein content Those mice which did not develop tumours were killed 90 days from the start of the experiment. Mice which died in the interim or were generally debilitated and therefore killed, were examined by post mortem and histopathological examination of organs in detail for evidence of disease including metastasis.

Tumour Transplantation and Re-culture.

A number of tumours arising from inoculum of MCF-7 cells were transplanted into further thymectomised irradiated mice. Tumour from a freshly killed mouse was cut into fragments of less than $3mm^3$. A mouse was anaesthetised with ether and one tumour fragment placed in each flank by tunnelling subcutaneously with the tumour bearing forceps through a single small incision in the mid dorsum. The wound was closed with a single steel clip and the oestrogen supplement injection administered as the mouse awoke. Thereafter the transplant bearing mice were treated as above, with oestrogen supplements every 3 weeks. Remaining tumour fragments were macerated and added to DMEM containing 12% FCS and tumour cells returned to culture in Nunclon flasks at 37^0C in an atmosphere of 5% CO_2, for 8 weeks before re-injection into mice or extraction of nucleic acid.

Tumour Therapeutic Manipulation

Two effects of tumour manipulation were examined in batches of 20 mice which had undergone thymectomy on the same day, received the same irradiation and been injected with the same preparation of cells on the same day and received the same oestrogen supplement.

1. Oestrogen withdrawal. Tumours were established to 100 mm^3; oestrogen supplementation was not replenished and the effects of oestrogen withdrawal on the tumours

observed by serial tumour measurement.

2. Antioestrogen treatment. The antioestrogen tamoxifen (Nolvadex, ICI 46,474) was dissolved in arachis oil and 1.25mg in 50ul arachis oil was injected daily, subcutaneously, into the hindquarters commencing on the same day as oestrogen supplementation to observe the effects in vivo of tamoxifen on tumours receiving the standard oestrogen supplementation. Tumours were measured as before, mice killed and tumours excised for study at 2, 4, 7, 14, and 21 days after commencing anti-oestrogen therapy. The dose of tamoxifen was estimated on mg tamoxifen per kg body weight from the human dose and tested for toxicity at 125mg/day on mice which had or had not received exogenous oestrogen.

Histopathology

Tumour material and tissue removed at post mortem was fixed in methacarn (60% methanol, 10% glacial acetic acid, 30% chloroform) for 1 hour then 95% ethanol and processed routinely to paraffin blocks by the Department of Pathology, University of Edinburgh and the Department of Pathology, Western General Hospital, Edinburgh. 10um sections were stained with haematoxylin and eosin and were examined for pathological features including tumour type, necrosis, lymphocyte infiltration, vascular invasion or metastasis. The mitotic index for each tumour was calculated from the mean number of mitoses in 50 randomly chosen high power fields (x400) by a single

pathologist (Dr David Patterson) examining the sections without knowledge of which mouse they had been excised from.

Cell DNA Synthesis

A monoclonal antibody BR9 directed against the halogenated nucleotide 5-Bromodeoxyuridine (BrdU), raised by Dr. D. Deane, MRC Human Genetics Unit, Western General Hospital, Edinburgh, was used for rapid S-phase measurements (Gonchoroff et al., 1985).

The BrdU (200ul of 6mg ml^{-1}) was injected into the peritoneal cavity of each mouse under examination at 0, 6, 12, 18, 24, 36, or 48 hours after injection of 50ug 17ß oestradiol.

Each mouse was killed 1 hour after administration of the BrdU, the tumours excised and finely minced in DMEM containing 1mg ml^{-1} dispase (neutral protease). Cells released by this digestion were fixed in 70% ethanol. For the simultaneous differentiation of human MCF-7 cells from invading host (mouse) tissues, a double staining procedure was used. Fixed cells were washed with PBS, then incubated with 10ug of an antihuman class I monoclonal antibody PE25 (Made by Dr. D. Deane) washed in PBS and 2ug of phycoerythin labelled anti-mouse conjugate added. To prevent cross-reactivity between this antibody and the BR9, the cells were then incubated in PBS containing 10ug ml^{1-} mouse IgG for 30 minutes. After a wash in PBS, 10ug of the monoclonal antibody BR9 was

added to the cell pellet of approximately 10^6 cells and incubated at room temperature for 40 minutes. The cells were washed in PBS and incubated for a further 40 minutes in the presence of 2ug Fluoroscein isothiocyanate (FITC)-labelled goat anti-mouse conjugate. After a further wash in PBS the cells were analysed on a FACScan flow cytometer for absolute number and proportion of tumour cells in S-phase.

Thymidine uptake was also used to determine DNA synthesis by isolated tumour cells. For each time point, intact cells were separated from the dispase/DMEM digests by density centrifugation over 5%-30% Ficoll 400 gradient and 150ul of cell suspension (10^6 cells per ml DMEM with 5% added FCS) added per well of a 96 well microtitre plate. 1uCi 3H thymidine was added to each well and the cells harvested 5 hours later onto glass fibre discs. The radioactivity emitted from each disc was counted in 5ml of scintillant in a Packard analyser.

Human Material

Patient Selection

Material for this study was obtained from female patients who attended the breast clinic, Department of Surgery, Royal Infirmary, Edinburgh. Criteria for patient selection were patients with untreated tumours (or treated with tamoxifen only) with a primary breast carcinoma proven by fine needle aspiration cytological examination, from whom sufficient quantities of very

fresh tumour material could be obtained. Small tumours (less than 1cm on mammography) and those lesions requiring needle localisation biopsy (where it is critical to determine all the calcified suspicious lesion has been excised) and patients who had previously received radiotherapy or chemotherapy for a breast lesion were excluded from the study. Consent was obtained from all patients approached and 20ml venous blood withdrawn prior to surgery.

Tumour Collection

Tumour material was obtained intra-operatively when the patient underwent tumour biopsy, local excision of the tumour or mastectomy by standard surgical procedures at Longmore Hospital, Edinburgh between August 1987 and July 1989.

The tumour size and method of resection for each tumour dictated the quantity of tissue removed and length of time for each tumour to be cut into 5 mm to 10mm blocks by the pathologist, placed in eppendorf tubes and frozen in liquid nitrogen. The time from excision to freezing ranged from 2 minutes to 10 minutes and was noted. Tumour material removed for the purposes of this work was taken adjacent to tissue processed for pathological examination and material sent to the Lister Surgical Laboratories, Department of Surgery, Royal Infirmary, Edinburgh for oestrogen receptor protein and other measurements.

Tumour tissue was transferred for long term storage to

two independent -70°C freezers and material retrieved as required.

Tumour material was obtained from three groups of patients:

1. Primary untreated tumours.
2. Primary tumours from whom tissue was available before treatment was commenced and following endocrine or chemotherapy.
3. Patients who had received a course of tamoxifen only but required subsequent surgery due to inadequate control of the tumour.

Clinical Correlation

Patient Data

Detailed patient data (full personal, family and clinical history and examination prior to therapeutic intervention) was obtained for every patient in this study. This included clinical staging of the disease according to the TNM tumour classification system (Hermanek and Sobin, 1987), tumour size on pathological examination of the excised breast or on radiological evidence where tumour biopsy rather than excision was performed, nodal involvement with tumour (pathological evidence) and biochemical parameters (such as oestrogen receptor protein).

Patient Follow-up

All patients studied were closely monitored in the

routine follow up clinic at Longmore Hospital, Edinburgh every 3 months post operatively, at which time a full clinical examination was performed, supplemented by annual mammography, chest and pelvic radiographs. The site and timing of tumour recurrence or metastasis (and consequently disease free interval, subsequent treatment and cause of death) was noted. This follow up will continue on an annual basis following the second anniversary of the patient's surgery.

Normal Tissue

Normal breast tissue was obtained from ten patients who underwent cosmetic reduction mammoplasty under the care of Mr. A.C.H. Watson, Consultant Plastic Surgeon, Bangour General Hospital, Broxburn, West Lothian. These patients had no personal or family history of breast carcinoma. A limited clinical history including age and menopausal status was obtained from each patient.

Normal uterine and ovarian tissue was obtained fresh from a single premenopausal women who underwent hysterectomy and oophorectomy for menorrhagia under the care of Dr. G. Smart, Consultant Gynaecologist, Royal Infirmary, Edinburgh.

Tonsils free from acute disease were obtained from 5 female patients who underwent tonsillectomy at the Royal Hospital for Sick Children, Edinburgh.

Breast tissue adjacent to cancer tissue was not examined since it has been established that breast tissue which

appears to be grossly normal may even so contain substantial amounts of tumour (Whittaker et al., 1986).

Pathology

Pathological examination of each tumour, surrounding breast tissue and axillary nodes was carried out by several different pathologists at the time of surgery who noted the size of the tumour (measured in 3 dimensions). The histological features of each tumour were noted by a single pathologist (Dr D. Patterson, Department of Pathology, University of Edinburgh, Edinburgh) who reviewed all the tumour material at a later date. The features noted included the tumour type, the presence of in situ disease, invasion into vessels, histological grade (Bloom & Richardson, 1957), degree of lymphocyte infiltration, extent of tumour necrosis and spread of tumour within the breast and/or to the lymph nodes.

Oestrogen Receptors

The cytoplasmic oestrogen receptor protein content was measured by Dr R A Hawkins and his collegues, Lister Surgical Laboratories, Royal Infirmary, Edinburgh, using the Dextran coated Charcoal method (DCC method; Hawkins et al., 1975) or for patients who had received hormonal therapy (and in whom the DCC method would therefore not accurately assess oestrogen receptor protein) and for mouse tumour material and cell line pellets, the Enzyme Immunosorbent Assay (EIA; Hawkins et al., 1987) and expressed in fmol mg protein^{-1}.

Tumour material was considered to be oestrogen receptor moderate or rich at 20 fmol mg protein^{-1} or greater, but oestrogen receptor poor or negative at less than 20 fmol mg protein^{-1}, these being the cut-off levels used in clinical practice (Anderson et al., 1989).

Nucleic Acid Studies

Ribonucleic Acid Extraction

Throughout the RNA extraction procedures strenuous efforts were made to avoid ribonuclease contamination and consequent RNA degradation. The recommendations of Maniatis and collegues (1982) were followed. Ribonuclease activity during the initial stages of extraction was minimised and subsequent introduction of trace amounts of ribonuclease were avoided. A designated laboratory area was devoted to RNA work. Gloves were worn at all times to avoid skin contamination of sterile disposable plastic ware which is free of ribonuclease (Maniatis et al., 1982) or laboratory glassware which was autoclaved, washed in 0.1% diethylpyrocarbonate (DEPC), a strong inhibitor of ribonuclease, then in distilled water and baked in an oven for one hour at 80^{o}C to remove traces of DEPC (Maniatis et al., 1982). All solutions were made up with high grade chemicals reserved for RNA work and autoclaved DEPC-treated distilled water, using baked sterilised glassware and bottles.

No exogenous inhibitors of ribonuclease (vanadyl-ribonucleoside complexes or RNasin; Maniatis et al., 1982) were used.

Total ribonucleic acid (RNA) was extracted from frozen tumour using a modification of the method described by Auffrey & Rougeon (1980) which disrupts cells, inactivates ribonuclease and extracts RNA by differential

precipitation. A frozen tumour fragment was weighed on a balance, cut into thin slices, placed in liquid nitrogen in the teflon capsule of a mikrodismembrator and pulverised within 10 seconds. The resulting tumour powder was finely disrupted in 13ml polypropylene tubes using a disposable plastic pipette in the presence of 2ml 3M Lithium chloride / 6M urea for every 100mg tumour and left at 4^0C overnight. Cells cultured <u>in vitro</u> were washed in PBS and 10^8 cells disrupted in 8ml 3M Lithium chloride / 6M Urea with a disposable plastic pipette and left to precipitate nucleic acid at 4^oC overnight. The DNA was sheared using a microprobe in an ultrasonic disintegrator with an ice jacket around the sample tube. The RNA was recovered by centrifugation at 12000 rpm (5000g) in a Sorval 3MK centrifuge, the pellet collected and resuspended in 8ml 3M lithium chloride/6M urea and spun again at 12,000 rpm.

The pellet was taken up in 6ml 10 mMol Tris buffer pH 7.0 /0.5% sodium dodecyl sulphate (SDS), and incubated with 300ug proteinase K at 37^0C for 20 minutes.

Proteins were extracted from the aqueous phase by vigorous mixing with an equal volume of glass distilled grade phenol equilibrated with 0.1M Tris pH7.0 and separated by centrifugation at 3000rpm (1000g) for 5 minutes in the swing out rotor of a Wifug 500E centrifuge. Two further extractions with 24:1 chloroform : isoamyl alcohol were performed using the same

centrifugation. The aqueous phase was then divided into two 13ml polypropylene tubes, 2 volumes of 100% ethanol added to each and the RNA precipitated at -20°C for 24 hours. The RNA was recovered by centrifugation at 12,000 rpm (5000g) for 30 minutes, air dried for 10 minutes and taken up in 20 to 500 ul of DEPC treated sterile water and, following spectrophotometry, stored in aliquots at -70°C in sterile eppendorf tubes.

Poly A+ RNA Purification

Poly A+ RNA was purified from total MCF-7 RNA by a protocol derived from Maniatis and colleagues (1982) and Aviv & Leder (1972). A 1cm column of oligo(dT) cellulose equilibrated in sterile loading buffer (1x loading buffer comprises 20mM Tris hydrochloride pH7.6, 0.5M sodium chloride, 1mM EDTA, 0.1% SDS) was poured in a pasteur pipette.

The column was washed in 3 column volumes each of sterile DEPC-treated water then 0.1M sodium hydroxide then sterile DEPC-treated water such that the pH of the column effluent was less than pH 8 and further flushed through with 5 volumes of sterile loading buffer.

1mg of total RNA extracted from MCF-7 cells in 400ul DEPC-treated water was heated to 65°C for 5 minutes and an equal volume of 2xloading buffer, the sample cooled to room temperature and applied to the olig(dT) cellulose column. The eluent was collected, heated to 65°C for 5

minutes, cooled to room temperature and reapplied to the column. The column was washed with 5 volumes of loading buffer then washed with 4 column volumes of low salt buffer (20mM Tris pH7.6, 0.1M sodium chloride, 1mM EDTA, 0.1% SDS).

The poly A+ RNA was eluted from the column with 3 volumes of sterile elution buffer (10mM Tris hydrochloride pH7.5, 1mM EDTA and 0.05% SDS).

The poly A+ RNA was precipitated by adding 3M sodium acetate pH 5.2 to the elute to a final concentration of 0.3M and 2.5 volumes of 100% ethanol and cooling to $-70^{0}C$ for 15 minutes. The precipitate was collected by 15 minutes spinning in a eppendorf centrifuge, rinsed in 70% ethanol and the pellet dissolved in 20ul sterile DEPC-treated water.

Electrophoresis of RNA.

The method of electrophoresis and Northern blot transfer of RNA was modified from Southern, 1975, Maniatis et al., 1982 and Fourney et al., 1988.

Electrophoresis was used to separate RNA species by molecular weight since the migration of fully denatured RNA is in linear proportion to the Log_{10} molecular weight (Lehrach et al., 1977).

20ug of total RNA (up to 10ul volume with DEPC-treated water) was denatured with 10ul formamide deionised by mixing with mixed bed resin pH7 for 30 minutes, filtered

and stored in aliquots in tightly capped tubes at -20^{0}C) and 2.5ul 37% (12.2M) formaldehyde (pH>4.0) in the presence of 2.5ul 10x MOPS buffer (0.2M morpholinopropanesulphonic acid (MOPS) pH 7.0, 50mM sodium acetate pH7.0, 5mM EDTA) at 55^{0}C for 20 minutes in a waterbath, 2ul loading buffer (50% glycerol, 1mM EDTA 0.4% bromophenol blue, 0.4% xylene cyanol) and 1ul 1ug/ul ethidium bromide were added to each sample (total volume 28ul). The denatured specimens were loaded onto a 0.66M formaldehyde , 1.1% high purity agar gel which allowed accurate RNA molecular weight determinations in a 20cm x 30cm gel tank, submerged beneath 1x MOPS buffer and the RNA species separated by electrophoresis at 60v, 0.4amps from an EC 105 power source until the dye fronts Were 4cm (Xylene) and 10cm (Bromophenol blue) from the loading pit. The MOPS buffer was circulated through ice using a 10rpm Crouzet pump.

At least six standard RNA specimens were run on each gel to allow comparison by densitometry at a later date.

Northern Blotting

The gel was washed in 2 changes of 10x standard saline citrate with gentle shaking on a Luckham skaker and photographed.

To blot the RNA, a blotting tank made from a seed tray, sheet of plate glass, 17mm Whatman chromatographic paper dipped in the 10x SSC to act as a wick. The gel was inverted such that the RNA lay uppermost in the gel and

the a Hybond-N nylon filter placed on top with care taken to exclude bubbles. The assembly was completed with 2 sheets of 3mm Whatman chromatographic paper and 30 paper towels on top as the salt wick. Blotting proceded for 8-12 hours, the Hybond was rinsed in 2x SSC, air dried and the RNA covalently fixed to the Hybond membrane using 3 minutes and 45 seconds exposure to ultra-violet light from a UV transilluminator. The Hybond and remaining gel were photographed to check for adequate transfer of ethidium stained material (Denis et al., 1988) and to mark the position of the ribosomal RNA bands.

Hybond filters were labelled and stored in sealed plastic bags at $4^{o}C$.

Hybridisation and washing conditions

Filters sealed in a heavy duty plastic bag were prehybridised in 7% SDS, 0.5M disodium hydrogen phosphate pH 7.2 and 1mMol EDTA pH7.0 (modified from Church & Gilbert, 1984) for 30 minutes at $65^{0}C$ in a shaking water bath. To this was added ^{32}P cytidine triphosphate (CTP) labelled cDNA probe labelled to 1 x 10^{7}cpm ml^{-1} using the Randomprime DNA labelling kit (Feinberg & Vogelstein, 1983).^{32}P CTP incorporated probe was separated from unincorporated radionucleotide by passage down a sephadex G-50 column to give incorporated probe in 400ul 10mM Tris hydrochloride pH7.5 containing 1mM EDTA and denatured by heating to $98^{o}C$ for 2 minutes and then put on ice before addition to the hybridisation solution.

Hybridisation proceded at 65^{o}C for 24 hours in a shaking water bath.

Following hybridisation, filters were stringently washed to remove nonspecifically attached probe in 2 changes of 500ml of 0.1% SDS 10mMol disodium hydrogen phosphate wash buffer at 65^{0}C with gentle agitation in a Gallenkamp Orbital Shaker for 5 minutes then, with the second change of buffer, for 30 minutes. Filters were blotted dry, wrapped in cling film and autoradiographed. The size of mRNA species was calculated from the position of ribosomal RNA markers. Filters were reprobed upto six times with different cDNA probes; before reprobing, filters were stripped of residual probe by washing at 80^{o}C for 60 minutes in 0.1% SDS in a Grant SS40-2 waterbath and the filter checked by autoradiography for 48 hours. Care was taken not to allow the filter to dry out.

<u>DNA Extraction from blood and tumour</u>

Each patient from whom tumour tissue was to be obtained consented to donating 20ml of venous blood prior to surgery or therapy other than tamoxifen. The blood was spun in a swing out rotor of a Wifug 500E centrifuge at 3000 rpm (1000g) for 5 minutes and the plasma aspirated. DMEM was added to the blood to total 20 ml, gently mixed, 19ml of lysis buffer and 1ml 20%SDS added, the blood mixed and placed at 4^{o}C for 30 minutes. The lysed blood was gently mixed with an equal volume of glass distilled

grade phenol and spun at 3000rpm for 10 minutes. The supernatant was saved and a half volume of ammonium acetate and two volumes of isopropylalcohol added, mixed and allowed to stand for 12 hours at $4^{o}C$. The DNA strands were spooled out using hooked glass rods made in house from glass pipettes and allowed to air dry for 20 minutes. The blood DNA was redissolved in 10ml resuspension buffer 10ug ml^{-1} RNAase was added to the resuspended DNA and incubated at $37^{o}C$ for 30 minutes. Tumour DNA was extracted from frozen tissue by placing finely chopped tumour tissue in lysis buffer containing 1% SDS and lysis allowed to occur at $4^{o}C$ over 12 hours. To this tumour DNA in lysis buffer, 10ug ml^{-1} RNAase was added and the mixture incubated at $37^{o}C$ for 30 minutes. To blood DNA or tumour DNA incubated at $37^{o}C$ with RNAase for 30 minutes, 50ug ml^{-1} freshly dissolved proteinase K was added and the mixture further incubated in a waterbath at $37^{o}C$ for 60 minutes. An equal volume of phenol was mixed with the aqueous DNA containing solution and left to stand for 20 minutes on ice. The phenol/resuspension buffer were separated by centrifugation at 3000 rpm (1000g) for 5 minutes, the supernatant was aspirated and mixed with 24:1 chloroform/isoamylalcohol and again centrifuged at 3000 rpm for 5 minutes. The supernatant was aspirated and a half volume of ammonium acetate and 2 volumes 100% ethanol gently mixed in and left at $-20^{o}C$ for 12 hours.

The DNA was then spun out on hooked glass rods, air dried for 30 minutes, and redissolved in 500ul TE buffer. The smaller fragments of DNA were not recovered. Following spectrophotometry, DNA was stored in screw top eppendorf tubes at 4°C or in aliquots at -70°C for long term storage.

DNA Digestion

5ug DNA was digested with 5 units per ug DNA of the restriction endonuclease appropriate for the probe to be used. Digestion was carried out in total volume 40ul or 60ul made up with distilled water in eppendorph tubes with the appropriate buffer. Spermidine was routinely added to all digestions and triton X added, in addition, to Msp 1 digests. Digestions were carried out at 37°C (except for Taq 1, 75°C) in a water bath over 4 hours and further enzyme (2 units per ug DNA) added and allowed to digest for a further 2 hours. 4ul orange G was added to the digested DNA and the DNA was immediately used for electrophoresis.

DNA Electrophoresis

A 0.8% agarose gel (2.4g agar in 300ml 1x TAE buffer) a 20 x 40cm gel tank was loaded with up to 22 samples (11 tumour/blood pairs) and digested lambda markers. 1x TAE buffer was used to submerge the gel and the gel run for a suitable time to ensure separation of the DNA species of interest (18 hours for 2 kb species), at 60v 0.2 amperes from a E-C power pack. Buffer was circulated through ice

using a Crouzet 10rpm pump to ensure the temperature and pH of the buffer remained satisfactory.

On completion of the electrophoresis, the gel was placed in a plastic tray, 1 litre of the TAE buffer used in the electrophoresis poured into the tray and the DNA in the gel stained with 20ul ethidium bromide 10ug ul^{-1} for 30 minutes. The gel was destained in distilled water for 10 minutes and photographed under ultraviolet light. The resulting photograph was studied to ensure that the gel had run correctly (gel tracks straight),to determine whether adequate samples had been loaded and whether or not digestion of the samples had occurred.

DNA Blotting

The DNA fragments were transferred to a hybond-N by an alkali blot modification of the Southern Blotting technique (Southern, 1975). A photographed gel was placed in denaturing solution for 10 minutes, placed on a single layer of denaturing solution soaked 17mm Whatman chromatography paper, itself on cling film on a benchtop. Hybond was placed on top of the gel followed by two layers of denaturing solution moistened 3mm Whatman chromatography paper and 30 dry paper towels, maintained in place with a 7mm thick perspex sheet.

Blotting proceded over 6 hours, after which the Hybond was removed and placed in neutralising solution for 30 minutes, air dried and the DNA bonded to the Hybond membrane by exposure to ultra-violet light from a UV

transilluminator for 4 minutes followed by baking at 80^0C under vacuum in an oven for 1 hour.

Hybridisation and washing conditions

The membrane was prehybridised in 30ml hybridisation buffer in a sealed polythene bag in a 65^oC shaking water bath for 45 minutes. To this, 10^7 cpm ml^{-1} ^{32}P CTP-labelled probe insert was added and allowed to hybridise for 24 hours. Probes were labelled by the randomprime labelling method (Feinberg & Vogelstein, 1983) using a kit and unincorporated probe removed by ethanol precipitation. To 20ul of labelling reaction, 7.5ul 3M sodium acetate, 25ul 10mg ml^{-1} sonicated salmon sperm (carrier) DNA and 250ul ethanol were added and left at -20^oC for 15 minutes. The precipitated random primed probe was collected by centrifugation at 4^oC, 13000rpm (2000g) for 15 minutes in an eppendorf centrifuge and redissolved in 200ul 10mg ml^{-1} sonicated salmon sperm DNA in TE, heated to 98^oC for 2 minutes and placed on ice for 5 minutes then added to the prehybridisation solution.

Following precipitation, the pMCT 35.1 and pBHp53 probes were taken up in 180ul 1.5mg ml^{-1} sonicated tonsil (total human) DNA, boiled for 10 minutes and the probe reannealed to total human DNA at 66^oC for 45 minutes to reduce nonspecific hybridisation to the digested genomic DNA (modified from Sealey et al., 1985).

Excess probe was washed from the membrane using 3 successive 30 minute washes of 0.1% SDS with 0.5, 0.2 or

0.1xSSC and 0.1% sodium pyrophosphate at 65°C and the DNA fragments of interest were detected by autoradiography.

DNA Sequencing

DNA from selected patients in whom there were allelic losses on the short arm of chromosome 17 and/or increased expression of p53 mRNA was examined in detail for DNA base changes. The published literature gives the sequence for p53 exons but only to a very limited extent for p53 introns (Mowat et al., 1985, Zakut-Houri et al., 1985, Lamb & Crawford, 1986, , Tan et al., 1986, Buchman et al., 1988, Finlay et al., 1988). Evidence in other malignancies such as colon cancer (Baker et al., 1989) indicates that mutations in exon 5 and exon 6 may cause functional change from tumour suppressor gene to oncogene. Therefore, oligonucleotide primers were synthesised by the Oswell DNA Service, Department of Chemistry, University of Edinburgh, West Mains Road, Edinburgh to the 20 nucleotide 5'end of exon 5 (primer 1) and the 20 nucleotide end of the 3' end of exon 6 (primer 2). The DNA amplification and sequencing techniques were adapted from published techniques by Dr J Prosser, MRC Human Genetics Unit, Western General Hospital, Edinburgh. From 1ug DNA extracted from a tumour or from the patient's blood, exons 5 and 6 were selectively amplified using the polymerase chain reaction (Saiki et al., 1985a, Saiki et al., 1985b, Erlich et al., 1988), and DNA

purified from this reaction sequenced using dideoxy labelling (McGraw et al., 1988).

Polymerase Chain Reaction

1ug DNA from each patient's blood or tumour was incubated with primer 1 (5'TTCCTCTTCCTGCAGTACTC), primer 2 (5'AGTTGCAAACCAGACCTCAG) buffer (1.5mM $MgCl_2$, 0.1mM dNTP's) with water added to 100ul and 0.5ul Taq polymerase. The mixture was submerged beneath 90ul paraffin to prevent evaporation and placed in the heat block of a Techne PHC-1 Dri-block.

Twenty five cycles of 94°C for 2 minutes (to separate the double strands of the DNA), 55°C for 2 minutes (to reanneal) then 72°C for 3 minutes (to allow polymerisation) were used with a final 5 minutes at 72°C after the 25 cycles (total time approximately 5 hours). 10ul from the aqueous phase was then run on a 1% agarose TE gel, stained with ethidium bromide and photographed to check DNA had been amplified and the resultant fragment size of 408 base pairs was as expected. Up to 20 individual samples were amplified in this way at one time.

DNA Purification

The remaining 90ul of polymerised DNA was loaded onto a 1.0% low melting point agarose gel and run at 60v for 4 hours.

The gel was again stained with ethidium bromide and photographed under ultraviolet light. The agarose bearing

the single band of interest for each specimen was excised under ultraviolet light control and placed in a labelled eppendorf tube.

The DNA was purified from the low melting point agarose with a Geneclean kit, which uses a method described by Vogelstein and Gillespie (1979). The weight of the DNA-bearing agarose was calculated by subtracting the weight of an empty eppendorf tube from that containing a sample. Two volumes of 6 M sodium iodide Solution were added to the agarose and the tube placed in a 45^{o}C water bath for 5 minutes to melt the agar.

To the resulting approximately 1000ul of sodium iodide and agar, 8ul of glassmilk suspension was added, the eppendorf contents vortexed and replaced in the 45^{o}C water bath for 5 minutes. The sample was then placed on ice for 5 minutes with vortex mixing every minute. The glass milk (silica pellets to which the DNA is bound) was pelleted by a 5 second spin in a microcentrifuge and the supernatant poured off.

The pellet was washed 3 times in ice cold NEW wash (sodium chloride, tris, EDTA in water/ethanol mixture). After each wash the glass milk was pelleted by 5 second centrifugation and the supernatant discarded, with the final few microlitres aspirated from the eppendorf tube with a micro pipette.

The DNA was then eluted from the silica by the addition of 10ul 1x TE buffer (pH 7.5), incubated at 45^{o}C and the

supernatant containing the DNA aspirated from the glass milk and transferred to a labelled eppendorf tube. This was repeated with a fresh 10ul of 1xTE buffer to remove the remaining DNA from the glass milk to a total 20ul sample. 3 ul of this sample were run on a check 1% agarose gel, the gel stained with ethidium and photographed to confirm good recovery of DNA without contaminants. The remaining sample was stored frozen at -20°C until used for sequencing.

DNA Sequencing

Both alpha ^{35}S and gamma ^{32}P were utilised with the sequenase DNA sequencing kit using dideoxynucleotide labelling (modified from Winship et al., 1989).

Annealing Reaction

DNA obtained by use of the polymerase chain reaction was annealed to either primer 1 or primer 2. To 50ng DNA, sequencing buffer (2ul), primer 250ng (1ul), 1ul 10% DMSO made up to total 10ul with distilled water was placed in a hot block at 100°C for 2 minutes and then chilled on ice.

Sequencing Reaction

To the 10ul annealed DNA was added 0.1m DTT (1ul) 1:15 diluted appropriate labelling mix (2ul), 1:6 diluted sequenase enzyme (1.5ul), ^{35}S or ^{32}P labelled ATP or CTP (0.5ul) and the mixture incubated at room temperature (20°C) for 1 to 5 minutes

Termination Reaction

3.0 ul of sequencing reaction mixture was added to each of four stop reactions in separate labelled eppendorf tubes containing 2ul of ddGTP, ddATP, ddTTP or ddCTP diluted with 1 in 10 parts 10% DMSO. The termination reaction was allowed to proceed for 5 to 10 minutes at 37°C, 4ul stop solution was added to each tube and the samples stored frozen until loaded on a sequencing gel.

Sequencing Gels

Sequencing was performed on Sequi-Gen nucleic acid sequencing cells using a Sequigel sequencing system. A gradient gel of 0.5x to 5x Tris borate EDTA (TBE) of uniform 0.4 mm thickness was used, which gives good resolution of sequences up to 150 nucleotides long (Maniatis et al., 1982).

A plug of 40% bis/acrylamide (38g acrylamide, 2g bisacrlyamide in 100mls) was made by adding 9.5 ml 40% bis/acrylamide and 0.5ml 20x TBE. To this 125ul ammonium persulphate and 50ul TEMED were added and the quick setting gel drawn up to form a plug between the two glass plates in the bottom of the gel apparatus. The gradient gel was made from:

	0.5X TBE	5X TBE
40% bis/acrylamide	6ml	1.5ml
20x TBE	1ml	2.5ml
urea	17g	4.25g
sucrose	nil	2g
distilled water to	40ml	10ml
ammonium persulphate (10%)	240ul	60ul
TEMED	35ul	7ul
Bromophenol blue	nil	few grains

To pour the gel, 19ml 0.5 TBE was drawn up into a 50 ml syringe, then a further 6 ml of the blue 5xTBE drawn up into the same syringe and the two mixed. The gel was then poured taking care not to incorporate any bubbles to form a gradient gel with the 5xTBE (blue) at the lower part of the gel. A sharks tooth comb was introduced into the top of the gel and the gel left to solidify for at least 30 minutes at an angle of 30 degrees above the horizontal.

Loading Gels

Gels were prerun at 25 watts (with amperage and voltage non limiting) for 30 minutes. Sequenced samples were denatured by heating to 100^{o}C for 2 minutes and then placed on ice until loaded. 1.0 to 1.5 ul per sample were loaded using a 10ul Hamilton syringe and the tip (made from a plastic tip drawn out in a flame) flushed with water between loadings. The samples were then run in until the dye front was 1 cm above the bottom of the gel. The gel was then carefully lifted on one glass plate and placed in 1 litre of gel fixative (10% methanol, 10% acetic acid in water) for 30 minutes then dried in a vacuum gel drier and autoradiographed.

Probes

Probe Growing

cDNA probes were used for the identification of complementary RNA sequences in the total or poly(A+) RNA bound to Hybond filters. Individual probes were grown

with the assistance of Mrs. I McKenzie, from stocks held at the MRC Human Genetics Unit, Western General Hospital Edinburgh and stored in glycerol in -70°C or as stabs in agar maintained at 4°C.

Plasmid DNA (0.1ug) was used to transform competent E. coli DH1.

10ml T-broth was inoculated with a single colony, and incubated with shaking at 37°C overnight. From this preculture, 0.1ml was inoculated into 4x25ml T-broth until the optical density at 600nm reached 0.6 and 4x250ml prewarmed T-broth innoculated with 12.5ml of this late log phase culture and incubated at 37°C vigorously shaking in an orbital shaker for 150 minutes, 1.875ml of 20mg ml^{-1} chloramphenicol added to each flask and shaking continued overnight.

From 500ml of this culture, the cells were harvested at 6,000 rpm (3000g) for 10 minutes at 4°C and the pellet resuspended in 2 ml of glucose mix (50mM glucose, 25mM tris pH 8.0, 10mM EDTA, filtered and autoclaved) and 10mg lysozyme dissolved in 18ml glucose mix added and placed on ice for 30 minutes. 40ml of alkaline-SDS (0.2M sodium hydroxide, 1% SDS) was added and the mixture gently mixed on ice until the homogenate was clear then left to stand on ice for 10 minutes.

30ml 5M potassium acetate pH 4.8 was added, the mixture stirred and incubated on ice for 30 minutes while a course white precipitate formed. The supernatant was

collected after 10 minutes centrifugation at 12000 rpm (5000g) and 4°C, transferred to a clean bottle and 2 volumes (10ml) ethanol at -20°C added and the precipitate formed at -20°C for 30 minutes. The precipitate was collected by centrifugation at 10000 rpm (4500g) for 10 minutes and resuspended in 10ml TE.

5ml of 7.5M ammonium acetate was added and the mixture left for 20 minutes on ice, the precipitate separated by centrifugation at 12000 rpm (5000g) for 20 minutes, 2 volumes of ethanol at -20°C added, the precipitate collected by centrifugation at 12000rpm for 30 minutes and the pellet taken up in a total 4ml TE.

10ug ml^{-1} RNase was added and incubated at 37°C for 15 minutes; 200ul 20% SDS then 100ug ml^{-1} proteinase K added and the mixture incubated for at least 1 hour at the same temperature.

Following phenol/chloroform (1:1) then chloroform extraction, 1/10 volume of 3M sodium acetate then 2 volumes of ethanol were added and the plasmid DNA precipitated at -20°C overnight. The precipitate was collected by centrifugation at 12000rpm (5000g) for 30 minutes and the pellet finally dissolved in 1 ml 10mM Tris/1mM EDTA.

A small aliquot of plasmid DNA was run on a 1% agarose in TAE gel alongside HindIII/EcoR1 digested lamda DNA as a marker, stained with ethidium and photographed to ensure it was of the expected size and that there was no

remaining protein or RNA.

Half the plasmid DNA was digested with a suitable restriction enzyme to cut out the probe insert and 10ul of the resultant digest run on a 1% agarose in TAE buffer gel alongside lamda DNA digested with HindIII and HindIII/EcoR1, stained with ethidium and photographed. The correct digestion of the plasmid / cDNA was confirmed. The remaining digested plasmid was run on a 1% low melting point agarose gel, and following ethidium staining the appropriate band excised from the gel. The agarose bearing the desired fragment was placed in the upper chamber of a Spinex column and the insert DNA collected following centrifugation at 13000rpm (2000g) for 10 minutes in a eppendorf centrifuge. The collected solution was extracted with phenol/chloroform (1:1) and Chloroform and precipitated by the addition of a half volume of 3M sodium acetate and 2 volumes of ethanol. The probe was redissolved in 100ul TE and the concentration of cDNA probe insert estimated by running an aliquot of the purified insert alogside 1ug HindIII lamda DNA on an agarose gel and ethidium staining the resultant gel. A dilution of the insert was then made up such that 25-50ng of insert DNA was contained in 2ul TE, suitable for randomprime labelling.

Probes

Probes used for Northern blots:

c-erbB2 : Semba et al., (1985)

pEJ : Shih & Weinberg, (1982)

c-myc : Land et al., (1983)

p53 : Zakut-Houri et al., (1985)

TGF-a : Derynck et al., (1984)

TGF-ß : Derynck et al., (1985)

Epidermal growth factor receptor : Ullrich et al., (1984)

OR3 : Walter et al., (1985)

pS2 : Masiakowski et al., (1982)

Actin : Minty et al., (1981)

Probes used to examine 17p allele loss:

YNZ 22.1 : Nakamura et al., (1988)

pHp53Bam : Zakut-Houri et al., (1985)

pBHp53 : Hoyheim et al., (1989)

pMCT 35.1 : Carlson et al., (1988)

Spectrophotometry

The quantity and the purity of the extracted RNA was assessed by spectrophotometry at 260nm and 240nm with ultraviolet light (Maniatis et al., 1982) from a deuterium source. The quantity of RNA extracted was calculated using a dilution of 2 to 5 ul RNA sample in 700ul water from the formula:

RNA Extracted =

optical density at 260nm	x	dilution factor	x	correction factor (40)	x	volume of RNA sample

and the purity from the 260nm/240nm ratio (Maniatis et al., 1982).

Similarly, the quantity of DNA was calculated from:

DNA Extracted =

optical density at 260nm	x	dilution factor	x	correction factor (50)	x	volume of DNA sample

and the purity from the 260nm/280nm ratio (Maniatis et al., 1982).

The total quantity of RNA or DNA extracted from a given piece of tissue was calculated.

Photography

RNA and DNA gels and Northern blot Hybond membranes were photographed under a UV transilluminator using a 15 second exposure to 4inch x 5 inch Kodak Professional Film with a Polaroid MP4 land camera and the film developed in an X-ray film processor.

Autoradiography

Filters or dried sequencing gels were exposed to Kodak XAR preflashed film for 48 hours at -70°C in a light tight cassette using intensifying screens and the film was developed in an X-ray film processor. A further exposure to preflashed Kodak XAR film was then made to obtain a radiograph of optimal definition using the same exposure conditions, for up to 14 days exposure.

Densitometry

The extent of hybridisation of radiolabelled probe to the mRNA species was determined from densitometry using a laser densitometer constructed by the Medical Research Council Human Genetics Unit and expressed with respect to hybridisation to the actin probe.

Statistical Analysis

The appropriate statistical analyses were determined in consultation with Dr. A Carrothers, Statistician, MRC Human Genetics Unit, Edinburgh. Statistical analysis was performed using the "Kwikstat" and "Epistat" IBM compatible statistical software packages.

RESULTS

Patients

Gene expression

Clinical and pathological correlation

DNA allele loss

Patient prognosis

DNA sequencing

Mouse xenograft model

- -histopathology
- -serum oestradiol concentration
- -oestrogen receptors

Xenograft gene expression

- -presence of mRNA species
- -changes in gene expression after oestrogen stimulation

Tamoxifen treatment of xenografts

Tamoxifen treated patients

Preoperatively systemically treated patients

Patients

Fresh breast tumour tissue and venous blood was collected from 80 female patients. Of the 80 patients 15 had received tamoxifen prior to surgery and 12 yielded both pretreatment and, following chemotherapy or endocrine therapy, post-treatment (mastectomy) specimens. Full patient documentation and all relevant pathological and biochemical data were available for every specimen.
Good quality, undegraded total RNA and DNA was extracted intact from all 92 tumours and DNA extracted from the corresponding 80 blood samples. Total RNA was also extracted intact from 10 reduction mammoplasty specimens, from other normal human tissues including tonsils, ovary and myometrium and from the human breast cancer cell lines MCF-7, T-47D and MDA MB 231.

Gene expression

mRNA for p53, c-myc, c-erbB-2, H-ras, TGF-ß and pS2 was detected on Northern blots of breast tumour RNA, breast cancer cell line RNA and normal human RNA (figures 3 & 4). Using laser densitometry, autoradiographs of Northern blots bearing up to 48 samples were examined to quantitate the expression of these mRNA species relative to actin mRNA. On each Northern blot, 3 breast cancer cell lines 4 normal human tissues and 4 tumours were used as internal controls to standardise between the

individual blots. Each filter was successfully reprobed up to 5 times. Samples that gave reproducible levels of gene expression with respect to actin (the internal control) on laser densitometry on more than one blot were used for analysis of mRNA levels in relation to clinical and pathological data.

Quantitative differences were noted between different tumours for all the probes compared with the actin control and qualitative differences between the tumours/normal tissues and the breast cancer cell lines were identified using the probes for p53, c-erbB-2 and pS2.

Gene expression (mRNA) was either undetectable, detectable and upto 1.5 times that found in control (reduction mammoplasty) breast tissue (if gene expression was detected in the control tissue), or overexpressed (at least twice the level found in control breast tissue). Where no mRNA was detected in the control breast tissue, then the detection of an mRNA species was considered on a "present" or "absent" basis in the tumours.

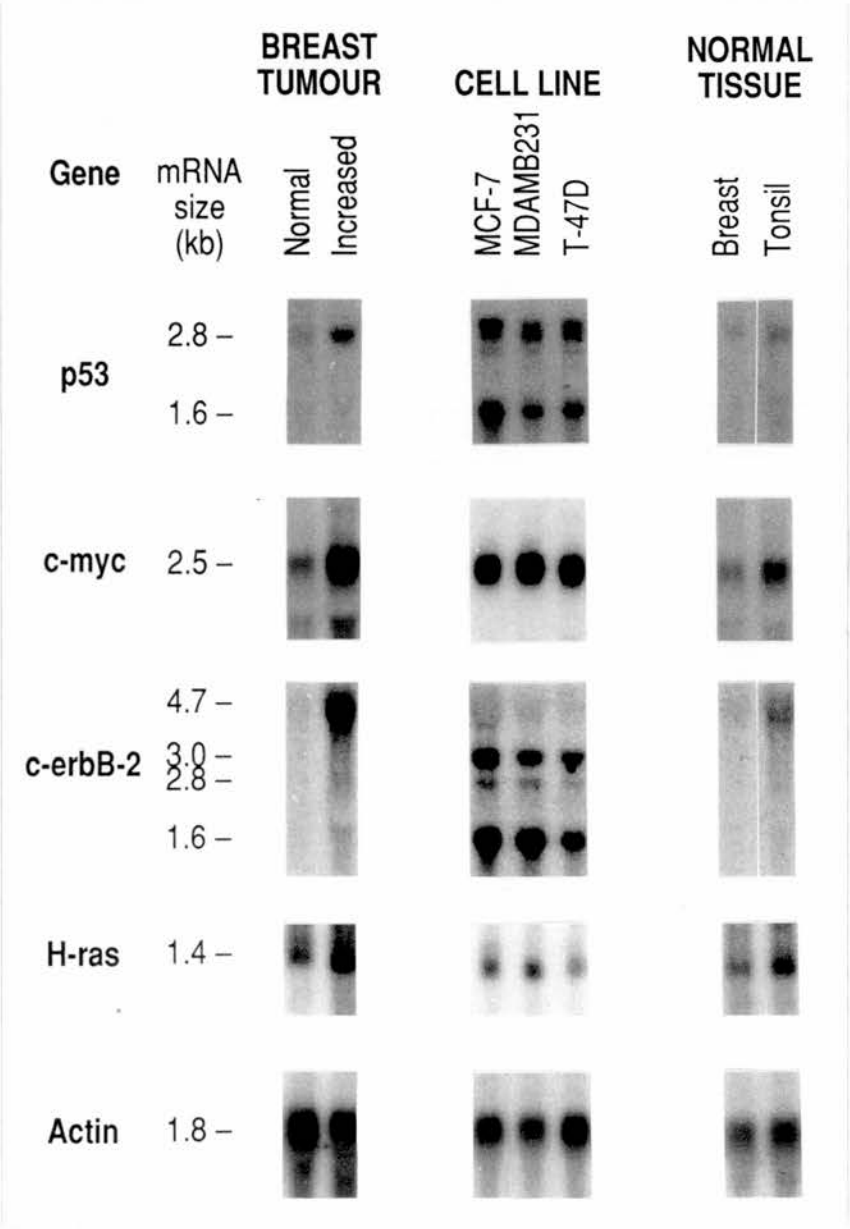
For example, a 2.8 kilobase p53 mRNA was detected in 43 of the 76 tumour specimens (57%), in all 3 breast cancer cell lines and in six of the ten reduction mammoplasty specimens (figure 3). Low levels of this p53 mRNA (comparable to those found in the six positive mammoplasty specimens) were also detected in normal human tonsil, uterus and ovarian tissue (data not shown). There

were quantitative differences between the tumour specimens and qualitative differences between the tumours and the cell lines. Thirty-three of the 76 patients had no detectable p53 mRNA in their tumour tissue, 19 patients had detectable p53 mRNA of 2.8kb similar in quantity to the reduction mammoplasty specimens that gave a positive signal. 24 patients had increased levels of the 2.8kb p53 mRNA approaching those found in the cell lines.

The 3 breast cancer cell lines each yielded four p53 mRNA species. Three closely related species were of approximately 2.8kb, with differences between cell lines in the amounts of mRNA for each of these species. A fourth (1.6kb) p53 mRNA was strongly expressed in all three lines.

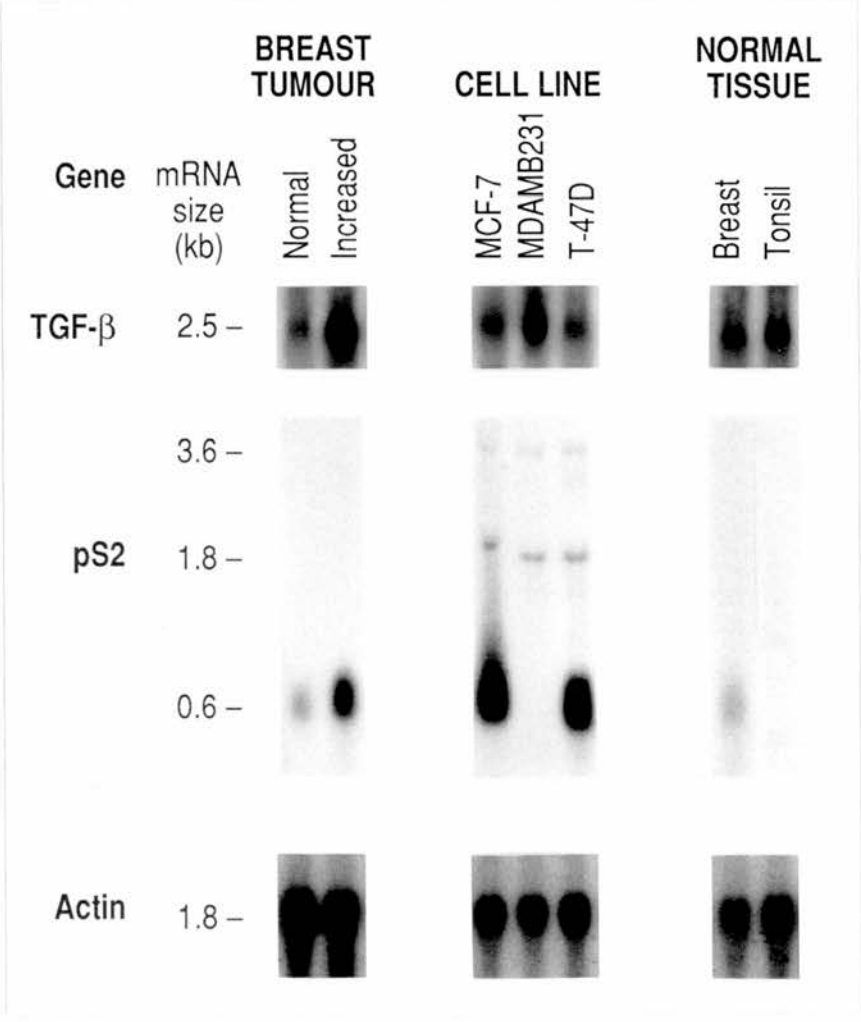
A single mRNA for c-myc of 2.5kb, a single mRNA for H-ras of 1.4kb and one species of 2.5kb for TGF-ß were each identified in a proportion of the tumours, normal tissues and the breast cancer cell lines (figures 3 & 4). In breast tumours, c-erbB-2 expression was detected as a 4.8kb mRNA, although no expression was demonstrated in normal breast tissue and mRNA species of 3.0kb, 2.8kb and 1.8kb were noted in the MCF-7 cell line (figure 3). Similarly, a 600 base pair single mRNA for pS2 was identified in some of the tumours, but not in the normal tissue and two additional mRNA species of 3kb and 1.6kb were found in the MCF-7 cell line (figure 4).

Figure 3 : Representative Northern blot results of oncogene expression in human breast cancer tissue, breast cancer cell lines and normal tissues.



Gene expression of p53, c-myc, c-erbB-2, H-ras and actin control; sizes in kilobases (kb) calculated from ribosomal RNA size markers of 4.7kb (28s) and 1.8kb (18s). Autographs obtained by probing Northern blots of total RNA with the exception of MCF-7 samples which were polyA+ RNA.

Figure 4 : Representative Northern blot results of growth factor and oestrogen regulated gene expression in human breast cancer tissue, breast cancer cell lines and normal tissues.



Gene expression of TGF-ß, pS2 and actin control; sizes in kilobases (kb) calculated from ribosomal RNA size markers of 4.7kb (28s) and 1.8kb (18s). Autographs obtained by probing Northern blots of total RNA with the exception of MCF-7 samples which were polyA+ RNA.

Clinical and pathological correlation

For each tumour, the quantity of mRNA expressed for each gene under study was assessed in relation to clinical and pathological parameters.

Overexpression of mRNA for the oncogene c-myc was significantly associated with small tumour size ($p < 0.05$; table 1). There were no significant associations between p53, c-erbB-2, H-ras, TGF-ß or pS2 mRNA expression and tumour size.

Table 1 : Gene expression compared to clinical tumour size at time of diagnosis.

	PROBE															
	p53			c-myc			c-erbB-2		H-ras			TGF-ß		pS2	
	0	N	+	0	N	+	0	+	0	N	+	N	+	0	+
	(No.Tumours examined)														
	(76)			(76)			(71)		(79)			(56)		(78)	
TUMOUR SIZE															
<5cm	16	12	15	15	16	12	24	16	20	12	11	9	25	27	17
>5cm	17	7	9	20	4	9	26	5	19	14	3	2	20	26	8
	NS			p=0.028*			NS		NS			NS		NS	

0, no expression detected;
N, gene expression as in normal tissue;
+, increased gene expression.

* Chi square test (Yates correction).
NS Not Significant.

Overexpression of mRNA for p53 was associated with clinically insignificant levels of oestrogen receptor protein (p =0.049) and pS2 overexpression correlated with tumours showing significant levels (greater than 20 fmol mg protein^{-1}) of oestrogen receptor protein (p=0.02) (table 2). There were no significant association between overexpression of c-myc, c-erbB-2, H-ras, or TGF-ß and tumour oestrogen receptor protein content.

Table 2 : Gene expression compared with tumour oestrogen receptor protein content.

	PROBE																	
	p53			c-myc			c-erbB-2			H-ras			TGF-beta			pS2		
	0	N	+	0	N	+	0		+	0	N	+	N		+	0		+
	(No.Tumours examined)																	
	(76)			(76)			(71)			(79)			(56)			(78)		
OESTROGEN RECEPTOR fmol mg^{-1} protein																		
<20	13	5	15	14	7	13	26	8		15	12	8	4	22		27	6	
>20	20	14	9	21	13	8	24	13		24	14	6	7	23		26	19	
	p=0.049*			NS			NS			NS			NS			p=0.02+		

0, no expression detected;
N, gene expression as in normal tissue;
+, increased gene expression.

* Chi square test (Yates correction).
\+ Fisher's exact test.
NS Not Significant.

Overexpression of mRNA for c-myc was associated with tumours which had not spread to the regional lymph nodes (p=0.021) (table 3). There were no significant relationships between mRNA p53, c-erbB-2, H-ras, TGF-ß or pS2 and the presence or absence of metastatic tumour in the regional lymph nodes.

Table 3 : Gene expression compared with regional lymph node involvement with tumour.

	PROBE																	
	p53			c-myc			c-erbB-2		H-ras			TGF-beta		pS2	
	0	N	+	0	N	+	0	+	0	N	+	N	+	0	+
	(No.Tumours examined)														
	(76)			(76)			(71)		(79)			(56)		(78)	
NODES INVOLVED															
Nil	15	7	13	12	8	15	21	13	14	16	7	4	24	24	12
≥1	18	12	11	23	12	6	29	8	25	10	7	7	21	29	13
	NS			p=0.021*			NS		NS			NS		NS	

0, no expression detected;
N, gene expression as in normal tissue;
+, increased gene expression.

* Chi square test (Yates correction).
\+ Fisher's exact test.
NS Not Significant.

There were no significant associations between expression of mRNA for p53, c-myc, c-erbB-2, H-ras, TGF-ß or pS2 and the menopausal status of the patients at the time of diagnosis.

Table 4 : Gene expression compared with menopausal status

	PROBE															
	p53			c-myc			c-erbB-2		H-ras			TGF-beta		pS2	
	0	N	+	0	N	+	0	+	0	N	+	N	+	0	+
	(No.Tumours examined)														
	(76)			(76)			(71)		(79)			(56)		(78)	
MENOPAUSAL STATUS															
pre-menopausal	13	4	9	9	8	9	14	9	10	12	4	1	18	18	8
post-menopausal	20	15	15	26	12	12	36	12	29	14	10	10	27	35	17
	NS			NS			NS		NS			NS		NS	

0, no expression detected;
N, gene expression as in normal tissue;
+, increased gene expression.

* Chi square test (Yates correction).
NS Not Significant.

There were no significant correlations between expression of any of the genes under study and the histological features of the tumour including pathological grade.

c-erbB-2 mRNA expression was compared to c-erbB-2 antibody staining using the antibody 21N (Gullick et al., 1987) in tissue sections of 31 tumours examined by Miss Claire Reid, Department of Pathology, University of Edinburgh. Immunohistochemical data were recorded without prior knowledge of c-erbB-2 mRNA findings. Protein expression correlated strongly with mRNA expression ($p=0.0004$, Fisher's exact test; table 5) although c-erbB-2 mRNA expression was detected in some tumours in which no antibody staining was detected .

Table 5 : Comparison of c-erbB-2 mRNA expression with c-erbB-2 expression detected by imunohistochemistry.

		c-erbB-2 mRNA	
		Present	absent
Antibody staining	Positive	8	0
	Negative	6	17

Fisher's exact test $p=0.0004$

Overall, these data demonstrate that quantitative differences in gene expression were evident between tumours compared to control tissues and the expression of individual genes correlates significantly with clinical and pathological parameters.

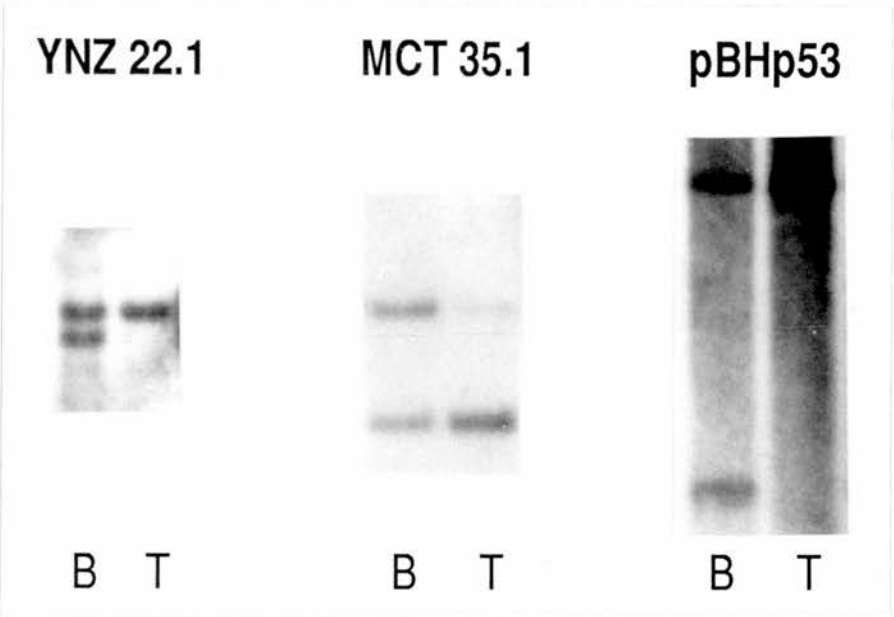
DNA allele loss

At the time these results were collated, three publications suggested that loss of genetic material from 17p was common (over 60%) in breast tumours (Mackay et al., 1988b, Devilee et al., 1989a Devilee et al., 1989b). These data were obtained using a VNTR probe (YNZ 22.1, Nakamura et al., 1988) that maps within the same chromosomal band (17p13) as the gene for p53 (Isobe et al., 1986). Since allele loss may be associated with loss of a tumour suppressor locus, and the dispute as to the function of p53 as oncogene (Editorial, Oncogene 1988) or tumour suppressor gene (Green, 1989) remained unresolved, p53 gene expression was compared with allele loss from 17p13.

Using the Southern blot technique, allele loss, or diminution of an allele signal to less than 50% of the predicted level, when the relative intensities of the unaffected allele band were measured in tumour and blood leukocyte DNA tracks by laser densitometry, was determined in blood/tumour pairs of DNA. Using probes YNZ 22.1 (17p13.3), MCT 35.1 (17p13.1), pBHp53 (17p13.1) and php53Bam (17p13), alleles were examined in the 80 untreated or tamoxifen treated tumours as far as the limited quantity of DNA allowed (figure 5). The cDNA probe used to detect p53 mRNA, php53Bam was not sufficiently informative for use on the blood/tumour

paired DNA; less than 10% of human tonsil samples were informative (i.e. heterozygous) with this probe.

Figure 5 : Representative allele losses for chromosome 17p.



Examples of autoradiographs of DNA from 3 patients comparing constitutional, blood leucocyte, DNA (B) with tumour DNA (T). Two bands (alleles) are present with each probe in the constitutional DNA and hence these patients are constitutionally heterozygous (informative). There is loss of an allele in the tumour DNA demonstrating loss of genetic material from 17p for each of the 3 loci examined. The faint second band in the tumour DNA detected with probe MCT 35.1 almost certainly represents heterozygous normal tissue DNA within the cancer tissue examined (Mackay et al., 1988b)

Overall, 72 of the 80 patients were informative for 1 or more of these 3 probes, with 46/72 (64%) exhibiting allelic loss from 17p13. Four patients who were informative for all three probes had allelic loss for all three loci. While there was no consistent pattern of allele loss, loss from 17p13 for each probe was over 50% for loci telomeric to p53 and 35% at the p53 locus (table 6).

Table 6 : Allele loss in human breast tumours

PROBE	BLOOD/TUMOUR PAIRS EXAMINED	No. INFORMATIVE (%)	ALLELE LOSS (%)
YNZ 22.1	80	54/80 (68%)	31/54 (57%)
MCT35.1	66	27/66 (41%)	14/27 (52%)
p53	74	43/74 (58%)	15/43 (35%)

Allele loss identified with each of these probes was related to clinical and pathological features of the tumours and to expression of the p53 gene.

YNZ 22.1 allele loss did not correlate with with tumour size, low level of oestrogen receptor protein, regional lymph node involvement or menopausal status (table 7).

Table 7: Correlation between YNZ allele loss and clinical and pathological parameters.

	TUMOUR SIZE		OESTROGEN RECEPTOR		NODES INVOLVED		MENOPAUSE	
	<5cm	>5cm	<20	>20	nil	>1	pre	post
Allele loss	17	14	20	11	12	19	13	18
No allele loss	14	9	8	15	11	12	8	15
	NS		NS		NS		NS	

pBHp53 allele loss did not correlate with tumour size, oestrogen receptor protein, regional lymph node involvement or menopausal status (table 8).

Table 8 : Correlation between pBHp53 allele loss and clinical and pathological parameters.

	TUMOUR SIZE		OESTROGEN RECEPTOR		NODES INVOLVED		MENOPAUSE	
	<5cm	>5cm	<20	>20	nil	>1	pre	post
Allele Loss	9	6	6	9	9	6	9	6
No allele loss	13	15	13	15	12	16	7	21
	NS		NS		NS		NS	

There were no correlations between MCT 35.1 allele loss and the clinical or pathological features of the tumours (table 9).

Table 9 : Allele loss of MCT 35.1 compared with clinical and pathological parameters.

	TUMOUR SIZE		OESTROGEN RECEPTOR		NODES INVOLVED		MENOPAUSE	
	<5cm	>5cm	<20	>20	nil	>1	pre	post
Allele Loss	9	5	7	7	8	6	7	7
No allele loss	8	5	5	8	5	8	4	9
	NS		NS		NS		NS	

p53 gene mRNA expression was compared to allele loss demonstrated with each of the three probes for informative patients (table 10). p53 mRNA overexpression was associated with YNZ 22.1 allele loss (p = 0.042, Chi square test) but not with pBHp53 or MCT 35.1 allele loss.

Table 10 : p53 mRNA expression compared to 17p13 allele loss.

p53 EXPRESSION	YNZ 22.1		P53		MCT35.1	
	Loss	No loss	Loss	No loss	Loss	No loss
Nil	11	13	6	15	8	4
Normal	6	7	6	4	1	4
Increased	14	3	3	9	5	5
Chi square test:	p=0.042		not applicable			
Fisher exact test:	p=0.017		p=0.32		p=0.16	

(for Nil/Normal v increased expression)

Four of the cancer specimens collected on histological examination contained only in situ (pre-invasive) breast cancer tissue (table 11).

Table 11 : p53 mRNA expression and 17p13 allele loss in 4 in situ cancer specimens.

SPECIMEN NUMBER	p53 mRNA EXPRESSION	ALLELE STATUS FOR PROBE YNZ 22.1	MCT 35.1	pBHp53
11	Overexpressed	no loss	no loss	no loss
18	No expression	no loss	not done	no loss
77	No expression	homozygous	loss	no loss
92	No expression	homozygous	homozygous	loss

Thus, one of the four specimens showed increased expression of p53 and allele loss at MCT 35.1 and pBHP53 was seen in one case each. These findings are in line with what would be predicted from the total tumour collection. A larger series of non-invasive cancers will need to be studied to establish whether the frequency of allele loss at YNZ 22 is genuinely reduced compared with more advanced tumours.

Patient prognosis

After 18 months mean clinical follow up from the definitive surgical procedure (range 4 to 24 months), 15 of the 80 patients (19%) had recurrence of their breast cancer and 5 of these patients (6% of the original 80) had died , all from the effects of metastatic breast cancer. The total number of such patients is comparatively small and the follow up, although meticulous, is short (18 months). However, a statistically significant correlation between failure to express mRNA for pS2 and disease recurrence was identified ($p=0.015$, Fishers exact test; table 12). There were no other statistical associations between expression of the genes examined and early prognosis or between allele losses and disease recurrence. The correlation between lack of pS2 gene expression and poor early prognosis was less significant than the stage of the disease at diagnosis or node status. Nevertheless pS2 does appear to be more useful than oestrogen receptor protein measurement for identifying patients with a very poor short term prognosis.

Table 12 : Tumour pS2 mRNA expression as a prognostic feature for breast cancer in 78 patients compared with known prognostic features in the overall group of 80 patients.

	pS2		Tumour stage		Nodes		Oestrogen receptor protein	
	0	+	T2	T3/T4	+	-	>20	<20
Disease recurrence	14	1	3	12	14	1	8	7
Disease free	39	24	42	23	29	36	35	30
	p=0.015+		p=0.002+		p=0.0005+		NS	

+ Fishers exact test.

NS Not Significant.

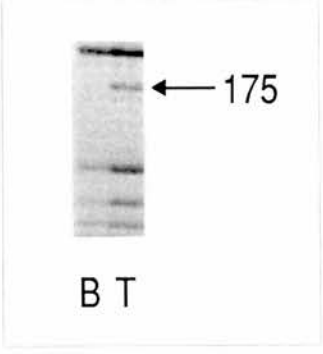
DNA sequencing

The results from mRNA studies of p53 gene expression and 17p13 allele loss demonstrate that expression of p53 mRNA is associated with loss of an allele telomeric to the p53 gene (as demonstrated by YNZ 22.1 loss; p=0.042) but not with allele loss in the immediate vicinity of the p53 gene (MCT 35.1 and pBHP53). A possible explanation for this is that the YNZ 22.1 allele loss may represent loss of a regulatory element for p53 gene expression (which would not necessarily require to be close to the p53 gene) that allows the overexpression of p53. It was therefore of importance to determine whether the p53 expressed was from a mutant (and hence potentially oncogenic) p53 gene. Alternatively, the absence of detectable p53 mRNA in 33/76 tumours may represent failure to express the tumour suppressive normal p53 gene. To address this problem DNA sequencing of part of the the p53 gene was used.

Four blood/tumour DNA pairs (tumour numbers 38, 43, 50 and 92) were examined to determine whether the sequence of the p53 gene in the tumours might be mutant. Exons 5 and 6 and the intervening intron were selectively amplified by the polymerase reaction and sequenced with 32P and 35S. A reproducable point mutation of Cytosine to Guanosine at amino acid 175 in exon 6 was demonstrated in the tumour of patient 43 (figure 6), but not in the

patient's constitutional DNA. This represents a change in the encoded protein from basic histidine to acidic leucine.

Figure 6 : A representative p53 sequencing autoradiograph



Part of a ^{35}S sequencing gel autoradiograph for blood and tumour of patient 43 demonstrating a guanosine band (indicated by the arrow) present in the tumour DNA (T) but not in the constitutional DNA (B) at amino acid 175 (numbering from Zakut-Houri et al., 1985).

No other base changes were identified in exons 5 and 6 or in the intervening intron in the remaining three blood/tumour pairs. However, if one considers the p53 expression, allele distribution and sequencing data for these four tumours together (table 13) the findings still support the thesis that either loss of normal p53 gene expression (tumours 50 and 92) or overexpression of mutant, oncogenic, p53 (tumour 43) may occur in breast tumours. Further, failure to detect a mutation in tumour 38 at exons 5 and 6 does not exclude the possibility of significant mutations elsewhere in the p53 gene.

Table 13 : p53 gene expression, 17p13 allele loss and p53 mutation in 4 breast tumours.

Tumour Number	p53 mRNA expression	Allele status YNZ	MCT	pBHP53	p53 Mutation
38	Overexpressed	loss	homo+	homo	none found
43	Overexpressed	loss	loss	loss	exon 6
50	Nil	loss	loss	loss	none found
92	Nil	homo	loss	homo	none found

+ homozygous

Mouse xenograft model

Using the regime of 50ug 17ß oestradiol benzoate in 50ul arachis oil injected subcutaneously every three weeks, xenografts of the oestrogen responsive human breast cancer cell line MCF-7 were established providing a renewable source of substantial amounts of tumour material. This could be transplanted into other mice, or the cells regrown from the xenografts in vitro and then reinoculated into further mice.

One hundred and forty-five CBA strain mice were injected with MCF-7 cells in this study (table 14). Tumour formation and growth of MCF-7 cells taken directly from tissue culture ("primary inocula") was observed at the site of inoculation in 30/57 (53%) mice. The take rate of transplanted tumour material was higher than that of primary inocula (23/32, 72%) and was significantly higher for tumour cells that had been re-cultured in vitro and then inoculated into fresh mice compared with primary inocula (16/18, 89% p=0.004 by Fishers exact test).

Tumours did not grow without oestrogen supplementation. The 50ug oestradiol benzoate in arachis oil, or the arachis oil alone were well tolerated by the CBA mice and the oestrogen delivered in this form promoted tumour growth. Withdrawl of oestrogen supplementation (failure to administer the supplement at 3 week intervals after

the tumours had become established) resulted in a decline in tumour volume.

Table 14 : The fate of MCF-7 cells inoculated into immunocompromised mice.

MCF-7 INOCULUM	OESTROGEN SUPPLEMENT	NUMBER OF CBA MICE	NUMBER OF MICE WITH TUMOURS	NO TUMOURS	DEATHS
Cells	Nil	20	Nil	20	Nil
Cells	50ug oestradiol benzoate	69	30 (53%)+	27	12
Transplant xenograft	50ug oestradiol benzoate	36	23 (72%)	9	4
Recultured xenograft cells	50ug oestradiol benzoate	20	16 (89%)	2	2

+ Number of live mice bearing tumours as a percentage of surviving animals.

Using the same regime of 50ug oestradiol benzoate or no oestrogen supplement, no tumour growth was obtained following injection of T-47D or MDA-MB-231 cells into the CBA mice.

Histopathology

Each MCF-7 tumour was firm, pale, solid and well circumscribed, not showing overt local invasion or ulceration of the overlying skin. All the tumours were examined histologically by Dr. David Paterson, Department of Pathology, University of Edinburgh and were compatible

with an origin from breast , although they did not show marked adenocarcinomatous differentiation. There were no areas of necrosis in the smaller tumours, although the tumours of 15mm diameter or greater did have evidence of central necrosis. No marked lymphocyte infiltration was noted in any tumour.

Microscopic and macroscopic examination of mice which died and of mice which were killed to obtain tumour showed evidence of metastasis in only one animal. In that instance, tumour cells were evident at the site of MCF-7 cell inoculation, as peritoneal seedlings and microscopically in the visceral pleura of the lung. No pathological evidence of oestrogen toxicity was found at post mortem in any animal on the 50ug oestradiol benzoate regime although there was some hair loss at the site of the oestradiol injection.

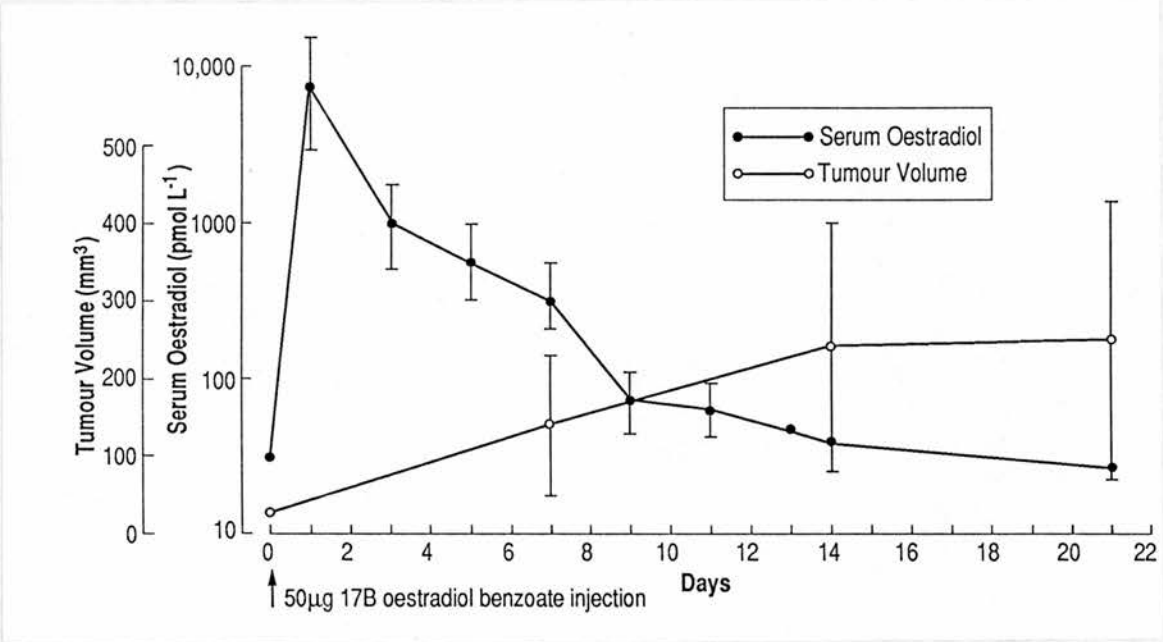
Serum oestradiol concentration

Oestradiol was not detected in the serum from thymectomised and irradiated mice prior to injection. After injection with 50ug oestradiol benzoate (figure 6), a sharp rise to a mean 7492 pmols L^{-1} (S.D. 3374 pmols L^{-1}) oestradiol occurred by 24 hours, declining exponentially to undetectable levels (less than 53 pmols L^{-1}) 2 weeks after the injection. Serum oestradiol detection and measurement was performed by S. Barnes and V. Sweeting, Department of Clinical Chemistry, Royal Infirmary, Edinburgh.

Tumour growth

The tumours became palpable during the first 3 weeks following 17 ß oestradiol benzoate injection and following the second and third injections of oestrogen, the tumour was observed to grow in size, but not in a uniform fashion (figure 7). In particular, during the first 14 days after injection, the tumour increased rapidly in size, then from day 14 to 21 slowed down or became static. By 3 to 6 weeks, all tumours were large enough for the studies described.

Figure 7 : Serum oestradiol and tumour volume in a mouse xenograft model.



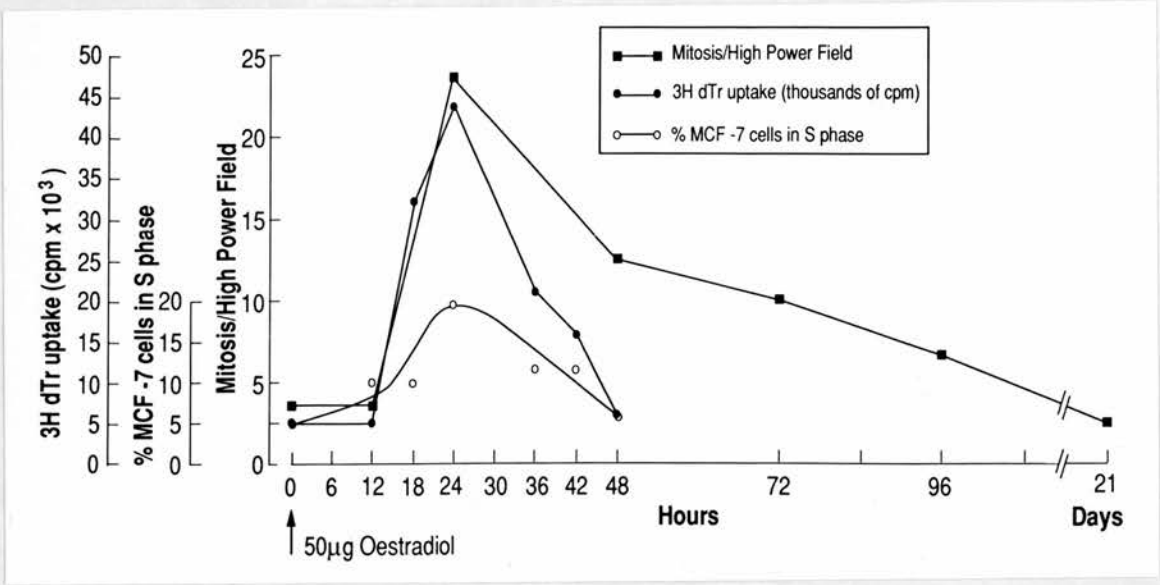
Serum oestradiol (mean ± standard deviation) in thymectomised and irradiated mice for 2-4 mice at each time point following subcutaneous injection of 50ug 17ß oestradiol benzoate. From undetectable levels (<53 pmol L^{-1}) prior to injection of oestradiol there is a rapid rise to a peak of 7492 $pmols^{-1}$ followed by a decline in serum oestradiol to undetectable levels by the third week post injection. Tumour volume (calculated from pi/12 x mean $diameter^3$) for a cohort of 12 mice (Mean volume ± standard deviation) measured during weeks 3 to 6 following injection of the MCF-7 cells. Only four time points are shown for clarity. There is a rise in tumour volume for the two weeks following injection of oestrogen, with little change in tumour volume once serum oestradiol becomes undetectable.

Cellular DNA synthesis and mitosis

Increased thymidine uptake was noted by 18 hours following the oestrogen injection (figure 8) and, in parallel with the percentage of S-phase cells, reached a maximum 24 hours following oestrogen injection, declining thereafter. Both these assays were performed by Dr. D L Deane, MRC Human Genetics Unit, Western General Hospital, Edinburgh.

The number of mitoses per high power field (x400) by Dr. D Paterson (figure 8) showed an increase, compared to the baseline value of 3 per x400 field, to 24 per x400 field demonstrable 24 hours following oestrogen stimulation of the tumour. The level fell to 5 mitoses per x400 field ten days later. The number of mitoses showed parallel changes to the biochemical indices of cellular DNA synthesis.

Figure 8 : Three indices of cellular proliferation in xenografts of MCF-7 cells following administration of oestradiol.



Cell proliferation measured by radiolabelled thymidine (^{3}H dTr) uptake, percentage of cells in S-phase and mitoses per x400 field showing mean value of 4 tumours for each time point. Standard deviation was $\leq$3.5 for thymidine uptake (except at time 24 hours, standard deviation 13.9), $\leq$3.7 for percentage of cells in S-phase (except at 24 hours, standard deviation 7.6) and $\leq$3.0 for mitoses per x400 field. The same tumours were used for all 3 indices of cell proliferation. Between 12 and 24 hours following injection of 17ß oestradiol benzoate (arrow at injection time = 0), MCF-7 cells are stimulated to divide, DNA synthesis returning to prestimulation levels by 48 hours and mitoses declining within days.

Oestrogen receptors

MCF-7 cells in vitro, tumour material taken immediately prior to oestradiol injection and cells cultured from xenografts contained a mean 120 fmols oestrogen receptor protein mg total protein^{-1}. There was a rise to mean 240 fmols mg protein^{-1} at 30 hours, but at 7, 14 and 21 days following injection, the level had returned to between 120 and 150 fmol mg protein^{-1}. Oestrogen receptor protein was assayed by Dr R.A. Hawkins, Department of Surgery, University of Edinburgh.

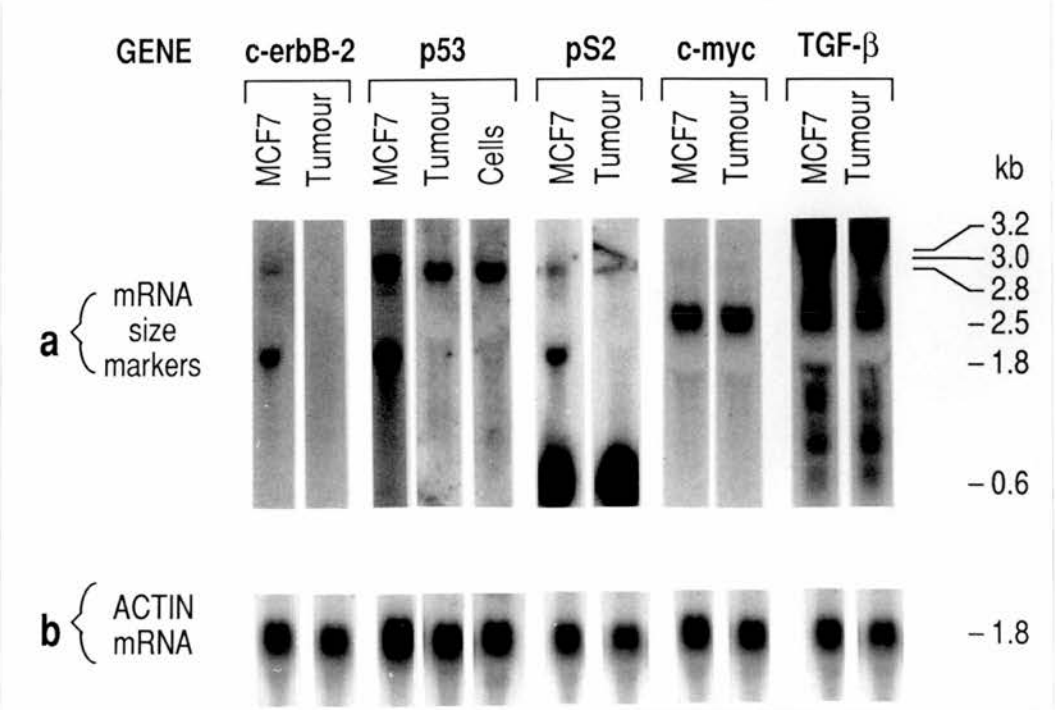
Xenograft tumour gene expression

Presence of mRNA species

Messenger RNA was detected in MCF-7 cells by seven of the nine probes (figure 9). No mRNA for EGFR or TGF-alpha was detected in the MCF-7 cells or xenografts, although both mRNA species were detected in two other breast cancer cell lines, MDA-MB-231 and T47-D (data not shown). mRNA for two genes (c-erbB-2 and OR3) detected in the MCF-7 cell line were not found in the xenograft material, but no mRNA species was detected in the tumours which was not present in the original cell line (figure 9). The mRNA for c-erbB-2 was detected at 3.0kb, 2.8kb and 1.8kb in the original cell line but not in the xenografts. Four mRNA species for p53 (three at 2.8kb, one at 1.8kb) were identified in the original cell line but only a single

mRNA for p53 was found in tumours or in re-cultured cells even after 56 days in vitro. Similarly, 3 mRNA species were detected with the pS2 probe in the original cell line but only the single 0.6kb mRNA in the tumours. Other cDNA probes such as those for TGF-ß and c-myc detected only a single mRNA (2.5kb in each case) present in both the cell line and xenografts. There were no differences in any mRNA species detected between xenografts, transplanted tumours and tumour cells recultured for 56 days.

Figure 9 : Examples of Northern blot autoradiographs of gene expression in MCF-7 cells in vitro and in vivo (xenografts).

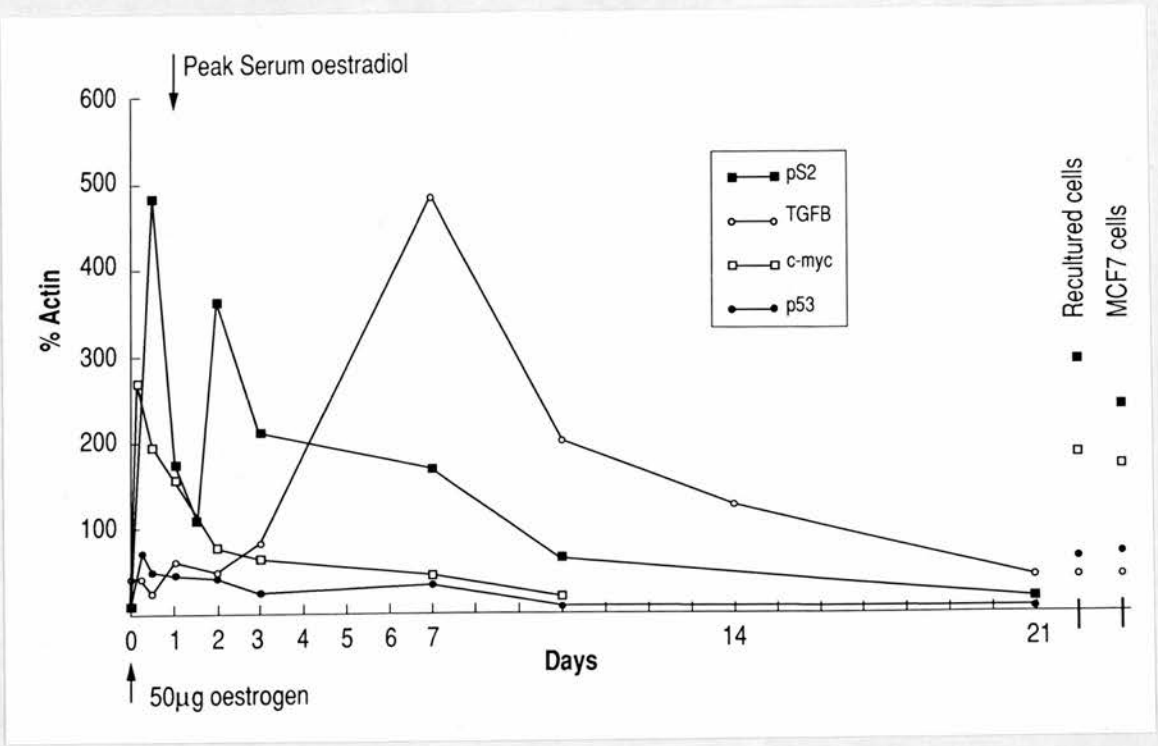


Representative autoradiographs of MCF-7 cells, MCF-7 xenografts and MCF-7 cells re-cultured from the xenografts after probing with cDNA probes for c-erbB-2, p53, pS2, c-myc and TGF-ß. In each case the actin-probed control for each lane is shown. Two c-erbB-2 species are evident in the cell line but are not seen in the xenografts. Four p53 mRNA species are demonstrated in the cell line but only one in the xenografts and re-cultured cells. Similarly, three species detected with the pS2 probe are seen in the cell line but only one species in the xenografts and cells cultured from those xenografts. The c-myc, TGF-ß and H-ras probes (H-ras blots not shown) detected identical species in both the MCF-7 cell line and the xenograft tumours.

Changes in gene expression after oestrogen stimulation

Densitometry permitted detection of changes in the levels of mRNA for c-myc, p53, TGF-ß and pS2 with respect to alpha-actin mRNA, following stimulation of the xenograft by oestrogen (figure 10). The mRNA species for c-myc and p53 both increased within the first 24 hours then returned towards the unstimulated level whilst that for TGF-ß was rapidly suppressed, increasing only as the oestrogen stimulus declined after one week. pS2 expression showed a biphasic response with an initial increase to 24 hours, then suppression for 12 hours, a less substantial increase by 48 hours and finally a decline as the expression of TGF-ß increased, peaked at day 7, and returned gradually to prestimulation levels by day 21. There was no change in H-ras expression following oestrogen stimulation.

Figure 10 : Changes in mRNA following oestrogenic stimulation of MCF-7 xenografts.

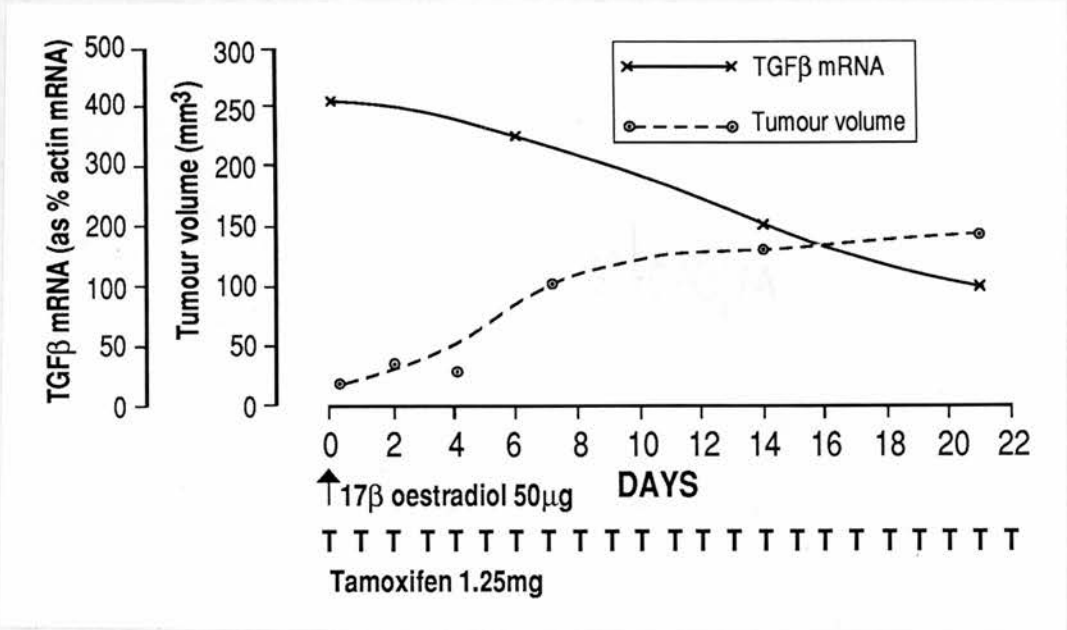


Host animals were injected with 17ß oestradiol benzoate at time 0 (arrow). The changes shown are for c-myc, p53, pS2 and TGF-ß mRNA species in xenografts, as detected by densitometry of autoradiographs, with respect to that for actin as a control. The percentage at each time point is the mean of 6 tumours (standard deviation $\leq$11%). mRNA expression for the same four species is shown for the original MCF-7 cells and cells recultured from xenograft tumours.

Tamoxifen treatment of xenograft tumours

In a group of ten tumours of mean volume 250mm^3, due to have the next dose of 50ug 17ß oestradiol administered, the oestrogen stimulus was administered and at the same time and at 24 hour intervals thereafter, 1.25mg tamoxifen in 50ul arachis oil administered subcutaneously at a different site. Sequential tumour measurement demonstrated tumour regression (figure 11) over the ensuing 21 days rather than the expected tumour growth observed following oestrogen injection alone to a mean 820 mm^3. Although no quantitative differences in tumour c-myc, p53 or pS2 expression relative to actin mRNA were observed during the first week compared to untreated tumours. TGF-ß expression, which peaked at 7 days in the untreated xenografts (figure 11), remained at elevated levels during the second and third weeks in the tamoxifen treated tumours and was associated with the decline in tumour volume.

Figure 11 : Effect of tamoxifen treatment on MCF-7 xenograft tumour TGF-ß mRNA.



Regression in MCF-7 xenograft tumours following tamoxifen administration, associated with a sustained rise in TGF-ß mRNA. Values for TGF-ß mRNA (as % actin mRNA) is the mean of densitometric measurement in 3 tumours at each time point. 17ß oestradiol benzoate 50ug administered at time=0 (arrow); daily injection of tamoxifen 1.25mg indicated by the letter "T".

Tamoxifen treated patients

A subgroup of 14 patients received tamoxifen prior to surgery. The tumour was excised at mastectomy either because it was increasing in size (8 patients) or the tumour was static in size despite 6 months treatment with tamoxifen (6 patients). Little difference was noted between the two groups for expression of p53, c-myc and c-erbB-2 genes (table 15). However, TGF-ß overexpression, low levels of pS2 expression and pEJ overexpression were features of the growing tumours but normal TGF-ß expression and pEJ expression and high pS2 expression features of the tumours static in size during tamoxifen treatment (table 15). It should be noted that mRNA quantification was only performed where the Northern blot autoradiographs were of good quality.

Table 15 : Gene expression in tumours from patients treated with tamoxifen prior to surgery.

PROBE		p53	c-myc	c-erbB-2	pEJ	TGF-ß	pS2
(No. tumours examined)		(14)	(13)	(14)	(14)	(11)	(14)
Tumour increasing in size	0	7	4	7	2	-	7
	N	1	-	-	2	-	-
	+	-	3	1	4	6	1
Tumour static	0	1	3	5	3	-	2
	N	4	2	-	3	4	-
	+	1	1	1	-	1	4

0 No gene expression detected
N Gene expression similar to that found in normal breast tissue
+ High level of gene expression

Preoperatively systemically treated patients

In tumour samples from 12 patients, mRNA was extracted from tissue obtained at biopsy prior to preoperative systemic therapy and following endocrine or chemotherapy treatment. Gene expression was compared in the paired samples (table 16).

Patients were grouped according to gene expression or response to therapy. No definite association was seen when comparing the pre-treatment to the post-treatment mRNAs detected either quantitatively or qualitatively.

Table 16 : Gene expression in pre-treatment and post-treatment tumour samples.

SPECIMEN NUMBERS	mRNA EXPRESSION DETECTED PRETREATMENT	POSTTREATMENT	TREATMENT	RESPONSE
63/73	Nil	Nil	Chemotherapy	Static
22/60	Nil	Nil	Endocrine	Progression
48/62	c-myc,p53	Nil	Chemotherapy	Static
80/94	c-myc,TGF-ß	Nil	Endocrine	Static
53/83	c-myc,pS2,TGF-ß	Nil	Endocrine	Response
81/93	c-myc,pEJ,TGF-ß	Nil	Endocrine	Static
Same levels pre and post treatment:				
54/67	c-myc,TGF-ß	c-myc,TGF-ß	Chemotherapy	Static
21/59	pS2	pS2	Chemotherapy	Response
64/82	c-myc,TGF-ß, p53,pEJ.	c-myc,TGF-ß, p53,pEJ.	Chemotherapy	Response
Change in levels pre and post treatment:				
35/71	Nil	Normal p53, c-myc	Endocrine	Response
52/68	High p53, pS2,pEJ	No p53, Normal pS2 High pEJ,c-myc	Endocrine	Static
56/70	Normal p53 high pEJ,c-myc	No p53 Normal pEJ High c-myc	Chemotherapy	Response

DISCUSSION

General considerations

Gene expression :

- c-myc
- c-erbB-2
- H-ras
- TGF-ß
- pS2
- p53

Chromosome 17p13

Patient prognosis

Mouse xenograft system

- mRNA differences *in vivo* and *in vitro*
- Changes in mRNA after stimulation with oestrogen
- Clinical implications
- Changes in mRNA with tamoxifen administration

TGF-ß and tamoxifen

Sequential tumour samples

Conclusions

Future prospects

General considerations

The work presented in this thesis examined primary breast cancers from a consecutive group of patients in whom there was sufficient material available both for routine diagnostic purposes and for experimental study. Therefore, some degree of patient and tumour selection has occurred in that impalpable cancers detected by mammography at breast cancer screening and tumours of less than 0.5 cm diameter have not been examined. However, the group of 80 patients of 240 presenting for surgery within the 18 month study period is a representative cross section of the disease spectrum presenting, with palpable disease, to a specialist breast cancer unit and is a higher proportion than in other studies from this unit (Miller et al., 1990). The control tissue examined for gene expression, reduction mammoplasty breast tissue, tonsil, normal uterus and ovary are representative of a variety of normal adult human tissues.

The method of RNA extraction used, differential precipitation by lithium salt, is now widely established as an excellent technique for extracting intact, high quality total RNA which can then be used, without further purification to poly A+ RNA, for the detection of messenger RNA species (Henry et al., 1988). The use of laser densitometry to quantify mRNA species with respect to a standard mRNA, of which actin is a well tried

example, has now supplanted the earlier technique of dot blotting in which serial dilutions of test RNA are probed (Travers et al., 1988) and has the immense advantage that the sizes and integrity of the mRNA species of interest can be individually assessed. Furthermore, the use of nylon filters allows more ready reprobing of northern blots, essential for work such as this where a limited amount of tissue and nucleic acid is available.

The DNA extraction, digestion, Southern blotting and probing techniques are now considered to be standard reliable methods for the detection of allele losses, representing loss of genetic material, from the chromosomal loci under examination (Vogelstein et al., 1989). By contrast, the methods used to examine the sequence of part of the p53 gene required considerable adaptation from the techniques for the polymerase chain reaction (Siaki et al., 1985a) and direct sequencing of double stranded DNA (Winship et al., 1989).

However, it should be noted that the tissues examined using these molecular techniques may contain stromal cells (fibroblasts, normal breast cells and adipocytes) and haemopoietic cells (lymphocytes, monocytes, polymorphs) in addition to breast cancer cells. It is possible that these contaminant normal tissues can express the genes under consideration. Given the high proportion of breast cancer cells and the absence of visible lymphocyte infiltrates in the tumours, the genes

detected in this work, as in previous studies (Mariani-Constantini et al., 1988, Travers et al., 1988), reflect predominantly the breast cancer cell expression. Conversely, it is conceivable that normal tissues may "dilute" the gene expression or allele losses identified (Slamon et al., 1989). Two factors suggest this is unlikely; first, the tissues from which nucleic acids were extracted were examined by a pathologist to ensure the predominance of tumour cells. Second, 100g of reduction mammoplasty (normal) breast tissue was required to yield the same amount of nucleic acid as 0.2g of tumour and indeed some workers have been unable to extract adequate samples of RNA from normal breast tissue (Mariani-Constantini et al., 1988).

The work incorporated into this thesis has therefore involved adaptation of the most modern molecular approaches to the study of a common, aggressive disease, breast cancer.

The decision to target the expression of specific genes for detailed examination on the basis of the rapidly evolving literature, has been justified and yielded data of considerable interest. Each of the genes examined: c-myc, c-erbB-2, H-ras, TGF-ß, pS2 and p53 merits individual consideration.

Gene expression

c-myc

The c-myc mRNA of 2.5kb detected in this work corresponds to that previously reported in MCF-7 cells (Zajchowski et al., 1988), the 2.3 kb to 2.7 kb mRNA for c-myc described in a variety of human tissues (Alitalo et al., 1983, Ar-Rushdi et al., 1983, Guerin et al., 1985, Erisman et al., 1985, Escot et al., 1986, Dubik et al., 1987, Monnat et al., 1987 and Finley et al., 1989) and confirms that c-myc overexpression is a feature of breast as well as other malignancies (Marcu, 1987).

The detection of mRNA for c-myc in 54% (41 of 76) tumours examined, represents a somewhat lower proportion than the 10/14 (Escot et al., 1986) and 87% of 23 breast tumours (Whittaker et al., 1986) previously described. This could be due to the examination of small numbers and a highly selected tumour population in the earlier studies. Certainly the high constitutive expression of c-myc in MCF-7 and T-47D (Escot et al., 1986) and the MDA MB 231 cell line (Dubik et al., 1987) were confirmed, the weight of tumour and cell line evidence thus arguing against the assertion of Kozbor & Croce (1984) that overexpression of c-myc mRNA in breast cancer was likely to be an unusual occurrence.

This study did not address the questions of transcriptional control or DNA changes in the c-myc gene.

Transcriptional control of c-myc is particularly complex, with multiple regulatory elements involved (Lang et al., 1988). Translocation of the c-myc gene (as in Burkitt's Lymphoma, Dalla-Favera et al., 1982, (Croce, 1986) and colorectal cancer, Alitalo et al., 1983) renders the gene unresponsive to normal transcriptional control. However, structural alterations in c-myc in small cell lung cancer (Kok et al., 1989) are thought to be secondary events linked to tumour progression. In any event, in breast cancer, high levels of c-myc mRNA do not require alterations in the c-myc gene (Escot et al., 1986) and may (Mariani-Constantini et al., 1988) or may not (Dubik et al., 1987) be related to amplification of the gene. Thus the detection of c-myc gene overexpression in human breast cancer is likely to be more important than the possible structural mechanisms which could have brought it about and which were not examined (Lang et al., 1988). While it has been claimed that elevated expression of exogenous c-myc is insufficient for transformation of established, but not late passage fibroblasts (Zerlin et al., 1987, Karn et al., 1989), SWB13-5 breast cancer cells with high c-myc expression, but not those with low expression, are tumorigenic (Lavialle et al., 1988) and 80% of transgenic mice with augmented mammary gland c-myc expression develop mammary cancer as early as 2 months after birth (Schoenenberger et al., 1988).

Just as the question of whether c-myc expression alone is

sufficient for mammary carcinogenesis remains open, the precise function of overexpressed c-myc remains speculative. Cells which overexpress c-myc may continue to proliferate despite the depletion of serum growth factors (Mulder & Brattain, 1988) with promotion of DNA replication (reviewed in Parkin & Sonenberg, 1989), shortening of the cell cycle (Karn et al., 1989) and inhibition of terminal differentiation (Dmitrovsky et al., 1986) all key factors.

The work presented in this thesis has demonstrated that overexpression of the c-myc gene is associated with small rather than large tumour size and with lack of metastasis to the regional lymph nodes. This suggests that c-myc overexpression may be a feature of breast tumorigenesis, perhaps being important in rapid early growth, accounting for the association with small tumour size rather than disease progression. In support of this, Spandidos and collegues (1987) demonstrated that high expression of c-myc protein could be identified in both in situ and invasive breast cancer; in colorectal cancer, they also identified overexpression of c-myc in both the cancer and precancerous polyps. While no correlation between c-myc expression and node status was found in an earlier study (Whittaker et al., 1986), other published evidence has suggested that c-myc expression correlates with nodal metastasis (Guerin et al., 1985) and the analogous n-myc expression in neuroblastoma certainly correlates with

poor prognosis (Grady-Leopardi et al., 1986). As already noted, c-myc overexpression may be a primary event with secondary amplification of the gene subsequently occurring, as in small cell lung cancer (Kok et al., 1989).

The association between c-myc amplification and large tumour size (Yokota et al., 1986a, Seshadri et al., 1989), poor histological grade (Nikiforova, 1988), tumour metastasis and recurrence (Lidereau et al., 1988) and poor short term prognosis (Varley et al., 1987a) therefore lends credence to the concept that amplification of the gene rather than overexpression may play a role in tumour progression. However, evidence from this work does suggest that c-myc overexpression may play a part in the early stages of breast tumorigenesis.

c-erbB-2

The mRNA for c-erbB-2 detected in 21/71 (30%) of tumours was identical in size to the 4.8kb transcript identified in previous studies (Coussens et al., 1985, Semba et al 1985, van de Vijver et al., 1987) and similar to that noted in others (4.4kb Slamon et al., 1989; 4.6kb Yamamoto et al., 1986, Yokota et al., 1988; 5kb King et al., 1985, Kraus et al., 1987). The present data confirm that c-erbB-2 protein is detected only where the mRNA is overexpressed (Berger et al., 1988) and that there is a very strong correlation between detection of

the mRNA for c-erbB-2 and immunohistochemical detection of the protein (Slamon et al., 1987, Slamon et al., 1989). However, as Slamon and colleagues point out, there may be loss of immunoreactivity during tissue fixation accounting for failure to detect c-erbB-2 protein at lower levels of gene expression.

Despite the good correlation between c-erbB-2 mRNA expression and c-erbB-2 gene amplification (van de Vijver et al., 1987, Kraus et al., 1987, Yokota et al., 1988, Slamon et al., 1989) there remains a clear group of tumours (18 of 187, Slamon et al 1989) in which there is overexpression of the gene at the mRNA and protein level but no c-erbB-2 gene amplification. This suggests that as for c-myc, amplification is not a prerequisite for overexpression of the c-erbB-2 gene. In addition, activating mutations in c-erbB-2 are rare in breast cancer (Garcia et al., 1989, Lemoine et al., 1990). Thus measurement of mRNA for c-erbB-2 remains the most sensitive method of evaluating the contribution of this gene to breast carcinogenesis.

Reports have suggested that c-erbB-2 mRNA expression correlates with advanced disease stage (Zhou et al., 1987, Seshadri et al., 1989) and immunohistochemical detection of c-erbB-2 protein with high tumour grade (Parkes et al., 1990) and node involvement with tumour (Berger et al., 1988). In this study, expression of c-erbB-2 mRNA did not correlate with any clinical or

pathological variable. It is interesting to note that c-erbB-2 expression is more frequently observed in non-invasive than invasive breast cancer (van de Vijver et al., 1988), perhaps providing a selective growth advantage for the cancer cells (Yamamoto et al., 1986).

H-ras

The 1.4 kb mRNA for H-ras detected in the breast tumours is similar in size to that identified in previous studies (Spandidos and Agnatis 1984, Slamon et al., 1984, Theillet et al., 1986, Biunno et al., 1988). This work confirms that a substantial proportion of breast cancers express H-ras, whether it is detected at the mRNA level (16/22, Theillet et al., 1986) by radioimmunoassay (66%, Horan-Hand et al., 1987) or using antibodies on histological sections (30%, Walker & Wilkinson, 1988) contrary to the findings of others (Whittaker et al., 1986, Biunno et al., 1988) who were unable to detect mRNA for H-ras in either normal or cancerous breast tissue.
In this work, no correlation was found between H-ras mRNA expression and the clinical and pathological parameters examined although immunohistochemical staining for p21, the H-ras protein, is associated with large tumour size and spread of the cancer to the regional lymph nodes (Lundy et al., 1986). Certainly, the detection of elevated levels of p21 is associated with the malignant

phenotype in breast cells (Horan-Hand et al., 1984, Ohuchi et al., 1986, Whittaker et al., 1986) and therefore the lack of clinical or pathological correlation in this study would fit with the concept that H-ras may provide a competence function (Kelekar & Cole, 1987). However, in the present study, no attempt has been made to differentiate between normal and mutant H-ras although it has been suggested that elevated expression of the normal H-ras allele may cause suppression of morphologically transformed and tumorigenic phenotypes (Spandidos & Wilkie, 1988). In addition, it has been demonstrated that H-ras allele loss predicts a poor short term prognosis (Garcia et al., 1989). Neither has the work presented here examined activation or amplification of the H-ras gene, although activation by mutation occurs very infrequently in breast tumours (Biunno et al., 1988, Rochlitz et al., 1989) and amplification is equally rare (Biunno et al., 1988).

TGF-ß and pS2

Detailed consideration of the implications of TGF-ß and pS2 gene expression is discussed below but, in brief, the 2.5kb mRNA for TGF-ß was of the size previously reported (Derynck et al., 1985, Knabbe et al., 1987, Travers et al., 1988, King et al., 1989). However, the minor TGF-ß species reported elsewhere of 1.9 kb (Derynck et al., 1986, Akhurst et al., 1988) or 4.8 kb (Derynck et al.,

1986) were not demonstrated. TGF-ß mRNA expression was found in all the tissues tested as previously described, with higher levels in the tumours (Derynck et al., 1987) and abundant expression of TGF-ß noted in 45 of the 56 tumours examined in this study, similar to the 76% of tumours examined by Travers et al (1988).

Similarly, the 600 base pair mRNA for pS2 confirms that described originally (Masiakowski et al., 1982) and found here in 33% of cancers, less than the the 46% (Luqmani et al., 1989) and 57% (Henry et al., 1990) previously reported. However, this work confirms that there is a correlation between oestrogen receptor protein and pS2 mRNA expression but not with menopausal status or the presence of nodal metastasis (Henry et al., 1990).

p53

A 2.8kb mRNA for p53 was detected in 43 of the 76 breast cancer specimens. This corresponds to the mRNA for p53 identified in previous studies of human tissue (Harlow et al., 1985, Lamb & Crawford, 1986, Buchman et al., 1988, Baker et al., 1989, Takahashi et al., 1989, Lubbert et al., 1989, Lavigeuer et al., 1989). The quantitative difference between individual tumours in p53 mRNA expression raises the possibility, discussed later in this thesis, that the p53 gene may fulfil different functions in different patients. The low level of p53 expression detected in normal breast, tonsil, ovary and

uterus confirmed reports that p53 is expressed at low levels in most adult tissues (Rotter 1983, Oren, 1988). High expression of the mRNA for p53 in the 3 breast cancer lines (MCF-7, T-47D, MDA MB 231) was clearly demonstrated in accordance with the high expression noted in other cell lines (De Leo et al., 1979, Rotter et al., 1981, Crawford et al., 1981, Benchimol et al., 1982).

If one considers the qualitative differences between the tumours and cell lines, the three p53 mRNAs of circa 2.8kb in size found in different quantities in each of the cell lines may result from multiple transcription initiation sites (frequently associated with the known absence of an upstream TATA box as for the p53 gene, Bienz-Tadmor et al., 1985), differential splicing (which could give rise to an altered protein with different biological properties, Reisman & Rotter, 1989) or other post-transcriptional modification. A similar situation has been found in lung cancer, where cell lines derived from lung tumours express 2.3, 2.6, and 3.7kb species in addition to the 2.8kb mRNA also found in normal lung tissue (Takahashi et al., 1989).

The 1.6kb mRNA (still large enough to encode a 53kd protein) could reflect reading frame differences and is similar in size to the single p53 mRNA species of 1.8kb previously described in NIH3T3 cells (Reich et al., 1983, Reich & Levine, 1984). This 1.6kb mRNA may correspond to the nuclear mRNA regulating translation detected by

Khochbin & Lawrence (1988) or the unusually stable 44kd p53 that accumulates to high intracellular levels (Rovinski et al., 1987).

The three cell lines examined are distinguishable on karyotype (Mr. G. Spowart, MRC Human Genetics Unit, Western General Hospital, Edinburgh, personal communication) on molecular analysis (using DNA probes; Dr J. Morten, ICI diagnostics, Macclesfield, Cheshire, personal communication) and they have different phenotypic characteristics; for example the MCF-7 line used in these studies had on average 120 fmol of oestrogen receptor protein mg^{-1} total protein, T-47D 40 fmol mg^{-1} and MDA-MB-231 0 fmol mg^{-1}.

These lines also show differing sensitivity tO oestrogens and antioestrogens. The consistently high level of p53 mRNA expressed in all three lines thus implies that, in this in vitro setting and by contrast with the clinical material, p53 mRNA expression is independent of oestrogen receptor protein content of the cells and of hormone sensitivity.

Cattoretti and colleagues (1988), using an antibody PAb1801 specific for human p53 protein in breast cancer specimens, noted a correlation between oestrogen receptor-negative tumours and elevated p53 protein expression ($p<0.05$). Using this antibody, no other significant correlation was identified, but with a mouse antibody, PAb 421, p53 staining correlated with other

additional poor prognostic parameters. Although the regulation of p53 expression may occur not only at the level of mRNA abundancy but also p53 protein stability, depending on the system under study (Rovinski et al., 1987), in both embryonal carcinoma cells (Reich et al., 1983) and in several tissues in transgenic mice (Lavigueur et al., 1989) p53 mRNA and p53 protein expression correlate.These results therefore agree with our findings in relation to p53 mRNA: p53 both at the protein and mRNA levels is associated with a low level of oestrogen receptor protein. If one considers oestrogen receptor protein to be a "luxury" protein, produced only by biochemically well differentiated cells, then those tumours expressing little or no oestrogen receptors, which also tend to have high p53 mRNA expression, may be at an early stage of differentiation.

Discussion of p53 gene expression leads on to considerations of DNA changes in the chromosomal material around the p53 gene on chromosome 17 in band 13.

Chromosome 17p13

The DNA studies confirm that the loss of one YNZ 22.1 allele from the short arm of chromosome 17 occurs in over half of the breast tumours studied (Mackay et al., 1988, Devilee et al., 1989a, Devilee et al., 1989b) and suggests for the first time that this allele loss

may be associated with low levels (under 20 fmol mg^{-1} protein) of oestrogen receptor protein. The tip of the short arm of chromosome 17 is thus of importance in breast as well as in colon cancer (Lothe et al., 1989, Vogelstein et al., 1989) where allele loss from 17p plays a role in the development of a major subset of colorectal tumours and also in a high proportion of other tumours such as lung cancer (Yokota et al., 1987, Takahashi et al., 1989), astrocytoma (James et al., 1989, Fults et al., 1989) and osteosarcoma (Toguchida et al., 1989).

The increasing allele loss (of YNZ and MCT) towards the tip of 17p13 suggests there may be a regulatory element for p53 in this region, p53 mRNA overexpression correlating as it does with YNZ allele loss. It is also possible that there may a second tumour suppressor gene in the region 17p13, although in colorectal cancer this does not appear to be the case (Baker et al., 1989). This issue is discussed more fully after consideration of the role of p53 itself.

The low level of polymorphism detected with the cDNA probe for p53 (Zakut-Houri et al., 1985) was as expected. However, although pBHp53 allele loss was not associated with menopausal status, Devilee et al (1989a) have suggested that patients with 17p allele loss have a high risk of early onset, bilateral, multifocal disease of familial type.

The possible roles for p53 merit discussion. Evidence from this thesis supports the proposal that p53 may behave either as a tumour suppressor gene or as an oncogene in breast cancer (Lane & Benchimol, 1990).

In those tumours where no p53 mRNA was detected, this may reflect deficiency of normal (unmutated) p53 and hence a reduced tumour suppressor function conferring a selective advantage for the development of the tumorigenic phenotype (Finlay et al., 1989). Supporting evidence for this comes from the observation that in 3 of the 4 in situ cancers, no p53 expression was detected. Thus, one "hit" along the carcinogenic pathway may be to stop expression of the p53 tumour suppressor gene as was found in myeloid leukaemias (Reisman & Rotter, 1989).

p53 gene deletion has also been reported in several mouse erythroleukaemia cell lines, and in this setting p53 has been likened to the retinoblastoma tumour suppressor gene (Mowat et al., 1985, Green, 1989). Indeed the similarities between retinoblastoma and p53, which both appear to be required for normal cellular growth, may merit study in the same tumours (Lane & Benchimol, 1990). Deletion of one copy of the p53 gene is also compatible with increased function of the other (abnormal) gene, perhaps enhancing the transformation process in concert with another oncogene. Thus, where there is normal or increased p53 mRNA expression, this may be of a mutated form (for example the point mutation identified in

patient 43) which therefore blocks the activity of any remaining wild type tumour suppressing p53 (Green, 1989) and/or acts as an oncogene, promoting carcinogenesis. Certainly, mutant p53 has a dramatically increased half life (typically 4 to 8 hours) compared to normal, wild type p53 (6 to 20 minutes) (Reihsaus et al., 1990, Lane & Benchimol, 1990). Mutant p53 also has altered protein and antibody binding characteristics with antibody binding further increasing stability (Reihsaus et al., 1990), rendering it less vulnerable to proteases, altered phosphorylation and an additional (extranuclear) cellular location (Jenkins et al., 1985, Sturzbecher et al., 1987, Oren, 1988, Van Roy et al., 1990).

Single tumorigenic point mutations, clustered in highly conserved regions of the p53 gene (Soussi et al., 1987, Nigro et al., 1989), particularly between amino acids 135 and 218 (Finlay et al., 1989, Baker et al., 1989) can account for the differences in tumour suppressor versus oncogene function of p53 (Matlashewski et al., 1987). In support of this, p53 point mutations have been described in colon cancer (Baker et al., 1989) 57% of lung cancer cell lines (Takahashi et al., 1989) and in two breast cancer cell lines but not in the single breast cancer examined which was homozygous for 17p (Nigro et al., 1989). When these mutants are overexpressed, they may act in a trans-dominant fashion to inactivate the endogenous

wild type p53, possibly by the formation of nonfuctional multimeric complexes (Rovinski & Benchimol, 1988, Eliyahu et al., 1988, Finlay et al., 1989) in a similar way to SV40 large T antigen, Adenovirus E1A and Human papilloma virus E6 protein (Lane & Benchimol, 1990). In support of this, wild type p53 suppresses co-transformation of REF cells by mutant p53 and ras (Finlay et al., 1989). However, it has been suggested that some additional oncogene activity of the mutant p53 may be required and only fully manifest in the absence of normal wild type p53 (Lane & Benchimol, 1990).

Alternatively, loss of one allele (resulting in failure to express detectable quantities of the p53 gene as found in three of the in situ cancers) may confer a minor growth advantage, with subsequent mutation of the remaining p53 required to initiate or promote carcinogenesis.

If mutation were to occur first, loss of the normal allele may be required to allow effective expression of the mutant p53, by analagy with co-transfection studies of normal and mutated H-ras (Spandidos & Wilkie, 1988). However, in the present work, the finding that in 2 of the 4 in situ breast cancers there was 17p deletion but no p53 expression suggests that loss of p53 tumour suppressor gene function (deletion) may be the first event and p53 mutation the second on the carcinogenic pathway.

Nigro and collegues (1989) have suggested the converse; they found that some tumours had mutant p53 but no allele loss although most tumours with 17p deletions contained mutant p53 in the allele that was retained. In the work presented here, one cancer, but not both of the tumours with p53 overexpression, had a mutation within exons 5 and 6 of the gene. This could be explained if there were other sites of important mutation in the p53 gene (exons 7,8 and 9) or possibly a second tumour suppressor gene on 17p13 or even if the p53 gene and 17p allele losses are co-incidental to important genetic abnormalities elsewhere (Nigro et al., 1989). However, the data presented here for breast cancer demonstrate a parallel with lung cancer (Takahashi et al., 1989) where 17p deletions and p53 mutations implicate p53 as an important tumour suppressor gene, the disruption of which is involved in the pathogenesis of cancer.

p53 abnormalities alone are insufficient to explain the aetiology of breast cancer as co-transformation and immortalisation studies with mutant p53 (Parada et al., 1984, Eliyahu et al., 1984, Eliyahu et al., 1985, Hinds et al., 1987) but not wild type p53 (Finlay et al., 1988) have demonstrated.

To summarise, loss of p53 expression may represent loss of tumour suppressor gene function (Lavigueur et al., 1989). Expression of mutant p53, either acting directly as an oncogene or indirectly by complexing with wild type

p53 may occur to render the cell susceptible to uncontrolled growth (Finlay et al., 1989).

The association between allele loss with probe YNZ 22.1 and overexpression of the p53 gene merits further consideration in that the tip of 17p13 may contain regulatory elements for p53 gene expression. This possibility is not excluded by the demonstration that the DNA 5' to the p53 gene is a conserved promotor region capable of negative transcriptional control (Bienz-Tadmor et al., 1985) and that there are 2 promotors, one upstream of exon 1 and the second within the first intron (Reisman & Rotter, 1989). In fact, full p53 gene promotor activity may reside within exon 1 of the p53 gene (Tuck & Crawford, 1989) and the DNAase I hypersensitive sites at the transcription initiation site may represent areas regulated by transacting proteins (Lubbert et al., 1989). Despite this evidence for gene regulatory elements close to the p53 gene, cis-acting or trans-acting loci near the tip of 17p13 or even more distant loci may be important. Further, initial data suggest (Devilee et al., 1990) that more detailed deletion mapping of 17p may demonstrate a common region of deletion and target regions of 17p, in addition to the p53 locus, worthy of further study.

Other future strategies may include screening all the tumours for p53 mutations with the amplification mismatch technique (Montandon et al., 1989). This may then be followed by sequencing all the important regions of the

p53 gene (particularly exons 5,6,7,8 and 9) either by direct DNA sequencing as described in this work, or using an asymetric polymerase chain reaction technique from mRNA (Iggo et al., 1990).

Prognosis

While at a mean 18 months follow up it remains too early to determine the full prognostic significance of the data, three pointers merit consideration even at this early stage.

Failure to detect pS2 mRNA expression appears to correlate with early disease recurrence, although the statistical significance of this finding remains less dramatic than for conventional prognostic indices such as tumour stage or involvement of regional lymph nodes with tumour. However, pS2 is oestrogen regulated and it is therefore of interest that failure to detect pS2 mRNA appears to be a more powerful predictor of early disease recurrence than oestrogen receptor protein content of the tumours. If this observation is carried through to 3 and 5 years follow up, it will indicate a group of patients with breast cancer who are unlikely to respond to endocrine therapy (Henry et al., 1990) but who should be offered additional systemic therapy. Eleven of 76 patients for whom p53 mRNA expression was determined had tumours showing loss of heterozygosity for YNZ 22.1,

increased expression of p53 mRNA and low levels of oestrogen receptor protein. With just 18 months mean follow up, four of these patients have already relapsed with metastatic disease. Continued follow up of the whole cohort will establish the prognostic significance of this combination of findings.

One might expect those patients with detectable c-erbB-2 mRNA to have a poor prognosis since it is now well established that c-erbB-2 amplification (which correlates well with mRNA overexpression, Slamon et al., 1989) and strong antibody staining for c-erbB-2 is an independent prognostic factor for shorter disease free interval and shorter overall survival (Slamon et al., 1987, van de Vijver et al., 1987, Berger et al., 1988, Slamon et al., 1989, Wright et al., 1989, Zeillinger et al., 1989, Ro et al., 1989, Thor et al., 1989). However, in the present group of patients, at 18 months follow up no prognostic value was observed for c-erbB-2 mRNA detection. Even if the relation between c-erbB-2 and poor prognosis is confirmed at 3 or 5 years follow up in the present group of patients, c-erbB-2 may prove to be less useful in clinical practice than pS2 since the c-erbB-2 gene is only amplified or overexpressed in a small minority of tumours (Barnes, 1989) and, unlike pS2, gives no indication of the most suitable adjuvant therapy.

Mouse Xenograft System

The MCF-7 breast cancer cell line has been established for the first time as a renewable xenograft system in CBA mice immunocompromised by thymectomy and whole body irradiation, a system previously used to test the tumorigenicity of lymphoblastoid cell lines (Morten et al., 1984). Tissue from these tumours can be transplanted to similar mice and cells cultured from the xenografts can be re-implanted to grow tumours. The use of an anatomically appropriate site for xenograft growth does not appear to be important in this CBA mouse system, unlike "nude" nu/nu mice (Price et al., 1990). This model therefore yields a large renewable supply of tumour material passaged *in vivo* and permits the *in vivo* study of tumours during hormonal manipulation. These MCF-7 tumours were clearly adenocarcinomata, with necrosis in only the larger tumours, as in nude mice (Osborne et al., 1988) and metastasis, as previously noted, a rare event (Busuttil et al., 1986).

As in nu/nu mice, oestrogen supplementation is a pre-requisite for MCF-7 tumour growth (Shafie & Grantham, 1981; Osborne et al., 1985; Gottardis et al., 1988). The absence of detectable serum oestrogen in female mice prior to injection confirmed that the mice had been "oophorectomised" by the irradiation. The serum oestrogen profile following a single subcutaneous injection of

oestradiol benzoate in arachis oil gave very high serum levels of oestradiol between 12 and 36 hours. From day 2 to day 8, levels of oestradiol were within the physiological range for non-pregnant premenopausal women (110-1630 pMol L^{-1}, data from Baxter Health Care, Newbury, U.K.) and they remained sufficient to sustain oestrogen-dependent tumour growth for 21 days. Serum oestradiol levels in surgically oophorectomised nude mice which have received the implantable pellet also fall within the same range (Shafie & Grantham, 1981, Blumenthal et al., 1988). There is no obvious explanation for the intolerance of the CBA mice to the implantable pellet, although the findings reported here comfirm previous experience with this strain in the Institute of Animal Technology, Western General Hospital, Edinburgh where this work was performed.

Cell proliferation as measured by DNA synthesis and mitotic activity of the tumour cells in response to oestradiol was similar to that observed in nude mice (Osborne et al., 1985) and, as expected, preceded the increase in tumour volume.

Thus, the CBA mouse xenograft system has similarities to the "nude" mice used previously for breast cancer studies, but has distinct advantages of low cost and ease of husbandry. These are important considerations where substantial numbers of animals are involved and daily handling for accurate measurements is a necessary part of

the protocol. Moreover, the characteristics of the model allow the ready study of molecular events in an oestrogen sensitive breast cancer system.

mRNA differences in vivo and in vitro

The original MCF-7 cells grown in vitro expressed several mRNA species not evident in the xenografts or in cells of these xenografts recultured for some time in vitro. If one can exclude contamination of the in vitro cultures, these findings suggest selection in vivo of a subpopulation of cells from the MCF7 culture.

The MCF-7 cells re-cultured from xenografts had an identical pattern of gene expression to the xenografts and did not, over an 8 week period, revert to the original MCF-7 cell line pattern. Serious consideration was given to the possibility that these findings could be due to a contaminant in the original cell line (such as mycoplasma) expressing the gene concerned or to plasmid contamination at some point in the RNA extraction or electrophoresis. Both these explanations are unlikely since tests on the MCF-7 culture (Barile, 1973) were persistently negative for mycoplasma, and no evidence of plasmid contamination was found in these or any other northern-blot RNA studies. Moreover, a range of different plasmids was used as vectors for the cDNA probes.

It therefore seems most likely that passage of the MCF-7 cells through mice does indeed select for a tumorigenic

subpopulation.

Using the oncogene probes to study the RNA from MCF-7 grown *in vitro*, 3 distinct mRNA species of 1.8kb, 2.8kb and 3.0kb were identified hybridising to the c-erbB-2 probe, although these were not present in the xenografts nor in recultured cells. These species were smaller than the single 4.8kb mRNA species previously described (Semba et al., 1985), detected in the human tumours and closer to the truncated 2.3kb transcript detected in MKN-7 gastric cancer cells (Yamamoto et al., 1986, Yokota et al., 1988). Moreover, no mRNA for erbB-2 of aberrant size was identified in a survey of breast cancer cell lines (Kraus et al., 1988). An amplified and rearranged epidermal growth factor (erbB-1) has been identified in epidermoid carcinoma cells generating a truncated 2.8kb mRNA that encoded only the extracellular EGF binding domain (Ullrich et al., 1984) and multiple mRNA transcription initiation sites have been demonstrated for c-erbB-2 (Tal et al., 1987). Thus, although increased expression of the c-erbB-2 gene has been found to provide a selective advantage in the formation of or progression of epithelial cell tumours (Yamamoto et al., 1986), this does not appear to be the case in the MCF-7 xenograft system developed here.

OR3, a probe for oestrogen receptor mRNA, detected two messages of 3.0 and 1.7kb, but no larger mRNA species. While mRNA of 6.3kb (Green et al., 1986, Rio et al.,

1987, Barrett-Lee et al., 1987, May et al., 1989), 6.2kb (Walter et al., 1985), 4.2kb (Parl et al., 1987) and 3.7kb (Barrett-Lee et al., 1987) have been reported in human tumour tissue and in the MCF-7 cell line, the species identified here do not correspond to any of these.

Henry and colleagues (1988) were unable to demonstrate oestrogen receptor mRNA in the MCF-7 cell line using this OR3 cDNA clone, but demonstrated hybridisation to a 6.2kb mRNA using their radionucleotide labelled riboprobe. The translated oestrogen receptor protein was certainly present when assayed by enzyme immunoassay. The slight rise in oestrogen receptor protein following oestrogen stimulation of the tumour, from 120 fmol mg^{-1} protein to 240 fmol mg^{-1} protein, and its subsequent return to basal level is consistent with the view that oestrogen can stimulate the synthesis of its own receptor.

The pS2 probe appears to cross-hybridise to the same 3kb and 1.7kb mRNA as the OR3 probe but hybridises most strongly to a small mRNA of about 600 base pairs, corresponding to the oestrogen induced mRNA of Masiakowski and collegues (1982).

Changes in mRNA after stimulation with oestrogen

The choice of internal control probe, actin, allowed demonstration of changes in transcription of the other genes studied, It is important to note that transcription of this standard (actin mRNA) is not influenced by oestrogen (Saceda et al., 1988, Barton & Shapiro 1988). Oestrogen-induced stimulation of c-myc expression, previously noted in breast cancer cells in vitro (Dubik et al., 1987), was confirmed. Although this probably represents a real increase in transcription, factors such as increased stability of the c-myc mRNA (Dubik et al., 1987) or post-transcriptional regulation (Morello et al., 1989) of the gene may also occur. The expression of c-myc and also p53 at elevated levels in the xenograft tumours in response to oestrogen confirm that in vivo the expression of these two nuclear genes may be involved in cell cycling (Kelly & Seibenlist, 1985, Lamb & Crawford, 1986). Recent evidence using conditional alleles of c-myc and the hormone binding domain of the oestrogen receptor gene suggests that oestrogen may act by binding to its receptor then interact with c-myc, thus activating DNA polymerase and DNA synthesis (Eilers et al., 1989). Similarly, the rise in p53 expression following oestrogen stimulation may reflect a function of p53 as a modulator of growth factor gene expression through DNA transcriptional activation (Braithwaite et al., 1987).

In vitro work has previously identified an increase of pS2 mRNA in response to oestrogen within 3 hours (Brown et al., 1984). The rapid rise in vivo in pS2 mRNA transcription confirms in vitro observations that pS2 is oestrogen regulated at the level of transcription (Masiakowski et al., 1982, Brown et al., 1984). The biphasic change in pS2 observed in the xenograft system described here suggests that the role of pS2 as a marker for oestrogen action in vivo may not be as simple as originally proposed on the basis of in vitro studies, with the very high peak of serum oestradiol perhaps causing transient inhibition of pS2 transcription. While pS2 behaves as an epidermal growth factor-like immunoreactive factor when secreted by MCF-7 cells (Kida et al., 1989), purified pS2 protein does not stimulate cellular DNA synthesis and hence may not be crucial to the effect of oestrogen on MCF-7 cells (Kida et al., 1989).

The initial decrease in TGF-ß transcription demonstrated in the xenografts confirmed the original in vitro studies of the TGF-ß response to oestrogen treatment of MCF-7 cells (Dickson et al., 1986). In the present xenograft system, TGF-ß transcription increased as the mitogenic stimulus of oestrogen declined, confirming in vitro studies (Knabbe et al., 1987) and compatible with the antiproliferative effects of TGF-ß noted on oestrogen receptor-positive breast cancer cell lines in vitro (Kerr

et al., 1989, Arrick et al., 1990)

While H-ras was expressed by the MCF-7 xenografts, no change in H-ras transcription was demonstrated, confirming that ras transcription is insensitive to the effects of oestrogen on MCF-7 (Dubik et al., 1987).

Clinical implications

Gene expression in the breast cancer cells, as detected by mRNA analysis, obviously changes when cells cultured in vitro grow as tumours in vivo. The physiological and clinical significance of in vitro observations have on occasion been controversial and may be difficult to interpret due to lack of host-related determinants that affect tumour behaviour in vivo (Shafie & Grantham, 1981). Certainly, different effects on cell kinetics have been observed using MCF-7 cells in vitro compared to nude mouse xenografts (Brunner et al., 1989).

The model described here provides information complementary to that obtained from in vitro work and from clinical studies, particularly in examining host - tumour cell interactions and in determining the role of gene expression in oestrogen-dependent breast tumour growth.

The MCF-7 xenografts in thymectomised and irradiated CBA strain mice therefore present a useful model for examining the in vivo behaviour of oestrogen-dependent breast cancer and has considerable potential to study the

mechanisms of action or failure of action of therapeutic agents *in vivo*. An illustration of this comes from studies of the effect on xenograft gene expression of the anti-tumour agent tamoxifen.

Changes in mRNA with tamoxifen administration

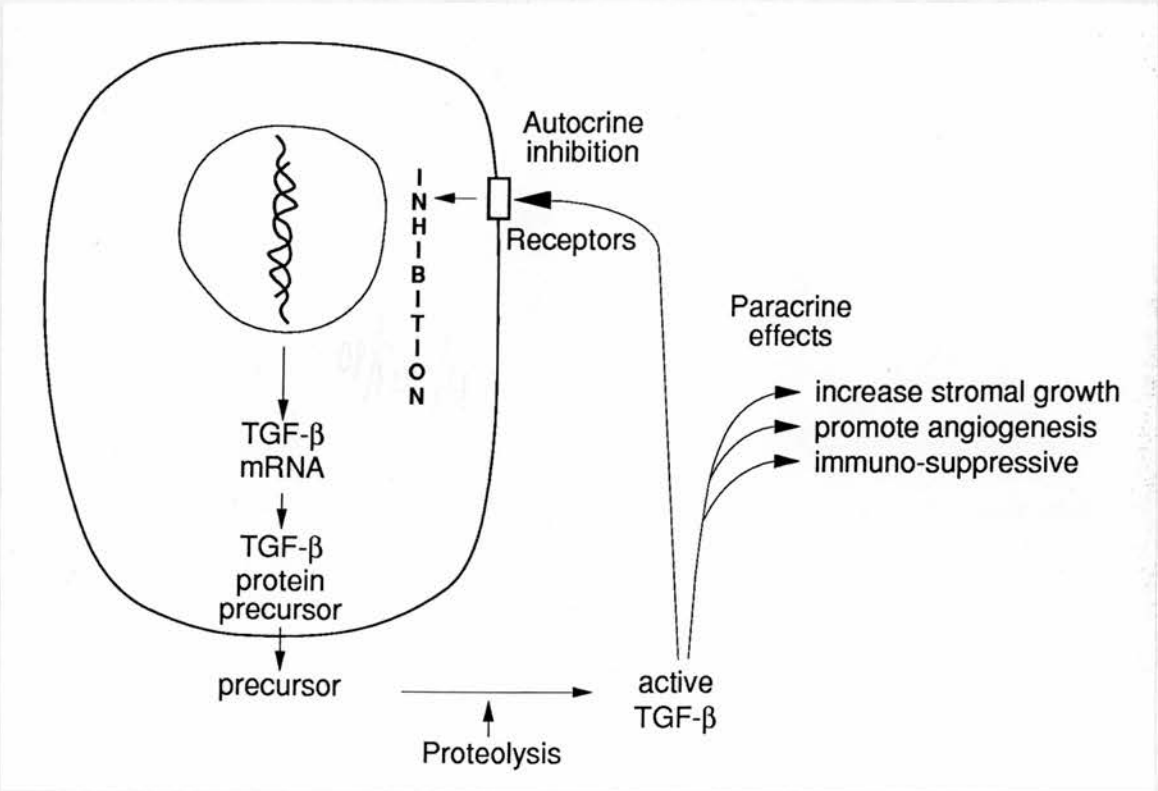
The sensitivity of MCF-7 cells grown as xenograft tumours to the antitumour effects of tamoxifen was independent of c-myc expression (Katsaros et al., 1988, Liu et al., 1989) or of p53 expression. The induction of pS2 in the xenografts by oestrogen was not antagonised by tamoxifen contrary to *in vitro* findings (May & Westley, 1987), possibly due to the excess of serum oestradiol during the first 24 hours. However, the effects of tamoxifen on breast cells are complex and other factors may well be involved (May & Westley, 1987, Johnson et al., 1989, Berry et al., 1989).

TGF-ß and tamoxifen

The MCF-7 oestrogen sensitive xenografts confirm the effect of tamoxifen on MCF-7 cells *in vitro* (Knabbe et al., 1987): oestrogen withdrawal leads to an increase in TGF-ß and decreased growth while tamoxifen treatment of the xenograft tumours results in a sustained rise in TGF-ß mRNA, associated with a reduction in tumour volume. Therefore, one might expect to find low levels of TGF-ß gene transcription in tumours growing despite tamoxifen

therapy, releasing the cells from proliferative constraints (Sporn & Roberts, 1985). However, high levels of TGF-ß mRNA and low levels of oestrogen receptor protein (Anderson et al., 1989) were found in this group of human breast tumours. By contrast, patients in whom tamoxifen therapy had induced tumour stasis (but not regression) had low levels of TGF-ß mRNA but oestrogen receptor protein of greater than 20 fmol mg^{-1} total protein. The association between high TGF-ß mRNA expression and failure of tamoxifen therapy may be coincidental given that these were oestrogen poor tumours. However, the lack of correlation between TGF-ß mRNA and oestrogen receptor protein, confirming a previous study (Travers et al., 1988), and the inhibitory effect of TGF-ß on oestrogen receptor poor MDA MB 231 cells (Knabbe et al., 1987) suggests that the association is not due purely to chance and in this situation TGF-ß induction is clearly not mediated through oestrogen receptors (Knabbe et al.,1987). The data are also unlikely to be due to a direct effect of tamoxifen on TGF-ß or due to TGF-ß degradation (Knabbe et al., 1987). The apparent tumour promoting effects of TGF-ß in these tumours may be due to an inability of the malignant cell to progress to an advanced state of differentiation in which it could respond to an inhibitory signal (Steel, 1989), as suggested by the poverty of oestrogen receptors in the tamoxifen unresponsive, progressing tumours.

Figure 12 : A diagramatic representation of the autocrine and paracrine pathways for TGF-ß.



The pathway for active TGF-ß production and the potential actions of TGF-ß in breast cancer tissue. TGF-ß may act by:
(i) the autocrine inhibitory pathway whereby TGF-ß produced by a breast cancer cell (as mRNA translated to protein precursor) is activated and acts via TGF-ß receptors to inhibit the cell
and/or
(ii) the paracrine stimulation of other cells and tissues or inhibition of immune response within the tumour.

The data suggest that escape from hormone dependence does not occur simply through failure of the cytokine release pathway.

The mechanism for this escape from the autocrine inhibitory effects of TGF-ß on the breast cancer cells (figure 12) may be due to failure to activate secreted TGF-ß (Hsaun, 1989) over 98% of which is secreted in inactive form (Wakefield et al., 1987) an established control point in MCF-7 in vitro (Knabbe et al., 1987). Alternatively cells may fail to respond to the negative stimulus (Roberts et al., 1985) or respond weakly (Parkinson, 1985) due to reduction or loss of receptor function (Roberts et al., 1988, Massague et al., 1988, Valverius et al., 1989). The tumour cells may be resistant to the growth inhibitory effects of TGF-ß as has been demonstrated in vitro (Valverius et al., 1989) perhaps due to a lack of functional TGF-ß receptors as occurs in retinblastoma (Kimchi et al., 1988). Alternatively, there may be a mechanism similar to that observed following viral transformation of cells, where increased TGF-ß secretion is associated with downregulation of the TGF-ß receptors in the same cells (Anzano et al., 1985). Although modulation of ligand-receptor binding may not be an important control mechanism in some systems (Wakefield et al., 1987), modulation of TGF-ß responsiveness may occur at the level of signal transduction (Valverius et al., 1989). TGF-ß

may even directly stimulate the breast cancer cells compatible with the *in vitro* observation that upregulation of TGF-ß mRNA is accompanied by apparent stimulation by the transcribed TGF-ß of T-47D cells rendered steroid insensitive (King et al., 1989).

The enhancement of stromal growth and angiogenesis (Roberts et al., 1986, Heine et al., 1987, Massague 1987, Sporn et al., 1987, Roberts et al., 1988) and the immunosuppressive effect of TGF-ß (Kerhl et al., 1986, Wrann et al., 1987, Sporn et al., 1987, Carel et al., 1990, Torre-Amoine et al., 1990) may also play a crucial role. Some combination of autocrine defect or paracrine effects on the supporting stroma may occur, as supported by the mRNA and protein distribution of epithelial derived TGF-ß (Akhurst et al 1988). The present study does not distinguish between the various isoforms of TGF-ß nor address the questions of TGF-ß activation and receptor function. Neither can one exclude interactions with other growth factors such as epidermal growth factor (Roberts et al., 1986, Fernandez-pol et al., 1987). A further indication that the *in vivo* situation may be more complex than *in vitro* studies have suggested is the significant correlation between high TGF-ß mRNA expression and pre-menopausal status. This may be explained by the local effects of TGF-ß, actively transcribed at a high rate in the breast tumour cells, dominating the effect of systemically circulating oestrogens which one might

expect from *in vitro* studies to inhibit TGF-ß transcription (Dickson et al., 1986).

Sequential tumour samples

In the small group of 12 tumours with both pre-treatment and post-treatment specimens, no distinct pattern of gene expression was detected to relate to the clinical behaviour of the disease. This may well be due to the small, heterogeneous nature of the group of 12 patients or the types of therapy administered. The measurement of expression of the multiple drug resistance gene might be of interest in a larger group of patients such as this.

Conclusions

This thesis presents three types of evidence for multiple genetic abnormalities occurring in breast cancer: oncogene overexpression (for example c-myc, c-erbB-2), tumour suppressor gene loss (YNZ allele loss, lack of p53 expression) and conversion of a tumour suppressor gene to an oncogene (by p53 mutational activation).

The data on gene expression in the 80 patients and the statistical correlations and associations with recognised clinical and pathological parameters suggest that overexpression of important genes (even of a normal rather than mutant gene) such as c-myc or c-erbB-2 may be significant pathogenic events in some tumours, possibly as early rather than late steps on the carcinogenic pathway. Furthermore, data on pS2 and TGF-ß expression suggest that direct measurement of expression of specific genes may give useful information for the clinical management of patients.

Those patients who do not express pS2 mRNA appear to have a comparatively poor short term prognosis. The breast cancer recurs at 18 months mean follow up in a significantly higher proportion of patients who fail to express the pS2 mRNA, even although there is no significant association between oestrogen receptor protein and early disease recurrence. The patients defined in this way may therefore from a group for whom

additional early adjuvant therapy would be particularly appropriate. The unexpected finding that TGF-ß mRNA is high in tumours taken from premenopausal women suggests that in vitro studies require support from direct investigation of patient material to confirm or refute in vitro findings.

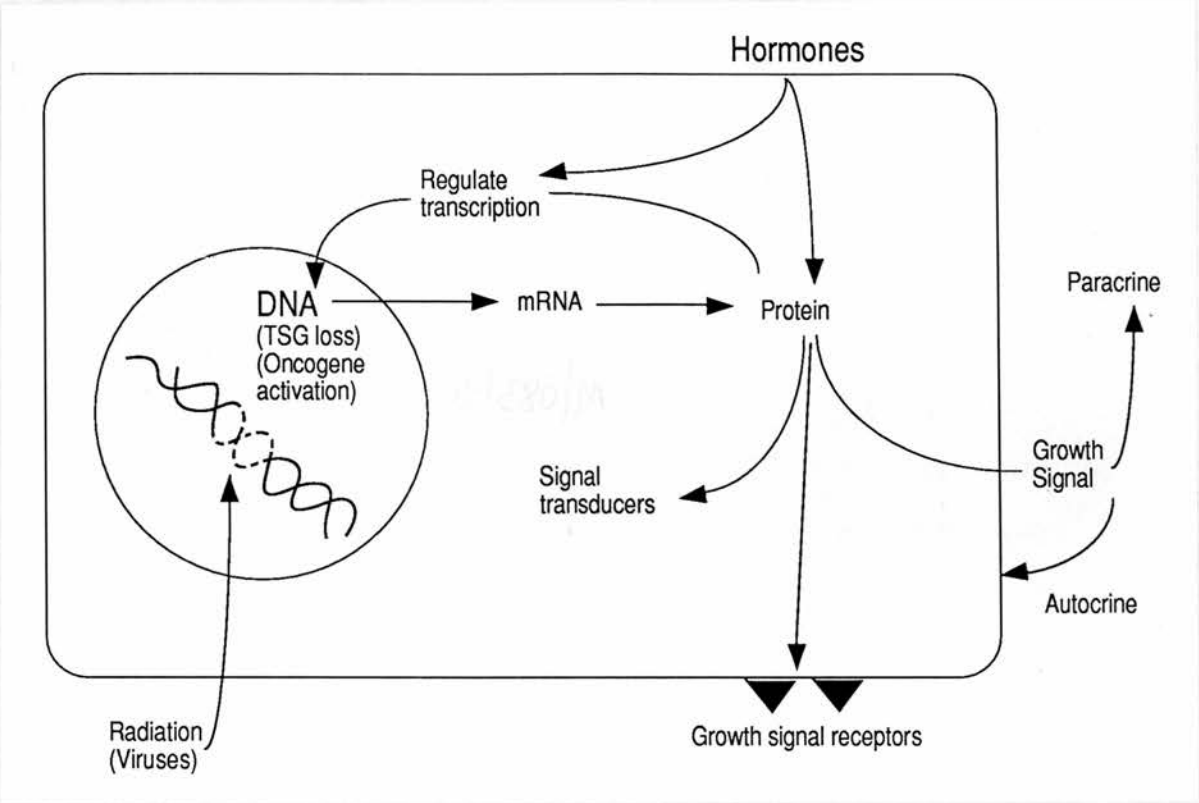
The xenograft studies have given useful information in terms of the changes in gene transcription (mRNA production) in response to an oestrogenic stimulus of oestrogen sensitive immortal human breast cancer cells. These studies also present evidence for the selection of a subset of increasingly tumorigenic cells from the initial cell population, as may occur in a patient's tumour. However, the increase in tumour TGF-ß expression in response to tamoxifen treatment of these xenograft tumours conflicts with the observed pattern in naturally occurring tumours in breast cancer patients, where it is proposed that tamoxifen insmnsitive tumours may subvert the normally autocrine inhibitory TGF-ß function, promote stromal growth and angiogenesis and suppress the local immunocompetent cells in a paracrine fashion.

This thesis also presents strong evidence that multiple events are involved in breast carcinogenesis. It does not give sufficient information to determine the likely sequence of the various genetic abnormalities in breast tumorigenesis and this will require molecular studies of breast tissue along the pathway from normal, through

atypia, hyperplasia and carcinoma in situ to overtly invasive breast cancer. The p53 oncogene/tumour suppressor gene will be of particular interest in this setting. The mouse xenograft model may also allow studies of the effects on tumour growth and characteristics of introducing normal (wild type) p53 into the MCF-7 cell line and/or deletion of the mutant p53.

Support for the genetic basis of human breast cancer from evidence presented in this thesis can be integrated with epidemiological data outlined in the introduction (figure 13). It is proposed that abnormalities in a cancer cell lie in the DNA. Loss of one or more tumour suppressor gene(s) and/or overexpression of oncogene(s) may result from radiation or viral damage to DNA or environmental interactions (including those with hormones) with the DNA. In familial breast cancer, one or more of these events may be inherited. The multiple DNA alterations are reflected in mRNA and protein changes, the gene products passing from the cell to act in an autocrine or paracrine fashion (for example TGF-ß), lodging in the cell membrane to receive growth modifying signals (c-erbB-2), coupling to second messenger systems (H-ras) or interacting directly with the DNA (c-myc, p53). Further tumour growth may then be facilitated by hormonal and other environmental influences, promoting the cancer cell in preference to neighbouring normal cells, resulting in a clinically identifiable cancer.

Figure 13: A diagramatic representation of interactions between genetic events and factors identified by epidemiological studies of human breast cancer.



The underlying DNA abnormalities, resulting from damage to the DNA by such factors as radiation and viral material, or interaction with the DNA by other factors results in tumour suppressor gene (TSG) loss or overexpression or activation of oncogenes. The protein products resulting from mRNA translation may act as a growth signal, growth signal receptor, signal transducer or, in a possible association with hormonal influences, regulating transcription. The net effect of these changes is to facilitate tumour cell growth.

Future prospects

In future, several oncogenes may be studied from very limited tumour material following amplification using the polymerase chain reaction. This concept has already been put into practice for the detection of oncogene mutation (Gonzalez-Cadard et al., 1989) and oncogene amplification in archival material (Frye et al., 1989). From studies such as that detailed in this thesis, important genes can be selected and sequential measurements of gene expression performed on the cells obtained by fine needle aspiration cytology. This may yield further in vivo information, during treatment regimes, on changes in gene expression occurring within the viable tumour cells which may then be used to complement clinical findings. Thus, early indications may be gained as to how a particular endocrine, chemotherapy or radiotherapy treatment is working at the molecular level (or how it is failing), or whether subsequent resistance to the treatment is likely.

While this thesis has examined selected oncogenes, tumour suppressor genes and growth factors, other genes such as the multiple drug resistance genes (Goldstein et al., 1989) also merit attention. New candidate oncogenes such as erbB-3 (Kraus et al., 1989) and further potential tumour suppressor loci (Devilee et al., 1989b) are emerging.

Future molecular studies will further address the problem of tumour metastasis. Oncogenes may act as tumour progression factors (Hall et al., 1989); established oncogenes, for example H-ras (Kyprianou & Isaacs, 1990) and novel genes such as pGM21 (Phillips et al., 1990) have been implicated in conferring a metastatic phenotype.

In the next decade, molecular technology will continue to contribute to greater understanding and improved therapeutic intervention in human breast cancer.

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APPENDIX

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Parts of the work submitted in this thesis have been awarded the following prizes:

Cheine Medal for Surgery 1989

William Leslie Prize for Surgery 1989

Lister Prize for Surgery 1989

Original Publications

p53 gene mRNA expression and chromosome 17p allele loss in breast cancer.

British Journal of Cancer (1990) 61, 74-78.

Gene expression in oestrogen-dependent human breast cancer xenograft tumours.

British Journal of Cancer (1990) 62, 78-84.

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p53 gene mRNA expression and chromosome 17p allele loss in breast cancer

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Summary p53 messenger RNA expression was examined using a cDNA probe in 76 fresh primary breast tumour specimens, 15 of which came from patients treated with taxoxifen prior to surgery. A 2.8 kb mRNA for p53 was expressed in 43 of the 76 specimens. In 19 tumours the levels were similar to those seen in non-malignant (reduction mammoplasty) breast tissue, but in 24 tumours over-expression of mRNA for p53, approaching that seen in three breast cancer cell lines, was found. The cell lines MCF-7, T-47D and MDA-MB-231 expressed three p53 mRNA species of about 2.8 kb and a fourth of 1.6 kb. Increased mRNA expression for p53 correlated ($P < 0.05$) with loss of genetic material from the short arm of chromosome 17 as demonstrated by allele loss with the VNTR probe YNZ 22.1. There was also statistically significant correlation between increased p53 mRNA expression and low oestrogen receptor protein content in the tumours ($P < 0.05$), but not with other clinical parameters. The findings support the view that p53 is involved in breast tumour biology, and suggest that its role may be complex.

p53 is a 53 kDa phosphoprotein with a short half-life (5–45 min) (Reich & Levine, 1984; Halevy *et al.*, 1988). The role of p53 protein remains obscure although the p53 gene has the structural features of a 'housekeeping' gene, including absence of a TATA box (Reynolds *et al.*, 1984; Bienz-Tadmor *et al.*, 1985). p53 may modulate transcriptional activation by binding to DNA in a similar way to the *myc* protein (Donner *et al.*, 1982), allowing cells to progress from a growth arrested state to an actively dividing state or to bypass the need for platelet derived growth factor in the induction of competence (Oren, 1986).

Despite persuasive evidence for its role as an oncogene (Eliyahu *et al.*, 1984, 1985; Parada *et al.*, 1984; Editorial, 1988; Oren, 1986) there is also reason to believe that p53 can act as a tumour suppressor gene (Green, 1989; Wang *et al.*, 1989). This paradox may be resolved if rearrangements of the p53 DNA alter the structure, expression (Masuda *et al.*, 1987) or stability (Jenkins *et al.*, 1985) of the 53 kDa protein product and if the function of a mutated p53 product differs from that of the normal gene (Green, 1989). The p53 gene maps to the 13.1 region of the short arm of chromosome 17 (Miller *et al.*, 1986). One previous study (Masuda *et al.*, 1987) did not find detectable changes in the p53 gene in breast cancer. However, using the YNZ 22.1 probe (Nakamura *et al.*, 1988), which also maps near the tip of 17p at 13.3, Mackay *et al.* (1988) found most patients had two alleles in their constitutional (blood) DNA and 61% of these informative patients had demonstrable loss of one allele (loss of heterozygosity) in the tumour DNA.

p53 mRNA and p53 protein levels may (Reich *et al.*, 1983) or may not (Richon *et al.*, 1989) correspond, since regulation of p53 expression can occur at the level of mRNA abundance or of p53 protein stability, depending on the system under study (Rovinski *et al.*, 1987).

While previous work on human breast cancer has examined p53 protein expression (Cattoretti *et al.*, 1988) or chromosome 17p allelic loss (Mackay *et al.*, 1988; Devilee *et al.*, 1989), no previous study has attempted to link the two by relating expression of p53 mRNA to clinical parameters and/or to deletions of chromosome 17p in breast cancer.

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Materials and methods

Seventy-six patients with fully documented history, examination, staging investigations and follow-up, who presented with breast cancer to the University Department of Surgery Breast Unit at Longmore Hospital, Edinburgh, were studied. They comprised 61 untreated and 15 tamoxifen-treated consecutive breast cancer patients from whom sufficient material was available for analysis. Tumour tissues (minimum 0.2 g) from patients who underwent wedge biopsy, local excision or mastectomy for carcinoma of the breast were frozen in liquid nitrogen and stored at − 70°C. Tissue immediately adjacent to that stored was fixed for histopathology and a further piece of tumour submitted for oestrogen receptor assay. For comparison with constitutional DNA, 20 ml of venous blood was withdrawn for DNA extraction from white blood cells. Breast tissue from 10 patients who underwent cosmetic reduction mammoplasty and who did not have a personal or family history of breast cancer was also obtained fresh and immediately frozen.

The breast cancer cell lines MCF-7 (Soule *et al.*, 1973), MDA-MB-231 (Cailleau *et al.*, 1974) and T-47D (Keydar *et al.*, 1979) were cultured and maintained under mycoplasma-free (Barile, 1973) standard conditions. They were harvested in the logarithmic phase of growth and the RNA was extracted for comparison with that from the tumours.

Ribonucleic acid extraction

From frozen tumour, total ribonucleic acid (RNA) was extracted using a modification of the method of Auffrey and Rougeon (1980). Briefly, a known weight of frozen tumour or a known number of cells washed in phosphate buffered saline was pulverised and then disrupted in 2 ml 100 mg^{-1} 3 M lithium chloride, 6 M urea and precipitated at 4° C overnight. The DNA was sheared using a Soniprep 150 ultrasonic disintegrator (MSE Scientific Instruments, Crawley, UK) with an ice jacket, the RNA was recovered by centrifugation at 12,000 r.p.m. and the pellet was taken up in 6 ml of 10 mM Tris buffer pH 7.0, 0.1% sodium dodecyl sulphate (SDS). Three hundred μg of proteinase K (Boehringer Mannheim, FRG) was added and the sample was incubated at 37°C for 20 min. Protein was extracted using phenol equilibrated with Tris (0.1 M, pH 7) and chloroform:isoamylalcohol (24:1).

Following ethanol precipitation of the aqueous phase at − 20°C, the RNA was recovered by centrifugation and dis-

solved in autoclaved distilled water treated with diethyl pyrocarbonate (DEPC, Sigma, USA) and stored in aliquots at − 70°C. The quantity and purity of the RNA was assessed by spectrophotometry at 260 nm and 240 nm.

Throughout the RNA extraction procedures, sterile disposable plastic ware was used where possible; all solutions were made up with autoclaved DEPC-treated water using baked glassware and gloves were worn to minimise exogenous ribonuclease contamination (Maniatis *et al.*, 1982).

Electrophoresis and transfer of RNA

Twenty μg of total RNA was denatured with formamide and formaldehyde at 55°C for 20 min; 2 μl loading buffer (50% glycerol, 1 mM EDTA 0.4% bromophenol blue, 0.4% xylene cyanol) and 1 μl 10 μg ul^{-1} ethidium bromide were added to each sample. The denatured specimens were loaded on to a 1.1% agarose gel containing 0.66 M formaldehyde, submerged beneath MOPS buffer (morpholinopropanesulphonic acid 0.2 M, pH 7.0, 50 mM sodium acetate pH 7.0, 5 mM EDTA) and the RNA species were separated electrophoretically (method modified from Fourney *et al.*, 1988). The gel was washed in two changes of 10 × standard saline-citrate (1 × SSC contains 150 mM sodium chloride, 300 mM sodium citrate, 1 mM EDTA, pH 7.4) and photographed under a UV transilluminator. The RNA was transferred to a nylon filter (hybond-N, Amersham, UK) by capillary action using 10 × SSC over 8 h (method modified from Southern, 1975). The filter was rinsed in 2 × SSC and air-dried, and the RNA was covalently fixed to the membrane using a UV transilluminator. The filter and remaining gel were photographed to check for adequate transfer of the RNA.

Probe hybridisation

Filters were pre-hybridised in 7% SDS, 0.5 M disodium hydrogen phosphate (pH 7.2) and 1 mM EDTA pH 7.0 (method modified from Church & Gilbert, 1984) for 30 min at 65°C. To this was added ^{32}P-cytidine triphosphate (CTP) labelled cDNA probe, with specific activity to 10^7 c.p.m. ml^{-1} using a random prime DNA-labelling system (Boehringer Mannheim, FRG). ^{32}P-CTP incorporated probe was separated from unincorporated radionucleotide using a Sephadex column (Nick column, Pharmacia, UK) and denatured before addition to the hybridisation solution.

To detect the p53 mRNA, the 2.1 kb cDNA clone php53Bam of p53 protein mRNA cut from pBR322 (Zakut-Houri *et al.*, 1985) was used. Following 24 h hybridisation, filters were washed to remove non-specifically attached probe in two changes of 0.1% SDS 10 mM disodium hydrogen phosphate wash buffer at 65°C with agitation. The filters were blotted dry, wrapped in clingfilm and exposed to pre-flashed Kodak XAR film at − 70°C for up to 14 days.

The extent of hybridisation of radiolabelled probe to the mRNA species was determined from densitometry (using a laser densitometer constructed by the Medical Research Council Human Genetics Unit) and expressed with respect to hybridisation to the actin probe. The size of each mRNA species was calculated from the position of ribosomal RNA markers.

The filters were stripped of residual probe by washing at 80°C for 60 min in 0.1% SDS and the filter was checked by autoradiography. As a standard probe, the Pst 1 insert cDNA of plasmid 91, detecting mouse a-actin mRNA-specific sequences (Minty *et al.*, 1981) was then hybridised and washed under the above conditions to quantify accurately the mRNA in each total RNA sample loaded.

DNA extraction

DNA was extracted from frozen tissue by disrupting finely chopped tissue in lysis buffer containing 1% SDS. Impurities were removed by using RNase and proteinase K, then phenol and chloroform, and the DNA was precipitated using ethanol in the presence of salt (Steel, 1984). Precipitated DNA was spooled from the alcohol, air-dried and redissolved in Tris/EDTA buffer, and the concentration and purity of the DNA were assessed using spectrophotometry at 260 nm and 280 nm. DNA was extracted from 20 ml venous blood in a similar way, but with an additional protein extraction and precipitation step prior to RNase treatment.

DNA (5 μg) from each patient's blood and tumour was digested using a bacterial endonuclease (for example, Tab I), the samples were separated electrophoretically alongside digested lambda markers on a 0.8% agarose gel, the DNA fragments transferred to a hybond-N membrane (Amersham, UK) using the Southern blot technique (Southern, 1975) and the DNA was fixed to the membrane with ultraviolet light and baking at 80°C for 2 h.

The membrane was incubated in hybridisation buffer (5 × Denhart's, 5 × SSC, 0.1% SDS, 10% dextran sulphate) to which 10^7 c.p.m. ml^{-1} ^{32}P-CTP-labelled YNZ 22.1 insert was added (Nakamura *et al.*, 1988) and allowed to hybridise for 24 h.

Excess probe was washed from the membrane using successive washes of 0.1% SDS and 1 × SSC and the DNA fragments were detected by autoradiography at − 70°C to pre-flashed Kodak XAR film.

Oestrogen receptors

The oestrogen receptor content was measured using the Enzyme Immunoassay (EIA; kit from Abbott Laboratories, North Chicago, IL, USA) and expressed in fmol per mg protein for both the tumours and the cell lines. Oestrogen receptor protein concentrations of 20 fmol mg^{-1} protein or greater were considered to be 'significant' (moderate to rich).

Results

A 2.8 kb p53 mRNA was detected in 43 of the 76 tumour specimens (57%), in all three breast cancer cell lines and in six of the 10 reduction mammoplasty specimens (Figure 1). Low levels of this p53 mRNA (comparable to those found in the six positive mammoplasty specimens) were also detected in normal human tonsil, uterus and ovarian tissue (data not shown). There were quantitative differences between the tumour specimens and qualitative differences between the tumours and the cell lines. Thirty-three of the 76 patients had no detectable p53 mRNA in their tumour tissue and 19 patients had detectable p53 mRNA of 2.8 kb similar to quantity to the reduction mammoplasty specimens that gave a positive signal. Twenty-four patients had increased levels of the 2.8 kb p53 mRNA approaching those found in the cell lines.

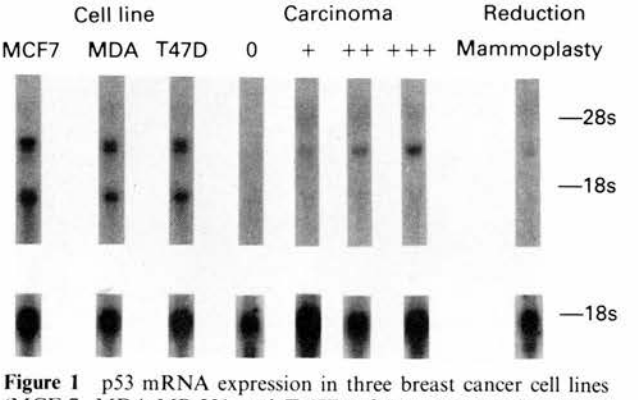

Figure 1 p53 mRNA expression in three breast cancer cell lines (MCF-7, MDA-MB-231 and T-47D), four representative breast tumours with no mRNA expression (0), normal mRNA expression (+) comparable to control (reduction mammoplasty) tissue and increased mRNA expression (++ or +++). In each case, upper part of plate shows p53 mRNA species and lower part of plate the control actin mRNA.

The three breast cancer cell lines each yielded four p53 mRNA species. Three closely related species were of approximately 2.8 kb, with differences between cell lines in the amounts of mRNA for each of these species. A fourth (1.6 kb) p53 mRNA was strongly expressed in all three lines.

There was a significant tendency for increased tumour p53 mRNA expression to be associated with clinically insignificant levels of oestrogen receptor protein ($P = 0.049$, χ^2 test; Table I). No statistically significant correlation was found between p53 mRNA expression and tumour size, spread of the tumour to lymph nodes, histolopathological features of the tumour, patient age or menopausal status.

The 76 patients all yielded sufficient DNA for analysis from both venous blood and tumour. Using the php53Bam cDNA probe for p53, polymorphic bands were detected in less than 10% of samples with BamHI, Bgl II, Sca I, Ban II, HinIII, EcoRI or Taq I and no rearrangements were identified. However, with the YNZ 22.1 cDNA probe and Taq I digests, 52 of the 76 (69%) blood DNA samples were polymorphic and the remaining 24 were not informative. Among the 53 informative patients there was unequivocal loss of heterozygosity (loss or marked diminution in intensity of one allele) in 30 tumours (58%) when compared to the constitutive (blood) DNA (Figure 2).

Loss of genetic material from the tip of the short arm of chromosome 17, as determined by loss of heterozygosity using the YNZ 22.1 probe, was significantly correlated with increased p53 mRNA expression (Table II, $P = 0.04$, χ^2 test).

Allelic loss was also correlated with low levels of oestrogen receptor protein ($P = 0.024$, Fisher's exact test). When the present data are combined with those from our earlier series (Mackay *et al.*, 1988) the association becomes highly significant (Table III, $P < 0.01$).

Table I p53 mRNA expression compared to oestrogen receptor protein in 76 breast cancer specimens (χ^2 6.04, $P = 0.049$)

Oestrogen receptor (fmol mg^{-1} protein)	*p53 mRNA expression*		
	Nil	*Normal*	*Increased*
Significant (>20)	20	14	9
Insignificant (<20)	13	5	15

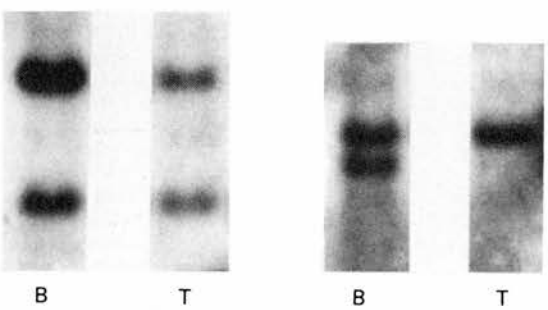

Figure 2 Detection of DNA alleles using cDNA probe YNZ 22.1 following digestion of blood (B) tumour (T) DNA pairs with the endonuclease Taq1, demonstrating no allelic loss (left pair) and loss of heterozygosity (right pair) from tumour DNA.

Table II p53 mRNA expression compared to allelic loss in 52 informative patients as demonstrated using the YNZ 22.1 probe for the short arm of chromosome 17 (χ^2 6.29, $P = 0.04$)

	p53 mRNA expression			
	Nil	*Normal*	*Increased*	
Allele loss	10	6	14	(30)
No allele loss	12	7	3	(22)

Table III Allelic loss in 52 informative patients with the YNZ 22.1 probe compared to oestrogen receptor protein ($P = 0.024$, Fisher's exact test)

	Oestrogen receptor (fmol mg^{-1} protein)	
	Significant >20	*Insignificant <20*
Allele loss	11 (19)	19 (32)
No allele loss	15 (24)	7 (11)

Figures in parentheses include 34 informative patients from our previously reported data (Mackay *et al.*, 1988; $P = 0.004$, Fisher's exact test).

Discussion

This study has examined p53 mRNA expression and loss of genetic material from the short arm of chromosome 17 in 76 patients. A 2.8 kb mRNA for p53 was detected in 43 of the 76 breast cancer specimens. This corresponds to the mRNA for p53 identified in previous studies of human tissue (Harlow *et al.*, 1985; Baker *et al.*, 1989). The quantitative difference between tumours in p53 mRNA expression raises the possibility that the p53 gene may fulfil different functions in the patients with no detectable mRNA by comparison with those in whom there was normal or increased expression. In those tumours where no p53 mRNA was detected, this may reflect deficiency of normal (unmutated) p53 and hence a reduced tumour suppressor function. Deletion of one copy of the p53 gene is compatible with increased function of the other (abnormal) gene. Thus, where there is normal or increased p53 mRNA expression, this may be of a mutated form (for example a point mutation), which therefore acts as an oncogene, promoting carcinogenesis. Alternatively, loss of one allele may confer a minor growth advantage, with subsequent mutation of the remaining p53 required to initiate or promote carcinogenesis. If mutation were to occur first, loss of the normal allele may be required to allow effective expression of the mutant p53, by analogy with co-transfection studies of normal and mutated H-*ras* (Spandidos & Wilkie, 1988). The step between normal and mutated p53 cannot readily be established using the Northern blot technique, although future use of the polymerase chain reaction (Saiki *et al.*, 1985) should clarify the situation.

The qualitative differences between the tumours and cell lines may reflect reading frame differences (likely to give rise to the 1.6 kb mRNA) or splicing, or even different adenylated tail lengths resulting in the three messages of about 2.8 kb in size. However, when MCF-7 cells are grown as xenografts in immunosuppressed mice, only a single 2.8 kb p53 mRNA species and no 1.6 kb mRNA is detected in the tumour tissue. This finding is independent of the rate of tumour growth (A.M. Thompson, manuscript in preparation). A single p53 mRNA species of 1.8 kb has been noted previously in NIH3T3 cells (Reich *et al.*, 1983; Reich & Levine, 1984) and this mRNA may correspond to the nuclear mRNA regulating translation detected by Khochbin and Lawrence (1988).

The three cell lines examined are distinguishable on karyotype and on molecular analysis (using cDNA probes), and they have different phenotypic characteristics; for example, the MCF-7 line used in these studies has on average 120 fmol mg^{-1} total protein oestrogen receptor protein, T-47D 40 fmol and MDA-MB-231 0 fmol. These lines also show differing sensitivity to oestrogens and anti-oestrogens. The consistently high level of p53 mRNA expressed in all three lines thus implies that, in this *in vitro* setting, p53 mRNA expression is independent of oestrogen receptor protein content of the cells and of hormone sensitivity. Although we have not confirmed the correlation noted *in vivo* between increased p53 mRNA expression and oestrogen poor tumours, this may be due to a myriad of factors including

the divergence of cell lines from the original tumour with time.

Cattoretti *et al.* (1988), using an antibody PAb1801 specific for human p53 protein in breast cancer specimens, noted a correlation between oestrogen receptor negative tumours and elevated p53 protein expression ($P < 0.05$). Using this antibody, no other significant correlation was identified. These results therefore agree with our findings in relation to p53 mRNA.

This study confirms that the loss of one YNZ 22.1 allele from the short arm of chromosome 17 occurs in over half the breast tumours studied (Mackay *et al.*, 1988, Devilee *et al.*, 1989) and establishes for the first time that this allele loss correlates with clinically insignificant levels of oestrogen receptor protein. The tip of the short arm of chromosome 17 is thus of importance in breast as well as in colon cancer (Lothe *et al.*, 1988; Vogelstein *et al.*, 1989).

Although of all the highly informative probes available for this study, YNZ 22.1, located at 17p 13.3, was the closest to the p53 (17p 13.1) locus, it is still several megabases telomeric to the p53 gene. The correlation between loss of heterozygosity for YNZ 22.1 and p53 mRNA expression provides evidence that there may be some link between a putative gene conferring increased susceptibility to cancer (Mackay *et al.*, 1988) and the oncogene or tumour suppressor gene function of p53.

Eleven of the 76 patients had tumours showing loss of heterozygosity for YNZ 22.1, increased expression of p53 mRNA and low levels of oestrogen receptor protein. With just 12 months mean follow-up, two of these patients have already relapsed with metastatic disease. Continued follow-up of the whole cohort will establish the prognostic significance of the present findings.

The observation that, in almost a third of tumours, p53 mRNA levels were elevated while, in a comparable proportion, the message was not detectable, supports the view that p53 is involved in breast tumour biology. Whether this is as an oncogene or as a tumour suppressor gene (possibly as either, depending on the individual tumour) remains to be seen. The advent of highly polymorphic probes for the p53 gene and the application of more recent technology, such as the polymerase chain reaction, should resolve these issues.

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Gene expression in oestrogen-dependent human breast cancer xenograft tumours

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Summary Xenograft tumours from an oestrogen-dependent human breast cancer cell line MCF-7 have been established and characterised in thymectomised, irradiated female CBA strain mice. There was evidence for selection in xenografts of a subpopulation of MCF-7 cells with an altered pattern of gene expression as measured by mRNA levels compared with the original cells *in vitro*. Tumorigenicity increased significantly on repeated animal passage but oestrogen dependence was retained. Following injection of the mice with oestrogen, mitosis was induced in the tumour cells with associated increases in thymidine uptake and percentage of cells in S-phase. In accord with these changes, c-*myc* and p53 expression were increased and TGF-beta was suppressed. Thereafter the expression of the c-*myc* and p53 genes fell whilst that of the TGF-beta gene was induced as the oestrogenic stimulus declined. The oestrogen-regulated mRNA pS2 showed a biphasic response to oestrogen and levels declined as the serum oestrogen fell to undetectable levels. This xenograft system demonstrates that changes in transcription of oncogenes, growth factor and oestrogen-regulated genes can be detected *in vivo* in response to oestrogen. It thus provides an *in vivo* model for studies of the biochemical and molecular basis for therapeutic manipulation of hormone-sensitive human breast cancer.

In studying the molecular biology of cancer cells, the significance of *in vitro* observations may be uncertain due to the absence of host factors that influence tumour behaviour *in vivo*. Recent work on experimental human breast tumours *in vivo* has made use, almost exclusively, of congenitally athymic ('nude') mice (Osborne *et al.*, 1988; Brunner *et al.*, 1989). We have previously reported the growth of a range of human tumours in thymectomised, irradiated mice (Busuttil *et al.*, 1986) which have advantages in ease of husbandry and cost (Steel *et al.*, 1978; Morten *et al.*, 1984; Hay *et al.*, 1985). We report here the characterization of an oestrogen-responsive tumour derived from the MCF-7 human breast carcinoma cell line grown *in vivo* in female thymectomised and irradiated CBA mice. In addition, we have examined the kinetics of the expression of a range of related genes (c-*myc*, p53, TGF-beta and pS2) following oestrogenic stimulation in this tumour model system.

Materials and methods

Twenty-one day old female mice from an established breeding colony of CBA/Ca strain mice at the Institute of Animal Technology, Western General Hospital, Edinburgh, maintained as described in Hay *et al.* (1985), were anaesthetised with ether and suction thymectomy performed. Three weeks after thymectomy, 200 mg kg^{-1} arabinoside C (Pfizer, UK) was injected by the intraperitoneal route and 48 h later the mice were irradiated to a total body dose of 7.50 Gy. Radiation was delivered from an X-ray source (250 kv: 0.3–0.4 Gy min^{-1}) with a Thoreus II filter. MCF-7 cells (Soule *et al.*, 1973) were cultured in Nunclon flasks (Nunc, Kamstrup, Denmark), fed regularly with Dulbecco's Modified Eagle Medium (DMEM; Gibco, Paisley, UK) containing phenol red (which has an oestrogenic effect on MCF-7 cells) supplemented with 12% fetal calf serum (FCS; Gibco) and maintained in an atmosphere containing 5% CO_2 at 37°C. All cultures were persistently negative for mycoplasma using conventional immunofluorescence techniques (Goding, 1983). The cells were harvested in the logarithmic (subconfluent) phase of growth and washed twice in phosphate-buffered saline (PBS). A single dose of 10^7 viable cells suspended in 50–100 μl PBS was injected into the fourth right mammary fat pad 1–3 weeks after irradiation. At this time 50 μg oestradiol benzoate (Intervet UK Ltd., Cambridge, UK) in 50 μl arachis oil was injected subcutaneously into the nape of the neck. This injection of 50 μg oestradiol benzoate in arachis oil was repeated every three weeks to the same site. MCF-7 cells were also injected into 20 CBA mice without oestrogen supplementation. A further 20 mice were tested with a second regime: either a 1.25 mg or a 0.5 mg oestradiol 6-week-release pellet (Innovative Research, Ohio, USA) was placed subcutaneously. Tumours were measured daily in two dimensions by the same author using calipers. The volume of the tumour was calculated using the formula $pi/12 \times (\text{mean diameter})^3$.

Mice were killed at selected times and the tumours were frozen immediately in liquid nitrogen or fixed for histology. Those mice which did not develop tumours were killed 90 days from the start of the experiment. Mice which died in the interim were examined in detail for evidence of disease.

Fragments of xenograft material arising from inocula of MCF-7 cells were further transplanted through a dorsal incision into additional thymectomised, irradiated mice under anaesthetic. An injection of 50 μg 17 beta oestradiol benzoate in arachis oil was administered as the mouse awoke. Thereafter the transplant-bearing mice were treated as above. Remaining tumour fragments were macerated and added to DMEM containing 12% FCS and the tumour cells returned to culture for 8 weeks before reinjection into mice or extraction of total ribonucleic acid (RNA).

Two additional human breast cancer cell lines MDA-MB-231 (Cailleau *et al.*, 1974) and T47-D (Keydar *et al.*, 1979) were cultured and similarly maintained. The cells were harvested in the logarithmic phase of growth so that the RNA could be extracted for comparison with that from the MCF-7 cells.

Serum oestradiol concentration

Serum was prepared from individual samples of blood (0.3–0.8 ml) taken by cardiac puncture from each of 30 mice at specified times following the 3-weekly oestradiol benzoate injections. The concentration of 17 β-oestradiol was determined in 50 μl samples of serum using a commercially available radioimmunoassay (Baxter Health Care, Newbury,

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rks., UK). Within assay CV was 5% and between assay / was 6%.

llular DNA synthesis

rcentage of cells in 'S' phase. A monoclonal antibody, R9, directed against the halogenated nucleotide 5-omodeoxyuridine (5BrdU) was raised by one of the thors (D.D.) and used for rapid S-phase measurements onochoroff *et al.*, 1985).

A total of 200 µl of 6 mg ml^{-1} 5-BrdU (Sigma) was ected into the peritoneal cavity of each mouse under amination at 0, 6, 12, 18, 24, 36, or 48 h after injection of µg 17 β-oestradiol. Each mouse was killed 1 h after ministration of the BrdU and the cells were released from tumours by dispase digestion and fixed in 70% ethanol. double staining procedure was used for the differentiation human MCF-7 cells from invading host (mouse) cells. xed cells were incubated with 10 µg of an antihuman class I onoclonal antibody PE25 (D.D.), washed in PBS and 2 µg phycoerythrin-labelled anti-mouse conjugate added outhern Biotechnology Associates Inc.). To prevent cross-ctivity of this antibody with the BR9, the cells were then ubated in PBS containing 10 µg ml^{-1} mouse IgG (Sigma) 30 min. After a wash in PBS, 10 µg of the monoclonal tibody BR9 was added to the pellet of 10^6 cells and ubated at room temperature for 40 minutes, washed in S and incubated for a further 40 minutes in the presence 2 µg fluoroscein isothiocyanate (FITC)-labelled goat anti-ouse conjugate (Sigma). After a final wash in PBS the cells re analysed on a FACScan flow cytometer (Becton Dick-n, Lincoln Park, NJ, USA) for the absolute number of ls and the proportion of tumour cells in S phase.

ymidine incorporation Thymidine uptake was also used as index of DNA synthesis in the isolated tumour cells. For ch time point, intact cells were separated from the dispase/ MEM digests by density centrifugation over 5–30% Ficoll 0 gradient (Pharmacia, Uppsala, Sweden) and 150 µl of cell spension (10^6 cells ml^{-1} DMEM with 5% added FCS) were ded per well of a 96-well mitcrotitre plate (Falcon 3072, cton Dickson). One µCi ^{3}H-thymidine (Amersham Interna-nal, Aylesbury, UK) was added to each well and the cells re harvested 5 h later on to glass fibre discs. The radio-tivity emitted from each disc was counted in 5 ml of scintil-at (Opti-Scint, Pharmacia, Sweden) in a Packard 1600CA alyser (Packard, Downers Grove, Illinois, USA).

stopathology

slice through the middle of each tumour was fixed in ethacarn for 1 h then 95% ethanol and then processed utinely. Paraffin sections were cut and examined after ining with haematoxylin and eosin. The mitotic index for ch tumour was calculated from the mean number of toses in 50 randomly chosen, high-power fields (× 400) by e author (D.P.).

strogen receptor concentration

e soluble oestrogen receptor concentration of xenograft sue or cells was measured following homogenisation by a ndard method (Hawkins *et al.*, 1981) and use of the zyme Immuno-Assay (EIA; Kit from Abbott boratories, North Chicago, Illinois). Both for the cells and the solid tumours receptor concentration was expressed in ol mg proteins^{-1} (Hawkins *et al.*, 1987).

traction of ribonucleic acid and northern blotting

om frozen tumour, the total ribonucleic acid (RNA) was racted using a modification of the method of Auffrey and ugeon (1980). A known weight of frozen tumour was lverised using a Mikrodismembrator II (Braun, FR Ger-ny) and the resulting powder was finely disrupted using a plastic pipette in the presence of 2 ml per 100 mg tissue of 3M lithium chloride/6 M urea and left at 4°C overnight. Alternatively, cells cultured *in vitro* were washed in PBS and then disrupted in 3 M lithium chloride/6M urea with a plastic pipette. The DNA was sheared using a Soniprep 150 ultrasonic disintegrator (MSE Scientific Instruments, Crawley, UK) with an ice jacket. The RNA was recovered by centrifugation at 12,000 r.p.m. and the pellet was taken up in 6 ml 10 mmol Tris buffer pH 7.0, 0.1% sodium dodecyl sulphate (SDS), 300 µg proteinase K (Boehringer Mannheim, FR Germany) were added and the tube incubated at 37°C for 20 min. Protein was extracted using phenol equilibrated with 0.1 M Tris at pH 7 and 24:1 chloroform:isoamylalcohol. Following ethanol precipitation of the aqueous phase at −20°C, the RNA was recovered by centrifugation and dissolved in diethyl pyrocarbonate (DEPC, Sigma, USA) treated, autoclaved, distilled water and stored in aliquots at −70°C. The quantity and purity of the RNA was assessed spectrophotometrically at 260 nm and 240 nm. Throughout the RNA extraction procedures, sterile disposable plastic ware was used where possible; all solutions were made up with autoclaved DEPC-treated water, using baked glassware and gloves were worn to minimise exogenous ribonuclease contamination (Maniatis *et al.*, 1982).

Twenty micrograms of total RNA was denatured with formamide and formaldehyde at 55°C for 20 min; 2 µl loading buffer (50% glycerol, 1 mM EDTA, 0.4% bromophenol blue, 0.4% xylene cyanol) and 1 µl of 10 µg µl^{-1} ethidium bromide were added to each sample. The denatured specimens were loaded on to a 1.1% agarose gel containing 0.66 M formaldehyde, submerged beneath MOPS buffer (0.2 M morpholinopropanesulphonic acid pH 7.0, 50 mM sodium acetate pH 7.0, 5 mM EDTA) and the RNA species were separated electrophoretically (modified from Fourney *et al.*, 1988).

The gel was washed in two changes of 10× standard saline citrate (1 × SSC contains 150 mM sodium chloride, 15 mM sodium citrate, 1 mM EDTA, pH 7.4), photographed under a UV transilluminator and the RNA was transferred to a nylon filter (hybond-N, Amersham, UK) by hydrostatic action using 10× SSC over 8 h (Southern, 1975). The hybond was rinsed in 2× SSC, air-dried and the RNA was covalently fixed to the membrane using a UV transilluminator. The hybond and the remaining gel were photographed to check for adequate transfer of the RNA.

Probe hybridisation

Filters were prehybridised in 7% SDS, 0.5 M disodium hydrogen phosphate pH 7.2 and 1 mM EDTA pH 7.0 (modified from Church & Gilbert, 1984) for 30 min at 65°C. To this was added ^{32}P cytidine triphosphate (CTP)-labelled cDNA probes labelled to 1 × 10^7 c.p.m. ml^{-1} using the randomprime DNA labelling system (Boehringer Mannheim, FR Germany). ^{32}P-CTP incorporated probe was separated from unincorporated radionucleotide using a Sephadex column (Nick column, Pharmacia, UK) and denatured before addition to the hybridisation solution.

cDNA probe inserts digested from their respective plasmids were used to detect messenger RNA (mRNA) species for three oncogenes (*erb*B-2; p53; c-*myc*) three growth factors or their receptors (epidermal growth factor receptor; transforming growth factor-beta; transforming growth factor-alpha) and two hormone-related genes (OR3, pS2).

For c-*erb*B-2, the KpnI-XbaI fragment of lamda 107 was used (Semba *et al.*, 1985); for p53, the 2.1 kb php53Bam cDNA (Zakut-Houri *et al.*, 1985); and for c-*myc*, pSV-c-*myc*-1 for exons 2 and 3 (Land *et al.*, 1983). Epidermal growth factor receptor (EGFR) was detected with the 3.9 kb pHER-A64-1 probe (Ullrich *et al.*, 1984), transforming growth factor-alpha (TGF-alpha) with a 1.05 kb insert from Sp64-BC1 (Derynck *et al.*, 1984) and transforming growth factor beta with the 1.3 kb insert from Sp65-C17N (Derynck *et al.*, 1985). The two hormone-related probes were the 1.6 kb OR3 oestrogen receptor cDNA (Walter *et al.*, 1985) and the pS2

0.56 kb cDNA for oestrogen-regulated mRNA (Masiakowski *et al.*, 1982). As a standard probe, the Pst 1 insert cDNA of plasmid 91, detecting mouse alpha-actin mRNA specific sequences (Minty *et al.*, 1981), was used to quantify accurately each total RNA sample loaded. It is of particular relevance in this oestrogen-sensitive model that transcription of actin mRNA in MCF-7 cells is not affected by oestrogen (Saceda *et al.*, 1988).

Following hybridisation for 24 hours, filters were washed to remove non-specifically attached probe in two changes of 0.1% SDS 10 mmol disodium hydrogen phosphate wash buffer at 65°C with agitation. Filters were blotted dry, wrapped in cling film and exposed to preflashed Kodak XAR film at −70°C for up to 14 days. The extent of hybridisation of radiolabelled probe to the mRNA species was determined from densitometry using a laser densitometer constructed by the Medical Research Council Human Genetics Unit and expressed with respect to hybridisation to the actin probe. The size of mRNA species was calculated from the position of ribosomal RNA markers. Filters were reprobed up to six times with different cDNA probes; before reprobing, filters were stripped of residual probe by washing at 80°C for 60 min in 0.1% SDS and the filter was checked by autoradiography.

Results

One hundred and forty-five CBA strain mice were injected with MCF-7 cells in this study (Table I). Of the 145 mice injected, 69 received both cultured MCF-7 cells and oestradiol benzoate. Twelve of these 69 mice died before 90 days leaving 57 for analysis, 30 of which (53%) grew a tumour. Of 36 mice into which tumour was transplanted, four died prematurely and 23/32 (72%) of the remaining mice grew tumours. The recultured cells grew as xenografts in 16 of the 18 mice injected which survived (89%). The take rate of transplanted tumour material was significantly higher than that of primary inocula ($P = 0.04$ by Fisher's exact test) and was higher still for tumour cells that had been recultured *in vitro* and then inoculated into fresh mice ($P = 0.004$ by Fisher's exact test).

Tumours did not grow without oestrogen supplementation. The 50 μg oestradiol benzoate in arachis oil or the arachis oil alone were well tolerated by the CBA mice and the oestrogen delivered in this form promoted tumour growth. The use of conventional pellets of 1.25 mg or 0.5 mg oestradiol resulted in 20/20 deaths within 10 days. No consistent cause for these deaths was evident at *post mortem*.

Histopathology

Each MCF-7 tumour was firm, pale, solid and well circumscribed, not showing overt local invasion or ulceration of the overlying skin. All the tumours were examined histologically and were compatible with an origin from breast, although they did not show marked adenocarcinomatous differentiaton. There were no areas of necrosis in the smaller tumours, although the larger tumours did have evidence of central necrosis. No marked lymphocyte infiltration was noted.

Microscopic and macroscopic examination of mice which died and mice which were killed to obtain tumour showed evidence of metastasis in only one animal. In that instance, tumour cells were evident at the site of MCF-7 cell inoculation, as peritoneal seedlings and microscopically in the visceral pleura of the lung. No pathological evidence of oestrogen toxicity was found at post mortem in any animal although there was some hair loss at the site of the oestradiol injection.

Table I The fate of MCF-7 cells inoculated into immunocompromised mice

MCF-7 inoculum	*Oestrogen supplement*	*Number of CBA mice*	*Number of live mice bearing tumours*	*Number of live mice without tumours*	*Deaths*
Cells	Nil	20	Nil	20	Nil
Cells	50 μg oestradiol benzoate	69	30 (53%)	27	12
Transplant xenograft	50 μg oestradiol benzoate	36	23 (72%)	9	4
Recultured xenograft cells	50 μg oestradiol benzoate	20	16 (89%)	2	2

Serum oestradiol concentration

Oestradiol was not detectable in the serum from thymectomised and irradiated mice prior to injection. After injection with 50 μg oestradiol benzoate (Figure 1), a sharp rise to a mean 7,492 pmol l^{-1} (s.d. 3,374 pmol l^{-1}) oestradiol occurred by 24 h, declining exponentially to undetectable levels (less than 53 pmol l^{-1}) 2 weeks after the injection.

Tumour growth

The tumours became palpable during the first 3 weeks following 17 β-oestradiol injection and following the second and third injections of oestrogen, the tumour was observed to grow in size, but not in a uniform fashion (Figure 1). In particular, during the first 14 days after injection, the tumour increased rapidly in size, then from day 14 to 21 slowed down or became static. By 3–6 weeks, all tumours were large enough for the studies described.

Cellular DNA synthesis and mitoses

Increased thymidine uptake was noted by 18 h following the oestrogen injection (Figure 2) and, in parallel with the percentage of S-phase cells, reached a maximum 24 h following oestrogen injection, declining thereafter.

The number of mitoses per ×400 field (Figure 2) showed an increase, compared to the baseline value of 3 per ×400 field, to 24 per ×400 field demonstrable 24 h following oestrogen stimulation of the tumour. The level fell to 5 mitoses per ×400 field 10 days later. The number of mitoses showed parallel changes to the biochemical indices of cellular DNA synthesis.

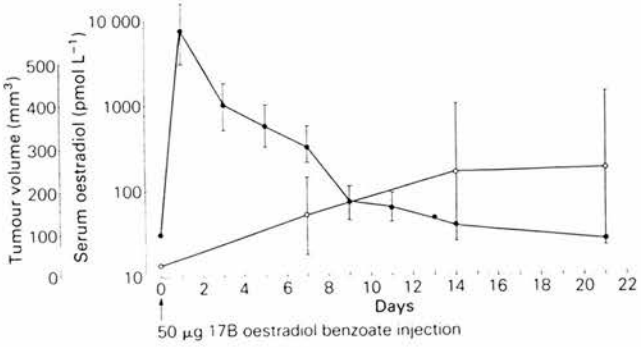

Figure 1 Serum oestradiol and tumour volume in a mouse xenograft model. Serum oestradiol (mean ± standard deviation) in thymectomised and irradiated mice for 2–4 mice at each time point following subcutaneous injection of 50 μg 17 β oestradiol benzoate. From undetectable levels (<53 pmol l^{-1}) prior to injection of oestradiol there is a rapid rise to a peak of 7,492 $pmol^{-1}$ followed by a decline in serum oestradiol to undetectable levels by the third week post-injection (solid dots). Tumour volume (calculated from pi/12 × mean $diameter^3$) for a cohort of 12 mice (mean volume ± standard deviation) measured during weeks 3 to 6 following injection of the MCF-7 cells. Only four time points are shown for clarity (open dots). There is a rise in tumour volume for the 2 weeks following injection of oestrogen, with little change in tumour volume once serum oestradiol becomes undetectable.

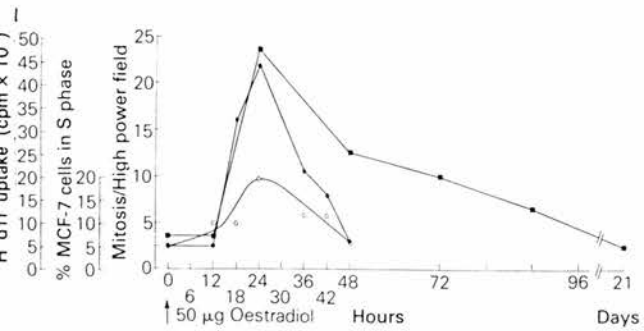

Figure 2 Three indices of cellular proliferation in xenografts of MCF-7 cells in mice following administration of oestradiol. Cell proliferation measured by ^{3}H-dTr uptake (solid dots), percentage of cells in S phase (open dots) and mitoses per × 400 field (solid squares) showing mean value of four tumours for each time point. The same tumours were used for all three indexes of cell proliferation. Between 12 and 24 h following injection of 17 β oestradiol benzoate, MCF-7 cells are stimulated to divide, DNA synthesis returning to prestimulation levels by 48 h and mitoses declining within days.

Oestrogen receptors

MCF-7 cells *in vitro*, tumour material taken immediately before oestradiol injection and cells cultured from xenografts had a mean 120 fmol (range 110–135 fmol) oestrogen receptors per mg protein. There was a rise to 240 fmol mg^{-1} protein at 30 h, but at 7, 14 and 21 days following injection, the level had returned to between 120 and 150 fmol mg^{-1} protein.

Tumour levels of mRNA

Presence of mRNA species Messenger RNA was detected in MCF-7 cells by seven of the nine probes (Figure 3). No mRNA for EGFR or TGF-alpha was detected in the MCF-7 cells or xenografts, although both mRNA species were detected in other breast cancer cell lines (MDA-MB-231 and T47-D). While some mRNA species (c-*erb*B-2 and OR3) detected in the MCF-7 cell line were not seen in the xenograft material, no mRNA species was detected in the tumours which was not present in the original cell line (Figure 3). The mRNA for c-*erb*B-2 was detected at 3.0 kb and 1.8 kb in the original cell line but not in the xenografts.

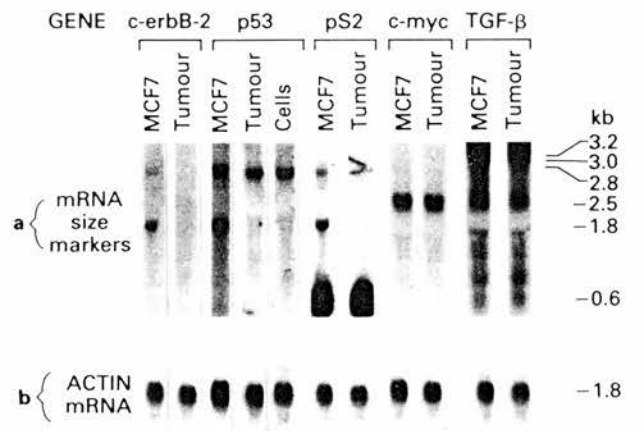

Figure 3 Gene expression in MCF-7 cells *in vitro* and *in vivo* (xenografts). Representative autoradiographs of MCF-7 cells, MCF-7 xenografts and MCF-7 cells re-cultured from the xenografts after probing with cDNA probes for c-*erb*B-2, p53, pS2, c-*myc* and TGF-beta (details in text). In each case the actin-probed control for each lane is shown. Two c-*erb*B-2 species are evident in the cell line but are not seen in the xenografts. Four p53 mRNA species are demonstrated in the cell line but only one in the xenografts and re-cultured cells. Similarly, three species detected with the pS2 probe are seen in the cell line but only one species in the xenografts and cells cultured from those xenografts. The c-*myc* and TGF-beta probes detect identical species in both the MCF-7 cell line and the xenograft tumours.

Four mRNA species for p53 (three at 2.8 kb, one at 1.8 kb) were identified in the original cell line but only a single mRNA for p53 was found in tumours or in re-cultured cells. Similarly, three mRNA species were detected with the pS2 probe in the original cell line but only the single 0.6 kb mRNA in the tumours. Other cDNA probes such as those for TGF-beta and c-*myc* detected only a single mRNA (2.5 kb in each case) present in both the cell line and xenografts. There were no differences in any mRNA species detected between xenografts, transplanted tumours and tumour cells recultured for periods of up to 56 days.

Changes in gene expression after oestrogen stimulation (Figure 4). Densitometry permitted detection of changes in the levels of mRNA for c-*myc*, p53, TGF-beta and pS2 with respect to alpha-actin mRNA, following stimulation of the xenograft by oestrogen. The mRNAs for c-*myc* and p53 both increased then fell back towards the unstimulated level within the first 24 h while that for TGF-beta was rapidly suppressed, rising only as the oestrogen stimulus declined after 1 week. pS2 expression showed a biphasic response with an initial increase to 24 h, then suppression for 12 h, a less substantial increase by 48 h and finally a decline as the expression of TGF-beta increased.

Discussion

General characteristics

We have established the MCF-7 breast cancer cell line as xenografts in CBA mice immunocompromised by thymectomy and whole body irradiation. Tissue from these tumours can be transplanted to similar mice, and cells cultured from the xenografts can be re-implanted to grow tumours. This model therefore yields a large renewable supply of tumour material passaged *in vivo* and permits the study of tumours during hormonal manipulation. These MCF-7 tumours were clearly adenocarcinomata, with necrosis in only the larger tumours as in nude mice (Osborne, 1988) and metastasis, as previously noted, a rare event (Busuttil *et al.*, 1986).

As in nude mice, oestrogen supplementation is a prerequisite for MCF-7 tumour growth (Shafie & Grantham, 1981; Osborne *et al.*, 1985; Gottardis *et al.*, 1988). The absence of detectable serum oestrogen in female mice prior to injection confirmed that the mice had been 'oophorectomised'

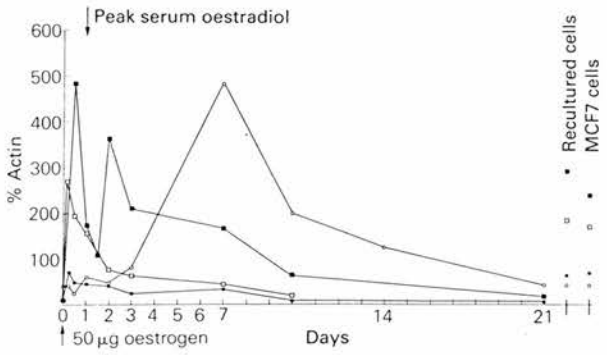

Figure 4 Changes in mRNA following oestrogenic stimulation of MCF-7 xenografts. Host animals were injected with 17β oestradiol benzoate at time 0. The changes shown are for c-*myc*, p53, pS2 and TGF-beta mRNA species in xenografts, as detected by densitometry of autoradiographs, with respect to that for actin as a control. The percentage at each time point is the mean of six tumours. mRNA expression for the same four species is shown for the original MCF-7 cells and cells recultured from xenograft tumours. Levels of c-*myc* (open squares) and p53 (solid dots) mRNA reach a peak within 12 h and decline to prestimulation levels by the second week. pS2 mRNA (solid squares) shows a biphasic response, with a peak at 12 h, apparent suppression of this peak to coincide with the peak serum oestradiol at 24 h and then a further substantial rise and then gentle decline to prestimulation values by the second week. In contrast to the other three species, TGF-beta is slightly suppressed in the first 24 h and peaks at day 7, returning gradually to prestimulation levels by day 21 (open dots).

by the irradiation. The serum oestrogen profile following a single subcutaneous injection of oestradiol benzoate in arachis oil (Figure 1) gave very high and possibly even inhibitory serum levels of oestradiol between 12 and 36 h. From day 2 to day 8, levels of oestradiol were within the physiological range for non-pregnant premenopausal women (110–1,630 pmol l^{-1}, data from Baxter Health Care, Newbury, UK) and they remained sufficient to sustain oestrogen-dependent tumour growth for 21 days. Serum oestradiol levels in surgically oophorectomised nude mice which have received the implantable pellet also fall within the same range (Shafie & Grantham, 1981; Blumenthal *et al.*, 1988). We have no explanation for the intolerance of our experimental mice to the implantable pellet, although the findings reported here confirm our previous experience with this strain (M.E.F. and C.M.S., unpublished data).

DNA synthesis and mitotic activity of the tumour cells was evident in respone to oestradiol (Figure 2). The increase in mitoses from a mean of 3 per ×400 field to 24 per field is in keeping with observations in nude mice (5 and 25 mitoses per ×400 field respectively; Osborne *et al.*, 1985). Similarly, despite possible influences on the thymidine pool by oestradiol, thymidine uptake and the % S phase cells confirmed the histological observation that, following oestrogen administration, there was an increase in cell proliferation, which preceded the increase in tumour volume.

The slight rise in oestrogen receptor protein following oestrogen stimulation of the tumour, from 120 fmol mg^{-1} protein to 240 fmol mg^{-1} protein, and its subsequent return to basal level is consistent with the view that oestrogen can stimulate the synthesis of its own receptor.

mRNA species

The mRNA species detected, particularly in the MCF-7 cells *in vitro*, merit comparison with published data. Using the oncogene probes to study the RNA from MCF-7 grown *in vitro*, two distinct mRNAs of 1.8 and 3.0 kb were identified hybridising to the c-*erb*B-2 probe, both smaller than the single 4.8 kb mRNA species previously described (Semba *et al.*, 1985) and identified in breast tumours and normal human tissues (data not shown). An amplified and rearranged epidermal growth factor receptor in epidermoid carcinoma cells generating a truncated 2.8 kb mRNA that encoded only the extracellular EGF binding domain has been reported previously (Ullrich *et al.*, 1984). Just as the c-*erb*B-2 gene may be rearranged in the MCF-7 cells studied, truncated transcription may occur. Certainly, alternative transcription mechanisms have been proposed for the c-*erb*B-2 gene (Tal *et al.*, 1987).

It is well recognised that a single gene can give rise to a variety of mRNA transcripts: the three p53 mRNA species of circa 2.8 kb similar to the 2.8 kb mRNA in human breast tumours (Thompson *et al.*, 1990) may result from such mechanisms as different transcription initiation sites, differential splicing or other post-transcriptional modification. Certainly, the mean size observed corresponds well to published data for the human p53 mRNA (Zakut-Houri *et al.*, 1985; Harlow *et al.*, 1985), and the additional 1.8 kb mRNA identified is probably still large enough to encode a 53 kDa protein. The c-*myc* mRNA of 2.5 kb corresponds to that previously reported in MCF-7 cells (Zajchowski *et al.*, 1988). The 2.5 kb mRNA for TGF-beta was of the expected size (Derynck *et al.*, 1985; Travers *et al.*, 1988).

Although mRNA for the epidermal growth factor receptor, and the mRNA for TGF-alpha, which acts upon it, have been reported in MCF-7 cells (Dickson *et al.*, 1986, Arteaga *et al.*, 1988), none was evident in the MCF-7 cell line tested here, suggesting that the MCF-7 cells in use in our laboratory may be variants of those used in some previous studies.

OR3, the probe for oestrogen receptor mRNA detected two messages of 3.0 and 1.7 kb, but no 6.2 kb mRNA. As in this study, Henry *et al.* (1988) were unable to demonstrate oestrogen receptor mRNA in the MCF-7 cell line using the OR3 cDNA clone, but demonstrated hybridisation to a 6.2 kb mRNA using their radionucleotide labelled RNA probe. While mRNA of 6.2 kb (Walter *et al.*, 1985), 4.2 kb (Parl *et al.*, 1987) and 3.7 kb (Barrett-Lee *et al.*, 1987) has been reported in human tumour tissue and in the MCF-7 cell line, the species identified here do not correspond to any of these. It is therefore possible that the probe used did not detect the oestrogen receptor mRNA (perhaps due to the experimental conditions) or alternatively that the MCF-7 cells used in this study produce oestrogen receptor mRNA smaller in size than that previously identified. However, the translated oestrogen receptor protein was certainly present when assayed by enzyme immunoassay.

The pS2 probe appears to cross-hybridise to the same 3 kb and 1.7 kb mRNA as the OR3 probe but hybridises most strongly to a small mRNA of about 600 base pairs (Figure 3), corresponding to the oestrogen induced mRNA of Masiakowski *et al.* (1982). The biphasic change in pS2 suggests that the role of pS2 as a marker for oestrogen action may not be as simple as originally proposed.

mRNA differences in vitro and in vivo

The original MCF-7 cells grown *in vitro* expressed several mRNA species not evident in the xenografts or in cells of these xenografts recultured for some time *in vitro*. These findings may indicate *in vivo* selection for a subpopulation of cells within the MCF7 culture.

The MCF-7 cells re-cultured from xenografts had an identical pattern of gene expression to the xenografts and did not, over an 8-week period, revert to the original MCF-7 cell line pattern. Serious consideration was given to the possibility that these findings could be due to a contaminant in the original cell line (such as mycoplasma) expressing the gene concerned or to contamination (perhaps by a plasmid) at some point in the RNA extraction or electrophoresis. Both these explanations are unlikely since tests on the MCF-7 culture (Barile, 1973) were persistently negative for mycoplasma, and no evidence of plasmid contamination was found in these or any other northern-blot RNA studies. Moreover, a range of different plasmids was used as vectors for the cDNA probes.

mRNA changes following oestrogen stimulation

Oestrogen-induced stimulation of c-*myc* expression, previously noted in breast cancer cells *in vitro* (Dubik *et al.*, 1987), was confirmed. The expression of c-*myc* and p53 at elevated levels in the xenograft tumours in response to oestrogen suggest that *in vivo* the expression of these two nuclear genes may be involved in cell cycling (Kelly & Seibenlist, 1985; Lamb & Crawford, 1986). *In vitro* work (Brown *et al.*, 1984) identified an increase of pS2 mRNA in response to oestrogen which was attributed to increased transcription. The biphasic response of pS2 mRNA noted in this study may reflect initial oestrogenic stimulus then, as the oestrogen achieves a peak, inhibition of pS2 transcription, with subsequent pS2 stimulation as the serum oestrogen returns to more physiological levels.

Similarly, a decrease in TGF-beta transcription has been noted *in vitro* in response to oestrogen treatment of MCF-7 cells (Dickson *et al.*, 1986). Both these effects were noted *in vivo* in response to oestrogen. In the present xenograft system, TGF-beta transcription increased as the mitogenic stimulus of oestrogen declined, compatible with the anti-proliferative effects noted on oestrogen receptor-positive breast cancer cell lines *in vitro* (Kerr *et al.*, 1989).

Clinical implications

Gene expression in the breast cancer cells, as detected by mRNA analysis, obviously changes when cells cultured *in vitro* grow as tumours *in vivo*. The physiological and clinical significance of *in vitro* observations have on occasion been controversial and may be difficult to interpret due to lack of

host-related determinants that affect tumour behaviour *in vivo* (Shafie & Grantham, 1981). Certainly, different effects on cell kinetics have been observed using MCF-7 cells *in vitro* compared to nude mouse xenografts (Brunner *et al.*, 1989).

The model we describe here provides information complementary to that obtained from *in vitro* work and from clinical studies, particularly in examining host–tumour cell interactions and in determining the role of gene expression in oestrogen-dependent breast tumour growth.

The MCF-7 xenografts in thymectomised and irradiated CBA strain mice therefore present a useful model for examining the *in vivo* behaviour of oestrogen-dependent breast cancer and has considerable potential for the study of the actions of therapeutic agents *in vivo*.

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