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Breast cancer is a disease where breast epithelial cells become refractory to appropriate growth and differentiation signals. It is likely that numerous genetic changes can contribute to malignant transformation, including mutations that alter the cell cylce regulatory machinery. We have therefore sought to characterize the function of both positive and negative cell cycle regulatory elements in normal and malignant breast epithelial cells. In particular, we have focused on cyclin E, a positive cell cycle regulators element already implicated in some breast malignancies and on a class of negative regulators of cyclin-dependent kinases (Cdks). Preliminary data indicate that both cylin E and Cdk inhibitors are important in breast malignancy.

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FOREWORD

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(5) INTRODUCTION

Breast cancer is a disease where breast epithelial cells become refractory to growth and differentiation signals. It is likely that numerous genetic changes can contribute to malignant transformation, including mutations that alter the cell cycle regulatory machinery. We have therefore sought to characterize the function of both positive and negative cell cycle regulatory elements in normal and malignant breast epithelial cells. In particular, we have focused on cyclin E, a positive cell cycle regulatory element already implicated in some breast malignancies and a class of negative regulators of cyclin dependent kinases (Cdks). To study the role of cyclin E in breast malignancy, we screened cell lines derived from breast carcinomas for cyclin E mutations. We have also created cyclin E mutations and studied their effects on cultured cells. Additionally, we have targeted cyclin E using antisense strategies to examine its essentiality. To investigate the role of Cdk inhibitors in regulation of proliferation of breast epithelial cells, we have (1) identified and studied Cdk inhibitors in other cell types where analysis is more straightforward and (2) attempted to extrapolate these results to breast epithelial cells to determine the relevance for cell cycle control in this system.

(6) BODY

(a) SOW Task 1: Characterization of Cdk inhibitor production in normal and malignant breast epithelial cells (months 1-24).

This project was terminated as of the previous reports.

(b) SOW Task 2: Characterization of cyclin/Cdk complexes in normal and malignant breast epithelial cells (months 1-36).

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This project was completed/terminated as of the previous reports.

(c) SOW Task 3: Test of essentiality of cell cycle regulatory components in breast epithelial cells by antisense (months 1-12).

We planned to use three antisense methods to test for essentiality of cyclin E and, eventually, other proteins: antisense deoxyoligonucleotides, antisense ribozymes and antisense-encoding adenoviruses. Although initial results using oligonucleotides were discouraging, we then obtained reasonable results using C-5 propyne-modified pyrimidine substituted oligonucleotides (1). The antisense oligonucleotide significantly reduced the level of cyclin E relative to the control oligonucleotide at all time points. However, FACS analysis of the same cells indicated that severe reduction in cyclin E levels was having little, if any, impact on rate of entry into S phase or progression through the cell cycle. However, when immunoprecipitation cyclin E/Cdk2 kinase assays were performed on the same lysates, it was clear that even though the antisense treated lysates had much less cyclin E than controls (<20%), the cyclin E-associated kinase activity was similar. Thus, the specific activity of the kinase was elevated to compensate for the reduction in cyclin E levels. It appeared that the specific activity of Cdk2 in cells deprived of cyclin E is modulated at the level phosphorylation of Y15. We are currently investigating this at the mechanistic level. However, it was clear from these results that more efficient antisense oblation of the cyclin E mRNA was necessary in order to have an impact on the kinase activity. We therefore turned to a new oligonucleotide chemistry known as the "G-clamp". Using such oligonucleotides directed against cyclin E, it was possible to eliminate virtually all of the cyclin E protein and to maintain cyclin E-associated kinase activity at no higher than basal G0 levels (Figs. 1 and 2). However, in

synchronized breast epithelial cell cultures, there was only a slight impact on the rate of entry into S phase (Fig. 3). These data suggest, contrary to current thinking, that cyclin E and cyclin E-associated kinase activity are not rate-limiting for entry into S phase.

Methods G-clamp anti-sense and mismatch oligonucleotides corresponding to the cyclin E cDNA were synthesized by Gilead Pharmaceuticals and transfected into asynchronous 184A1 cells using cationic lipid. The chemistry is proprietary at this point but will be published shortly. Cells were either harvested to prepare protein for Western blotting or for FACS analysis.

Since several reports suggest that abnormal accumulation of cyclin E may have a role in the etiology of breast cancer (2,3), we have also taken the liberty of investigating the physiology of cyclin E accumulation. Even though this was not explicit in the original proposal, we feel that it is well within the scope of the project.

We identified cyclin E mutations that lead to hyperaccumulation of cyclin E, analysis of which indicated that cyclin E ubiquitination and turnover are regulated by autophosphorylation of cyclin E/Cdk2 on Thr380 of cyclin E (4). Mutating this residue leads to increased half-life and persistent accumulation of cyclin E in mammalian cells. We have now shown that the consequences of this is perturbed progression through S phase, more importantly, genetic instability. Conditional constitutive expression of wild-type and stabilized mutant alleles of cyclin E in Rat-1 fibroblasts allows enhanced chromosome loss (Figs. 4 and 5, Table 1), although chromosome loss is greater for the mutant. Other cyclins, e.g. cyclin D1, do not cause chromosome loss in this system (Fig. 6). We are now performing similar experiments in 184A1 human breast epithelial cells.

Methods: Standard molecular biological approaches were employed. Mutant and wild type cyclin E alleles were expressed in Rat-1 fibroblasts and are being expressed in 184A breast epithelial cells using the tetracycline repressible system (5). Chromosome loss was measured by growing cells in the presence or absence of tetracycline for 4 weeks, photographing mitotic chromosome spreads, and directly counting the number of chromosomes per spread.

In order to determine if elevation of cyclin E in breast cancer was correlated with defects in proteolysis of cyclin E, cyclin E levels were measured in a panel of cell lines derived from human breast cancers. A significant portion of these were shown to express elevated levels of cyclin E compared to non-transformed breast epithelial cell lines (Fig. 7). However, most of these cell lines showed no evidence of elevation of cyclin E mRNA levels, suggesting a defect in cyclin E turnover (Fig. 8). In order to determine if the putative proteolysis defect corresponded to mutations in the C-terminal domain of the cyclin E protein, which is the target of the proteolytic machinery, an SSCP analysis was undertaken for these high cyclin E cell lines. Although a sequence polymorphism was found, no mutations were evident from this analysis. Therefore, we concluded that other alterations in the ubiquitin-mediated proteolysis machinery were responsible for the accumulation of cyclin E.

In the course of analysis of cyclin E biosynthesis, we discovered that folding of the cyclin E polypeptide depends on the eukaryotic chaparonin, known as CCT (complex containing T-polypeptides). Maturation of newly translated cyclin E was found to require interaction with the CCT in an ATP-dependent folding reaction in order to become stable and to bind Cdk2. However, we were not able to attribute any regulatory significance to this process (6).

Methods: Standard molecular biological methods were employed.

(d) SOW Task 4: Cloning and characterization of Cdk inhibitors from HeLa cells (months 1-36).

In conjunction with our investigation of the stoichiometry of the inhbition of cyclin/Cdk complexes by the inhibitor p27, we performed a parallel study on the related inhibitor p21, which is also expressed in HeLa cells. This is an important issue since it has been claimed that p21 requires a stoichiometry of 2:1 to inhibit Cdk complexes. Our data, using both biophysical (equilibrium sedimentation analysis) and biochemical methods on purified recombinant proteins, indicate that p21 is inhibitory for cyclin A/Cdk2 kinase complexes at a 1:1 stoichiometry (Fig. 9; 7).

Methods: Standard molecular biological and biochemical methods were employed. To measure molecular masses of p21/Cdk complexes, equilibrium sedimentation analysis was performed using a Beckman XL-1 analytical ultracentrifuge.

(e) SOW Task 5: Cloning and characterization of breast epithelial cell Cdk inhibitors (months 12-48).

This project was terminated as of the previous report.

(f) SOW Task 6: Characterization of Cdk inhibitors in vivo (months 24-48).

This project was terminated as of the previous report.

(7) CONCLUSIONS

We could provide no compelling evidence that our initial hypothesis that Cip/Kip family Cdk inhibitor proteins were important for regulation of the cell cycle in human breast epithelial cells. Although these cells express p21 and p27 to some degree, they do not seem to be implicated directly in the regulatory modes that we investigated. p57, and a probable inactive derivative, p25, were also present in breast epithelial cells, but appear to be a symptom of approaching senescence. On the other hand, p15, a member of INK4 family of Cdk inhibitors appears to be involved in cytokine-mediated cell cycle arrest of breast epithelial cells. However, since others are pursuing this aspect of inhibitor action, we have chosen not to.

With regard to analysis of Cdk complexes from breast tumor derived cell lines, we found that cyclin E/Cdk2 complexes from one MDA-MB-231, were resistent to inhibition by members of the Cip/Kip family. We cloned a cyclin E variant from this cell line that had a three-amino acid deletion. However, when recombinant cyclin E complexes were prepared containing this variant or wild type, there was no difference between the two, indicating that resistance to inhibitors of the endogenous complexes must be due to other, as yet, indeterminate factors.

Since we cloned human p27 in the context of this project, we have continued to investigate its regulation. In particular, we have determined that translational control rather than transcriptional control is the primary mode of regulation in many different cell cycle regulatory contexts. Correlative data suggest that regulation of ribosomal function by pp70 S6 kinase may be involved, although the regulation of p27 translation by this kinase would be the converse to what has been demonstrated for other translationally-regulated mRNAs so far. In addition, analysis of the 5' UTR of the p27 mRNA is

consistent with possible translational regulation. We have analyzed the inhibitory stoichiometry of both p27 and the related inhibitor p21, and found, contrary to prior reports, that one molecule of either inhibitor is sufficient to inhibit a kinase complex consisting of cyclin A and Cdk2.

A major effort is now being focused on understanding the function and regulation of cyclin E. Antisense work has suggested that cyclin E is not normally rate-limiting for the G1 to S phase transition in breast epithelial cell lines, and may not even be essential, which runs counter to accepted dogma for mammalian cells, in general. This is an important issue if one is to consider targeting cyclin E/Cdk2 in a therapeutic context. A complementary issue that we are focusing on is whether abnormal accumulation of cyclin E might be important in the context of malignant transformation and breast cancer, in particular. We have found that point mutations in cyclin E can stabilize the protein dramatically *in vivo* and, more interestingly, lead to genetic instability. In particular, chromosome loss, which we have observed in the context of persistent cyclin E overexpression in our model system, could lead to accelerated LOH and malignancy. This idea is consistent with studies on transgenic mice that overexpress cyclin E in the mammary epithelium during pregnancy and lactation. These mice develop a high incidence of mammary carcinoma after a latency period of about a year(3). Therefore, we are investing a significant effort in understanding how deregulation of cyclin E accumulation leads to loss of cell cycle control and genetic instability. We have also analyzed cyclin E from tumor derived cell lines and determined that stabilizing mutations are not present, suggesting alterations in the proteolytic machinery that normally targets cyclin E.

Bibliography

1. Wagner, R.W., Matteucci, M.D., Lewis, J.G., Gutierrez, A.J., Moulds, C., and Foehler, B.C. (1993) Antisense inhibition by oligonucleotides containing C-% propyne pyrimidines. Science 260:1510-1513.

2 . Keyomarsi, K., and Pardee, A.B. (1993) Redundant cyclin overexpression and gene amplification in breast cancer cells. Proc. Natl. Acad. Sci. USA 90: 1112-1116.

3. Bortner, D.M., and Rosenberg, M.P. (1996) Induction of mammary gland hyperplasia and carcinomas in transgenic mice expressing human cyclin E. Mol. Cell. Biol. 17: 453-459.

4. Won, K.-A, and Reed, S.I. (1996) Activation of cyclin E/CDK2 is coupled to site-specific autophosphorylation and ubiquitin-dependent degradation of cyclin E. EMBO J. 15:4182-4193.

5. Gossen, M., and Bujard, H. (1992) Tight control of gene expression in mammalian cells by tetracycline-responsive promoters. Proc. Natl. Acad. Sci. USA 89: 5547-5551.

6. Won, K.-A., Schumacher, R.J., Farr, G.W., Horwich, A.L. and Reed, S.I. (1998) Maturation of human cyclin E requires folding by the eukaryotic chaperonin CCT. Mol. Cell. Biol., in press.

7. Hengst, L., Lashuel, H.A., and Reed, S.I. (1998) Complete inhibition of Cdk/cyclin by one molecule of $p21^{Cip1}$, Genes Dev., in press.

PUBLICATIONS:

1. Hengst, L, Dulic, V., Slingerland, J.M., Lees, E., and Reed, S.I. (1994) A cell cycle-regulated inhibitor fo cyclin-dependent kinase. Proc. Natl. Acad. Sci. USA 91:5291-5295.

2. Hengst, L., and Reed, S.I. (1996) Translational control of p27Kip1 accumulation during the cell cycle. Science 271: 1861-1864.

3. Won, K.-A, and Reed, S.I. (1996) Activation of cyclin E/CDK2 is coupled to site-specific autophosphorylation and ubiquitin-dependent degradation of cyclin E. EMBO J. 15:4182-4193.

4. Won, K.-A., Schumacher, R.J., Farr, G.W., Horwich, A.L. and Reed, S.I. (1998) Maturation of human cyclin E requires folding by the eukaryotic chaperonin CCT. Mol. Cell. Biol., in press.

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Figure legends:

Fig. 1. Cylin E western blot of synchonized human breast epithelial cells treated with antisense and missense G-clamp oligonucleotides. 184A1 cells were rendered quiescent by withdrawal of serum and EGF for 48 hours, transfected with C-5 propyne substituted antisense or control oligonucleotides and then stimulated to reenter the cell cycle. Cyclin E levels were monitored by Western blot as a function of time after addition of serum and EGF. Cyclin E accumulation was strongly inhibited by the antisense but not the control mismatch (Mm) oligonucleotide.

Fig. 2 Cyclin E kinase activity in synchronized human breast epithelial cells treated with antisense and missense G-clamp oligonucleotides. Same is Fig. 1 except that kinase activity was measured in cyclin E immunoprecipitates using histone H1 as a substrate. Direct quantitation by PhosphorImager analysis is shown below.

Fig. 3. Reduction of cyclin E levels does not signifcantly affect entry into S phase. Flow cytometric analysis was performed on the experiment described in Fig. 1 to determine the rate of entry of stimulated cells into S phase after transfection with cyclin E antisense or control oligonucleotides. Although cyclin E accumulation was strongly inhibited by transfection with the antisense oligonucleotide, there was no signicant impact on the rate of entry into S phase.

Fig. 4. Constitutive over-expression of cyclin E in Rat-1 cells leads to elevated levels of chromosome loss. Cells expressing cyclin E under control of the tetracycline repressible promoter were cultured for four weeks either in the presence or absence of tetracycline. Mitotic chromosome spreads were then prepared. The histogram represents the data from

total chromosome counts for 100 mitotic spreads +/- tetracycline. Based on these data, approximately two out of three cells expressing the mutant cyclin E allele lost a chromosome.

Fig. 5. Constitutive overexpression of a stabilized mutant allele of cyclin E leads to elevated levels of chromosome loss. Same as Fig. 4., except that the T380A allele was expressed. In this experiment almost every cell lost a chromosome.

Fig. 6. Constitutive overexpression of a stabilized mutant allele of cyclin D1 does not lead to chromosome loss. Same as Fig. 4, except that cyclin D1 was expressed.

Fig. 7. Many human breast cancer derived cell lines have elevated levels of cyclin E. Western blot of equivalent amounts of protein developed with cyclin E specific antibodies. 184A1-1 corresponds to the non-transformed control. MDA-MB-157 has been shown to contain an amplification at the cyclin E locus.

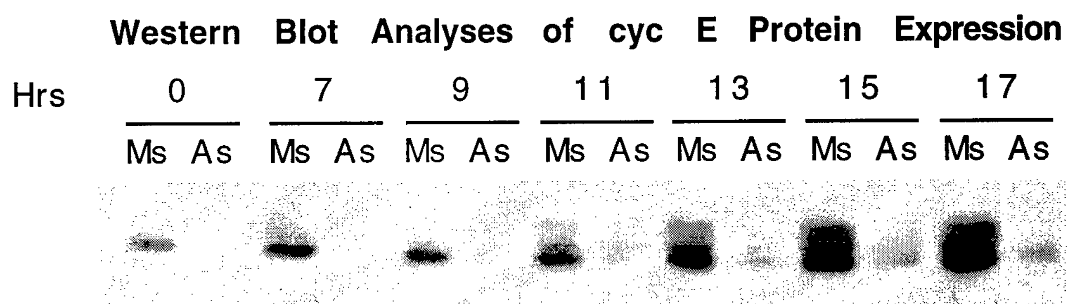
Fig. 8. Many of the human breast cancer derived cell lines that have elevated cyclin E levels do not have elevated cyclin E mRNA levels. Northern blot analysis of a subset of the cell lines analyzed in Fig. 7.

Fig. 9. Determination of the molecular weight of purified cyclin A/Cdk2 (left panels) and p21/cyclin A/Cdk2 complexes (right panels) by sedimentation equilibrium centrifugation. The apparent molecular weight of cyclin A/Cdk2 kinase complex was determined as 86 +/- 2 kDa and increases to 105 +/- 9 kDa after saturation of the complex with p21. The lower panels show the raw concentration data determined by measuring the absorbance at 235 nm (cyclin A/Cdk2) or 280 nm (p21/cyclin A/Cdk2). The solid line drawn through the data points was obtained by fitting the fringe displacement versus radial position to a

single species model. The residual difference between the experimental data and the fitted data for each point is shown in the upper panel.

Table 1. Compilation and error analysis of three independent chromosome loss experiments using wild-type cyclin E, as described in Fig. 4. On the average, one chromosome was lost per three cells.

Figure 1



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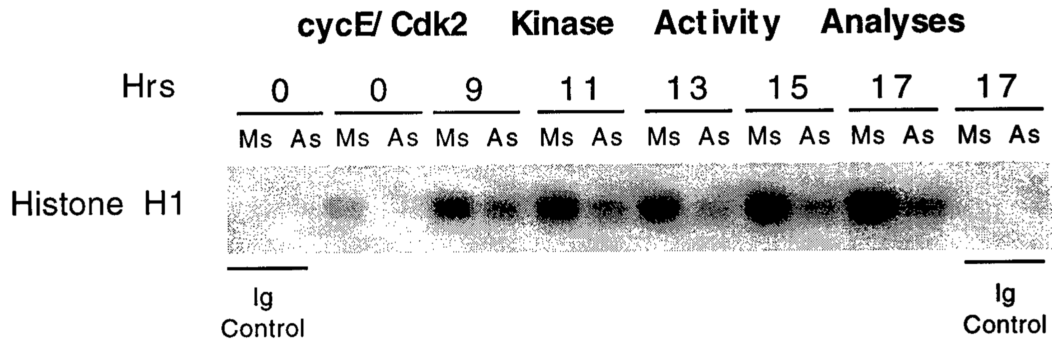


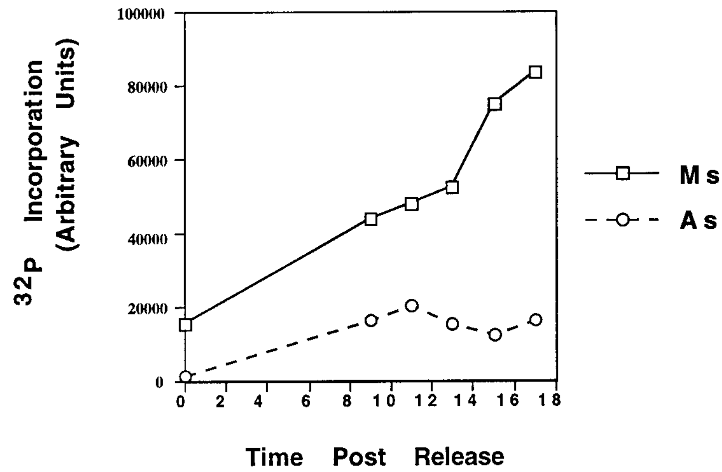
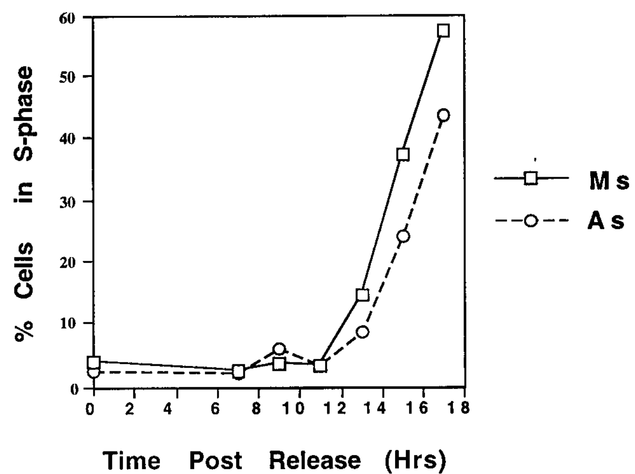


Figure 3

Analyses of the Rate of Entry into S-phase



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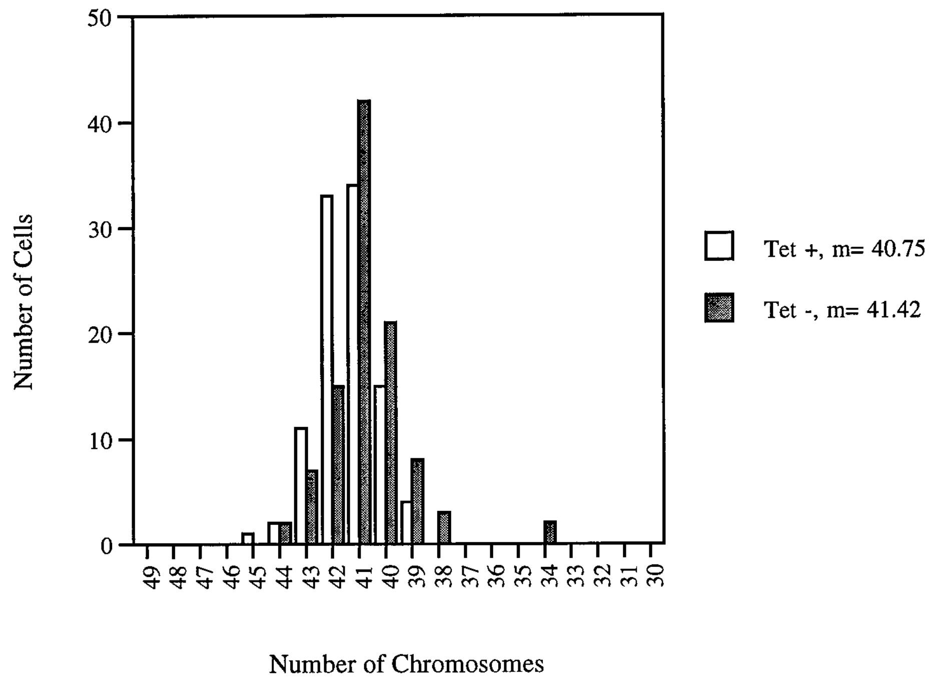
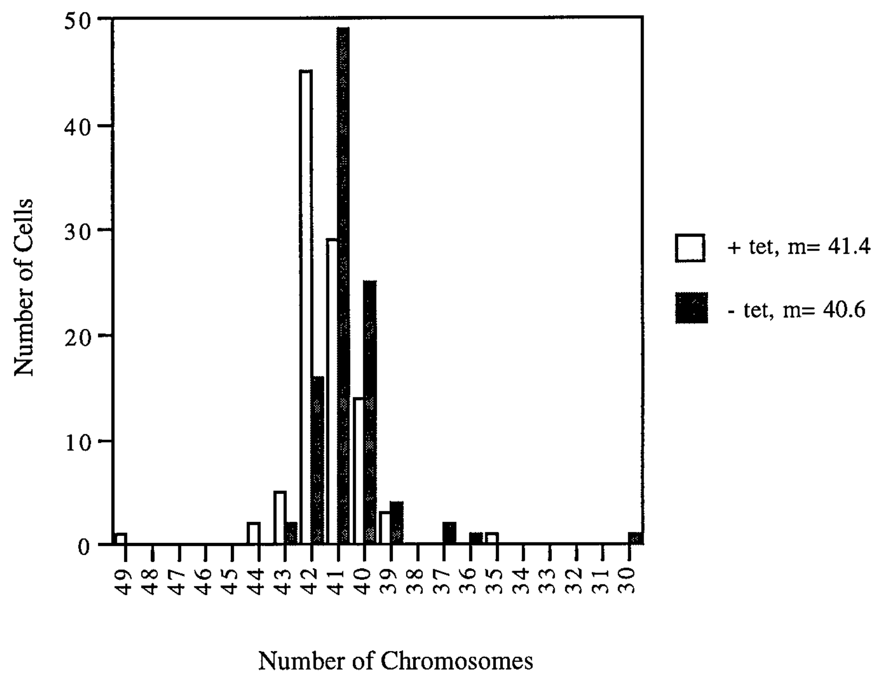


Figure 5



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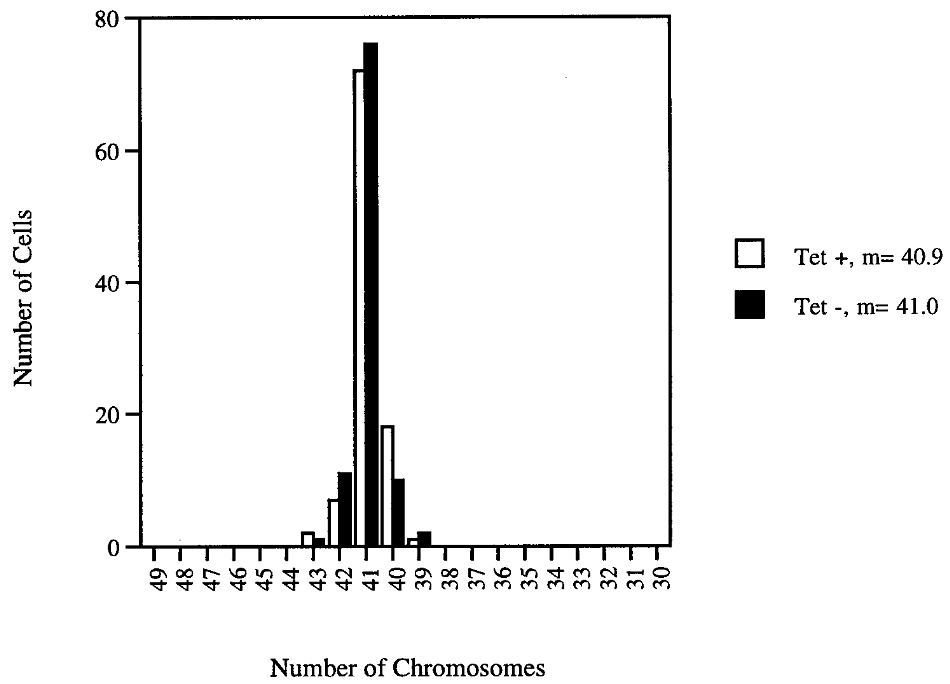

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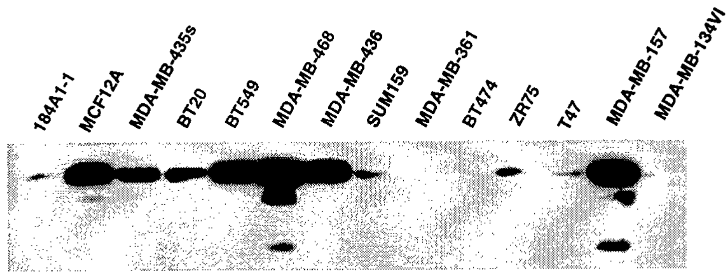


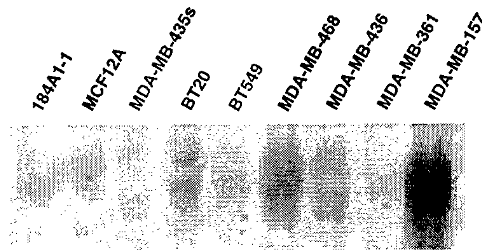
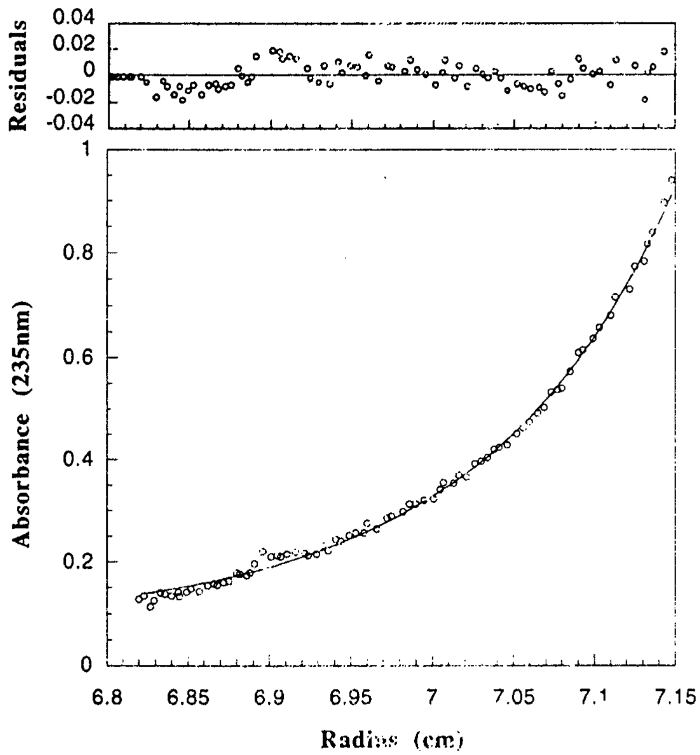


Figure 8

Figure 9



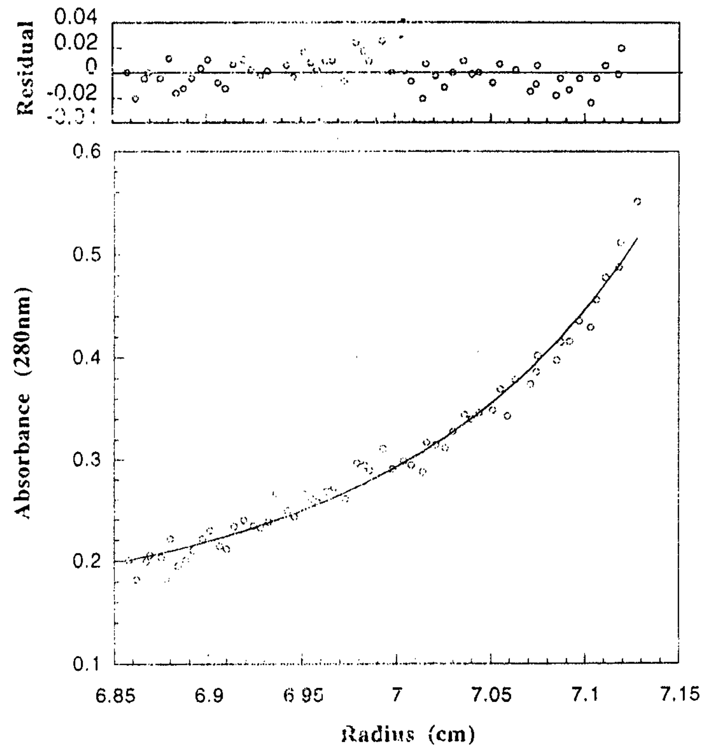


Table 1

Cyclin E Induced Chromosome Loss

	Average Number of Chromosomes	Mean ± σ
Cyclin E -	41.50 41.35 41.42	41.42 ± 0.08
Cyclin E +	41.35 41.09 40.75	41.06 ± 0.30