The Effects of the Deregulated Expression of the Cloned Transcription Factor E2F-1 on Chinese Hamster Ovary Cells

Thesis by

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In Partial Fulfillment of the Requirements
for the Degree of
Doctor of Philosophy

California Institute of Technology
Pasadena, California

1995
(Submitted April 28, 1995)

to my family

ACKNOWLEDGEMENTS

As a scientist and person, I feel very strongly that we are a product of our interactions with people as much as of our formal education. Therefore, it is important for me to give my thanks to the many people who have contributed directly and indirectly to this thesis. Most importantly, I would like to thank Mom, Dad, and Dave. The years in graduate school have not been easy for me and I know that I could not have conceived of writing a thesis without the support - both emotional and financial - and love from a family who understands not only the meaning of love, but also the meaning and purpose of education.

Jay. The trust that Jay Bailey has shown me has far exceeded the amount many advisors would give to their students. Jay has given me the opportunity to establish an entire mini-lab. Few graduate students can claim such responsibility as part of their education and fewer still can claim success. Thanks for everything Jay. Mike. Luckily, my education has crossed intellectual, cultural, geographic, and emotional borders. Without Mike, Liz, and the whole Harrington clan, I could not have crossed any of these bridges. I look forward to our coming years together with great anticipation and excitement. Adriana. Without Adriana Guerini's hands, this project would never exist. Adriana cannot be thanked enough for her help with transfections and cell culture technique, in general. I will never have an officemate, groupmate, or friend as sweet as you! Bernard. Two floors above where Adriana and I sit is Bernard Witholt. The personal

frustration that I felt at particular times while at ETH was tendered by you, among others. Thanks for listening...

"The gene for unbridled dedication to a lost cause will always overwhelm the pure logic genes."

- D. Koshland, Science, **265**: 1639 (1994).

...Vassily. Speaking of dedication: there isn't enough thanks for a person with your qualities - both bad and good. In the end, I know that you will be judged as one of the most exceptional scientists, friends, and humans around (and that's not a compliment, it's the truth). Conny. Conny your incredible talents in technical support (especially in purification of DNA and 2D gels), friendship, and patience can never be repaid. Thank you.

Beyond these most immediate people is another group of people and organizations which have directly contributed to my thesis work. Bob Grubbs and Julie Kornfield have provided the kind of support that each and every graduate student should have from committee members. The National Science Foundation and the Swiss Priority Program in Biotechnology have provided extensive financial support. Charles Weissmann, Hans Eppenberger, and Lee Hood have given their money, time, and reputation as support. Adria, Kathy, and Patricia have been exceptional staff members.

I would also like to briefly acknowledge the following people, in no particular order: Yevgeny for supporting Vassily, Todd for the talks about Switzerland, Phil, Becky, and Kyle for being truly decent people, Helen, Jina, and Miki for their technical expertise, Tom for helping me keep things in perspective, Karen McCarthy, Susanne, Erika, Lois, Jeanne, and Myrtha for being exceptional staff members and Helena for being even more than that, Wolfgang Renner for his ideas and friendship, Neilay, Wolfgang Minas (especially for help with the FACS, PCs and lots of other things), Wolfgang Krömer, Wilfred, Miguel, Pauli, Uwe, Kathy, Christian, Peter (especially for the confocal microscopy), Ellen (especially for help with the FACS), Pablo, Ferrucio, and Lisa for being more than groupmates need to be, Claire for being an American, Dana and Julie for the advice, friendship, and love when I needed it most, CBD, Brian, Laura, Holly, Jen, Barb, Rene, Deanna, JYC, Kevin, Bernadette, Celica, Susan Henderson, Morgan, and Susan Soong for the friendship, and Alex for going out of his way to help my work.

I look to the sea
Reflections in the waves spark my memory
Some happy, some sad
I think of childhood friends
And the dreams we had
A gathering of angels
Appeared above our heads
They sang to us this song of hope and
This is what they said
Come sail away, Come sail away
Come sail away with me
I thought that they were angels
But to my surprise
We climbed aboard their starship
And headed for the skies
-Styx, 1977

Mon 1/6/92

Kelvin,

I'd like to see you TODAY if at all possible. Please stop by my office ASAP to set a time. Thanks.

Jay

ABSTRACT

E2F-1 is one of a family of transcription factors known to be involved in cell cycle regulation at the G1/S phase transition. Several proteins intimately involved in DNA synthesis are regulated by the E2F transcription factor family. It is believed that the presence of free E2F-1 in mammalian cells is required to activate transition of cells through the G1-S restriction point of the cell cycle. Stable clones from CHO K1 cells transfected with an expression vector for human E2F-1 were created. All of the clones expressed significantly higher amounts of E2F-1 than control cells as determined by Western analysis. Several clones were further studied by confocal microscopy and Southern analysis. Both of these studies also provide evidence for the expression of cloned E2F-1 in these CHO cells. CHO K1:E2F-1 cells are able to proliferate in well-defined medium completely free from proteins or serum and flow cytometric analysis of CHO K1:E2F-1 cells indicates a prolonged S-phase compared to CHO cells grown by bFGF stimulation and by cyclin E overexpression. Two-dimensional gel electrophoresis (2DE) of CHO cellular proteins reveals increased expression of 236 spots indicating significant regulatory effects for cloned E2F-1 in CHO cells. These 2DE results also suggest new routes to identification of gene products regulated by a particular transcription factor. Expression of cloned E2F-1 is an important means for bypassing the serum requirement of mammalian cell culture. Metabolic engineering of cell cycle regulation

bypasses exogenous growth factor requirements, addressing a priority objective in economical, reproducible, and safe biopharmaceutical manufacturing.

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

APS	ammonium persulfate
bFGF	basic fibroblast growth factor
BME	2-mercaptoethanol
CDI	cyclin dependent kinase inhibitor
CDK	cyclin dependent kinase
CHAPS	3-([3-chol-amido-propyl)-dimethyl-ammonio]-1-propane sulfonate
CHO	Chinese hamster ovary
CSF	cerebrospinal fluid
DMF	dimethylformamide
DOTAP	N-[1-(2,3-Dioleoyloxy) propyl]-N,N,N-trimethyl-ammoniummethylsulfate
DTT	dithiothreitol
ECL	enhanced chemilluminescence
FCS	fetal calf serum
IEF	isoelectric focusing
IPG	immobilized pH gradients
NP-40	Nonidet P-40
PBS	phosphate-buffered saline
PBST	phosphate-buffered saline plus Tween 20
PDA	piperazine diacrylamide
pRb	retinoblastoma gene product
PTFE	polytetrafluoroethylene

PVDF	polyvinylidene difluoride
REF	rat embryo fibroblast
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
TBS	tris-buffered saline
TBST	tris-buffered saline plus Tween 20
TEMED	N,N,N',N'-tetramethylethylenediamine
t-PA	tissue plasminogen activator
2DE	two-dimensional electrophoresis

CHAPTER ONE

Introduction

The path of the righteous man is beset on all sides by the inequities of the selfish and the tyranny of evil men. Blessed is he who, in the name of charity and good will, shepherds the weak through the valley of the darkness. For he is truly his brother's keeper and the finder of lost children. And I will strike down upon thee with great vengeance and furious anger those who attempt to poison and destroy my brothers. And you will know I am the Lord when I lay my vengeance upon you.

-Quentin Tarantino, 1995

1.1 Cell Culture and the Biotechnologist

The production of biopharmaceuticals by animal cell culture is a multibillion-dollar-a-year industry that employs physicians, biologists, managers, and chemical engineers. Knowledge of fundamental principles of chemical engineering is required for the effective production of pharmaceuticals whether by chemical synthesis, purification from natural sources, or recombinant protein production. The existing knowledge base of the molecular biology of living organisms is expanding from basic research in biology, biotechnology, and the Human Genome Initiative. Consequently, recombinant protein production is becoming an increasingly important means for pharmaceutical production. Table 1 (from Peto et al. 1994) gives some examples of recombinant protein products derived from animal cell culture.

Chemical engineers have an important niche in the realm of recombinant protein production in both scale up and downstream

processing. Further, armed with expertise in molecular biology, genetic engineering, and biochemistry, the chemical engineer is prepared to discern where potential problems may arise in cell culture and to address these problems at many different levels. An illustrative but incomplete list of these problems includes the creation of novel lead compounds, the design of new instrumentation to study biological systems, the metabolic engineering of new organisms better suited for recombinant protein production, the purification of a desired compound, and quality control.

This thesis work begins to address a problem fundamental to recombinant protein production by a particular mammalian cell culture system: the Chinese hamster ovary (CHO) cell (Puck et al. 1958). This particular cell type has been investigated in many research and industrial laboratories. Among the reasons for its use as a model system are its relatively fast growth rates, high cloning efficiency, ease of transfection and gene amplification, and satisfactory post-translational processing.

One of the most important elements of a CHO cell culture system is the growth medium used. The discovery that mammalian cells could be cultured in vitro led to attempts at defining the components necessary for sustained growth. Early attempts at media definition culminated in the basal medium of Eagle (1955). Other attempts at creating a defined medium include CMRL 1066 of Parker et al. (1957), 199 of Morgan et al. (1950), and Ham's F12 (1965). There are many characteristics of the medium which are important not only for maintaining growth, but also for optimizing growth

rates; these include pH, buffering capacity, osmolality, temperature, viscosity, and surface tension and propensity for foaming. However, all of the above "defined" media must be supplemented with between 5% and 20% serum (typically fetal calf serum) to obtain growth of cells in culture at any pH, temperature, etc. The importance of basal medium is thus reduced due to the presence of the serum, whose many growth promoting components ultimately stimulate proliferation. Even after years of exhaustive research into matching specific cell lines to a particular medium (without serum), the choice of medium is often empirical and requires serum (Freshney 1994, p. 83).

Serum is derived from the approximately 8 percent of cows which come to slaughter bearing a calf. The blood serum from these calves is extracted and pooled into lots of several liters each. Because these calves are in development, their serum contains large amounts of growth factors and hormones which promote growth and development. It is estimated that a single fetus yields approximately 200mL of serum (Hodgson 1991). The serum is then sterilized and filtered before being subject to quality control.

The demand for serum for cell culture worldwide is about 486,000 liters annually worth over $100,000,000 (Hodgson 1993). Of this total, about half is used in the United States and about 65% is consumed by companies involved in human and animal healthcare for the production of various biopharmaceuticals (Hodgson 1993). The world market can supply some 500,000 liters per year of serum; however, the supply source can greatly affect

the demand for a particular serum. For example, in countries where cattle are known to be infected with prion-borne diseases such as bovine spongiform encephalopathy, serum cannot be exported for use in research because the agents causing these diseases are transmissible and resistant to typical inactivating methods such as ethanol, formaldehyde, hydrogen peroxide, gamma irradiation, and heat (Hodgson 1990). Therefore, the cost of serum is related to the source. Serum from New Zealand, for example, is approximately $350 per liter while serum from France and Germany, which cannot be used for production in the US, retails for approximately $200 per liter (Hodgson 1991).

1.2 Motivation

Elimination of serum and other animal-derived proteins from mammalian cell culture processes is a current priority for biopharmaceutical manufacturing and would be a great convenience for biological research. The cell culture process is relatively slow and expensive and often requires the use of fetal calf serum in the medium in order to effect mitogenic stimulation of the cultured cells. Unfortunately, the addition of fetal calf serum to the culture medium carries a risk of contamination by viruses, mycoplasma, and other undefined infectious species such as prion agents. Heat inactivation of serum can inactivate many but not all of these infectious contaminants. Moreover, minute amounts of various other

animal proteins present in a production system can cause allergenic reactions in individuals treated with the particular biopharmaceutical.

The addition of serum also complicates process consistency and downstream processing. Serum is an undefined mixture of various growth-modulating components (see Figure 1). While the addition of serum generally enhances the overall growth rate of cells, the high batch-to-batch variability requires extensive testing to verify similar growth characteristics. An important example of the effect of changing the serum source comes from the production of tissue plasminogen activator (t-PA) at Genentech. A switch in serum batches between roller bottle production and suspension culture resulted in a change in protease activity which shifted the ratio between the one- and two-chain forms of t-PA. This, in turn, changed the dose requirement and side-effect profile (Hodgson 1991). The undefined and complex nature of serum also makes purification of the desired product protein a difficult and expensive task.

It is clear from the problems mentioned above that the removal of animal blood serum from production-based cell culture systems is an important and pressing problem in biotechnology. One method for addressing this problem is to select mutants capable of growth in a serum- and/or protein-free environment. However, this is a long and highly variable process that necessarily results in cell lines with unknown genotypes. A more rational approach to the creation of serum- and protein-free growing cells is to engineer the cell's metabolism in such a way as to

bypass the intracellular signals that halt the cell cycle's progression when no serum is present.

1.3 The Scope of This Thesis

Contemporary biotechnology is a field that combines two related but complementary research areas. The first area involves the creation of new organisms and systems for use as a technology while the second emphasizes the engineering of new and improved instrumentation for the study of biological systems. Metabolic engineering is a subset of the former wherein rational genetic engineering manipulations are made to improve cell functions (Bailey 1991) and thus address process limitations. Often the ability to perform such manipulations comes directly from developments in molecular biology, genetics, and biochemistry. This thesis addresses a limitation involved in the production of biopharmaceuticals. The tools of genetics and molecular biology were used to make a rational manipulation of cellular metabolism: Chinese hamster ovary cells were transfected with an expression vector for E2F-1, a subunit of a transcription factor, E2F, known to be intimately involved in the regulation of the cell cycle. By overexpressing cloned E2F-1 in CHO cells, cell proliferation was activated on media completely free from protein and serum.

This thesis also benefitted from research in the area of instrumentation for the study of biological systems. Principles learned and experiments done in the improvements of two-dimensional electrophoresis (2DE) of proteins (see Appendix 1 and Appendix 2) have lent themselves well to the study of cell cycle regulatory proteins (see Chapter 4). As a result of these studies and mathematical modelling (Hatzimanikatis et al. 1995), E2F-1 was chosen from among a subset of potential cell cycle regulatory proteins (see Chapters 2 and Chapter 4) as the particular gene to constitutively overexpress and thus create a cell line capable of growth on protein-free media (see Chapter 3).

1.4 References

Bailey, J.E., Towards a Science of Metabolic Engineering, Science, **252**: 1668 (1991).

Eagle, H., The Specific Amino Acid Requirements of Mammalian Cells (Strain L) in Tissue Culture, J. Biol. Chem., **214**: 839 (1955).

Freshney, R.I., Culture of Animal Cells, 3 ed., Wiley-Liss, New York, 1994.

Ham, R.G., Clonal Growth of Mammalian Cells in a Chemically Defined Synthetic Medium, Proc. Natl. Acad. Sci. USA, **53**: 288 (1965).

Hatzimanikatis, V., Lee, K.H., and Bailey, J.E., manuscript in preparation 1995.

Hodgson, J., BSE Scares Up a Host of Sourcing Worries, Bio/Technology **8**: 990 (1990).

Hodgson, J., Checking Sources: The Serum Supply Secret, Bio/Technology **9**: 1320-1324 (1991).

Hodgson, J., Fetal Bovine Serum Revisited, Bio/Technology **11**: 49-53 (1993).

Morgan, J.G., Morton, H.J., Parker, R.C., Nutrition of Animal Cells in Tissue Culture I. Initial Studies on a Synthetic Medium, Proc. Soc. Exp. Biol. Med., **73**: 1 (1950).

Parker, R.C., Castor, L.N., McCulloch, E.A., Altered Cell Strains in Continuous Culture, Special Publications, NY Acad Sci, **5**: 303 (1957).

Peto, T., Holtorf, A.P., and Hermans, H., Beneficial Effects of Animal Cell Technology, Animal Cell Technology Industrial Platform, Rotterdam (1994).

Puck, T.T., Cieciura, S.J., Robinson, A., Genetics of Somatic Mammalian Cells. III. Long-Term Cultivation of Euploid Cells from Human and Animal Subjects, J. Exp. Med., **108**: 945-955 (1958).

1.5 Tables

Examples of vaccines, therapeutic and diagnostic products on the market (as of December 1993) made using animal cell technology.

Vaccines:

rabies

polio

rubella

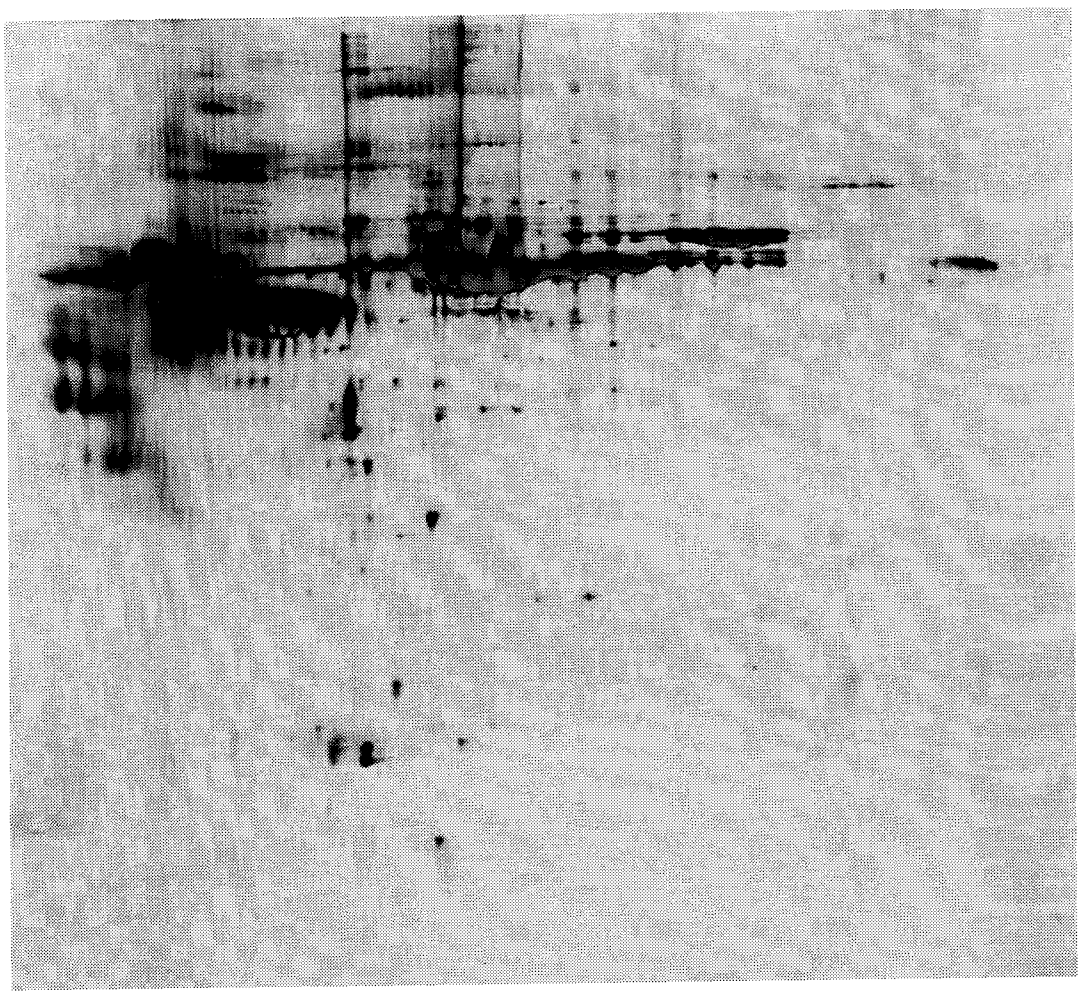
hepatitis-A

Therapeutics:	**Indication:**
tissue plasminogen activator	acute myocardial infarction
erythropoietin	anemia, cancer, AIDS
factor VIII	hemophilia A
DNAse	cystic fibrosis
diagnostic monoclonal antibodies	diagnosis of disease including colorectal and ovarian cancer
imaging	

1.6 Figures

Figure 1. Silver stained two-dimensional gel (2DE) of fetal calf serum from Life Technologies. This representative figure shows the complex nature of the protein content of fetal calf serum that is typically used in the culture of mammalian cells. Many of the components have unknown effects on the growth of cells in culture. The methods for 2DE are given in Chapter 4. Acidic proteins are to the left and basic proteins are to the right. Higher molecular weight species are at the top and lower molecular weight species are at the bottom.

Figure 1



CHAPTER TWO

Background:
Serum, The Cell Cycle, and Cancer

2.1 Essential Concepts

The primary function which serum serves in cell culture is to provide growth factors which are necessary to induce cell proliferation. Growth factors activate proliferation and regulate differentiation of mammalian cells through surface receptor-binding and subsequent signal transduction cascades which ultimately lead to DNA synthesis and cell division (reviewed in Nurse 1994, Sherr 1994, Heichman and Roberts 1994, King et al. 1994, Hunter and Pines 1994). Deregulated expression of some of the controlling molecules–from surface receptors to cell cycle regulators–correlates with tumorigenicity and cancer (reviewed in Hartwell and Kastan 1994, Marx 1994, Cross and Dexter 1991). While the detailed biochemical pathway between a particular external stimulus and the corresponding progression through a cell-cycle transition remains obscure for many growth factors, progression through the cell cycle is known to be regulated by cyclin-dependent kinases (Nurse 1994). Figure 1 (adapted from Hunter and Pines 1994) is a schematic representation of the interactions of several of the key proteins which control progression through the cell cycle.

The proteins from Figure 1 most relevant to this work are those which play a role during the G1 phase. As depicted, growth factors send signals to the cell cycle regulatory machinery via signal transduction cascades that involve proteins such as Ras, Raf, Myc, Fos, and Jun, all of which have been implicated in cancer. Many of the known growth factor signal transduction cascades unite in modulation of D-type cyclin-cyclin

dependent kinase (cdk) complex activity. Activated cyclin D-cdk complexes are able to phosphorylate the retinoblastoma gene product (pRb) which is bound to an inactive transcription factor, E2F. Upon hyperphosphorylation of pRb, E2F dissociates and is free to initiate transcription of targeted genes. Many of these genes are known to be involved in DNA synthesis including thymidine kinase, dihydrofolate reductase, and cdc2 (LaThangue 1994, see also Chapter 3). As the cells complete the cell cycle, pRb becomes dephosphorylated and is able to complex with free E2F thus blocking entry into another S phase. pRb is a known tumor suppressor, and D-type cyclins and their cdk partners are also involved in tumorigenesis. When the cyclin E-cdk2 complex is functional, it can bind to p107 and E2F, and this interaction also acts to release free E2F at the end of the G1 phase (Lees et al. 1992).

Beyond this basic level of control are cyclin-cyclin dependent kinase inhibitors (cdi's) which have also been implicated in cancer. p16 blocks the activation of D-cyclin-cdk complex which consequently blocks phosphorylation of pRb and the onset of S phase. p21, another oncogenic product, can inhibit cyclin E-cdk2 activity and is a target of p53. p21 transcription is activated by wildtype p53 but not mutant p53. 70% of colorectal cancers, 50% of lung cancers, and 40% of breast cancers carry p53 mutations (Culotta and Koshland 1993).

It is evident from the mechanism described above that cell cycle biology, cancer research, and growth factor research are fields that intersect at

the biomolecular level. Therefore, it should be noted that studies of the control of proliferation necessarily address problems in cancer research such as the development of new therapeutics, and problems in biopharmaceutical manufacturing such as serum removal from cell culture systems.

2.2 Cyclin E Appears to Mediate bFGF Stimulation in Chinese Hamster Ovary Cell Culture (Source: Renner, Lee, Hatzimanikatis, Bailey, and Eppenberger, in press, 1995)

Previous studies have suggested that some of the complex physiological changes that are regulated by mitogenic stimulation are mediated by the action of cyclins. D-type cyclins were shown to be induced by colony-stimulating factor 1 in mouse macrophage cells (Matsushime et al. 1994). Human diploid fibroblasts and Rat-1 fibroblasts transfected with an expression vector for human cyclin E exhibited a smaller decline in the percentage of S phase cells than did untransfected controls following transfer to medium with low serum concentration (Ohtsubo and Roberts 1993). In this study cells mitogenically stimulated by different growth factors for cyclin E expression were analyzed, a cell line for cyclin E overexpression was engineered, and the resulting phenotype was investigated.

The effects of insulin and basic fibroblast growth factor (bFGF) on the ability of Chinese hamster ovary (CHO) K1 cells to proliferate and on the morphology of these cells were studied. CHO cells were selected because they are widely used in the manufacture of protein therapeutics, they have been very well characterized as a cultured cell system, and they can be cultured in a completely defined, protein-free basal medium supplemented only with a single growth factor.

CHO K1 cells, which normally cannot proliferate in serum-free basal medium, were directly transferred from Ham's F12 Medium supplemented with 10% fetal calf serum (FCS) to the CHO cell line-specific basal medium FMX-8 supplemented with either 20ng/mL bFGF or 1µg/mL insulin. Proliferation continued following such a transfer without a significant reduction in specific growth rate for at least seven days. In contrast, the population ceased to proliferate following transfer to unsupplemented FMX-8. After four days of growth in FMX-8 medium supplemented with bFGF, high levels of endogenous cyclin E expression could be detected by immunoblot analysis. Only low level cyclin E expression could be detected by the same assay in cultures growing by insulin-stimulation (Renner et al. 1995).

CHO K1 cells in serum-containing medium exhibit a spread, epithelial sheet-like morphology with extensive cell-cell contacts. In the serum-free medium, the morphology of these cells was affected by the type of mitogenic stimulation. Cells stimulated with insulin showed a

morphology similar to serum-stimulated cells (Figure 2i). After three days, cells growing in medium supplemented with bFGF showed an altered, rounded-up cell morphology and a loss of cell-cell contacts (Figure 2ii). At the end of the culture period, a considerable number of cells completely detached and grew in suspension.

To test whether cyclin E might be one of the cell cycle regulators important for proliferation and altered morphology due to bFGF stimulation, the effect of exogenous bFGF stimulation on morphology was simulated by expression of recombinant human cyclin E in CHO K1 cells. The cDNA for human cyclin E was expressed using the vector pRc:cycE constructed from the pRc/CMV vector (Invitrogen). CHO K1 cells were transfected by Lipofection (Gibco). One day after transfection, the cells were transferred to the serum- and protein-free FMX-8 medium. The populations transfected with pRc:cycE continued to proliferate on basal media, and cell clones with a rounded-up cell shape could be observed. Instead of forming cell-cell contacts and the typical epithelial sheet-like shape of the original cell line, cyclin E transfectants grew as single cells with a morphology indistinguishable from that of untransfected cells grown in basal medium supplemented with bFGF (Figure 2iii).

In the first four weeks following transfection, cells were diluted at ratios of 1/2 to 1/5 per week; later, this cell line could be split 1/40 per week. This cell line, designated CHO K1:cycE, expresses cyclin E without bFGF stimulation in FMX-8 alone as well as in FMX-8 supplemented with bFGF or

insulin or both together (Renner et al. 1995). Addition of insulin did not revert the morphological phenotype of CHO K1:cycE cells.

The experiments presented provide strong evidence for a key role of cyclin E in the growth phenotype of CHO cells. The similarity of the growth and morphology of CHO cells transfected with cloned cyclin E to the response of the cells stimulated by bFGF suggests that cyclin E plays a pivotal role in mitogenic stimulation by bFGF. These results clearly indicate that cyclin E is a major determinant of growth factor response and the contact behavior of CHO K1 cells. Cyclin E deregulation, as observed in many tumor cells (Keyomarsi and Pardee 1993), might therefore be an important element in the process of cellular transformation.

2.3 References

Cross, M. and Dexter, T.M., Growth Factors in Development, Transformation, and Tumorigenesis, Cell, **64**: 271 (1991).

Culotta, E. and Koshland, D.E., p53 Sweeps Through Cancer Research, Science, **262**: 1958 (1993).

Hartwell, L.H. and Kastan, M.B., Cell Cycle Control and Cancer, Science, **266**: 1821 (1994).

Heichman, K.A. and Roberts, J.M., Rules to Replicate By, Cell, **79**: 557 (1994).

Hunter, T. and Pines, J., Cyclins and Cancer II: Cyclin D and CDK Inhibitors Come of Age, Cell, **79**: 573 (1994).

Keyomarsi, K. and Pardee, A.B., Redundant Cyclin Overexpression and Gene Amplification in Breast Cancer Cells, Proc. Natl. Acad. Sci. USA, **90**: 1112 (1993).

King, R.W., Jackson, P.K., and Kirschner, M.W., Mitosis in Transition, Cell, **79**: 563 (1994).

LaThangue, N.B., DRTF1/E2F: An Expanding Family of Heterodimeric Transcription Factors Implicated in Cell-Cycle Control, TIBS, **19**: 108 (1994).

Lees, E., Faha, B., Dulic, V., Reed, S.I., and Harlow, E., Cyclin E/cdk2 and Cyclin A/cdk2 Kinases Associate with p107 and E2F in a Temporally Distinct Manner, Genes Dev., **6**: 1874 (1992).

Marx, J., How Cells Cycle Toward Cancer, Science, **263**: 319 (1994).

Matsushime, H., Quelle, D.E., Shurtleff, S.A., Shibuya, M., Sherr, C., and Kato, J.Y., D-Type Cyclin-Dependent Kinase Activity in Mammalian Cells, Mol. Cell Biol., **14**: 2066 (1994).

Nurse, P., Ordering S Phase and M Phase in the Cell Cycle, Cell, **79**: 547 (1994).

Ohtsubo, M. and Roberts, J.M., Cyclin-Dependent Regulation of G1 in Mammalian Fibroblasts, Science, **259**: 1908 (1993).

Renner, W., Lee, K.H., Hatzimanikatis, V., Bailey, J.E., and Eppenberger, H., in press 1995.

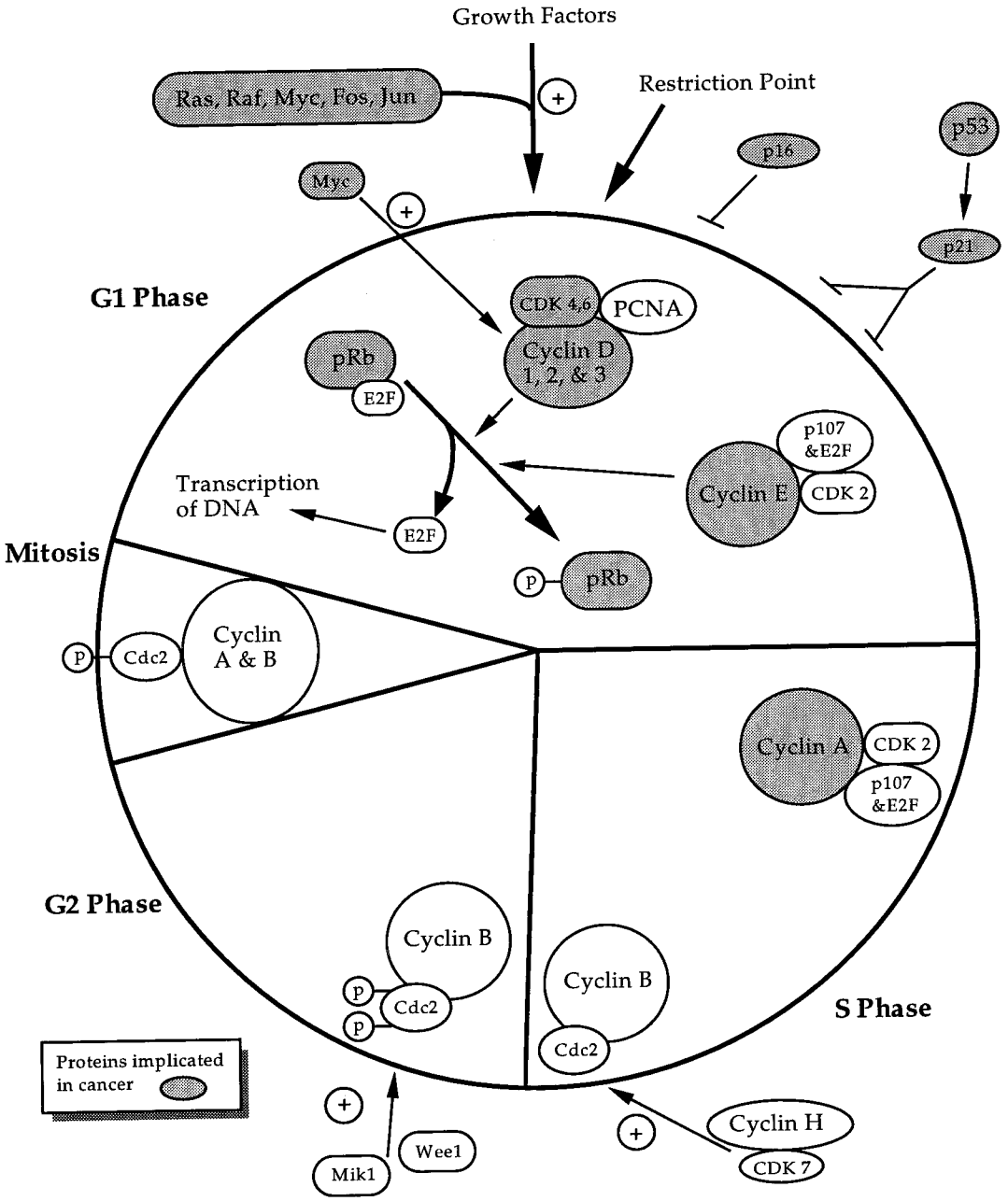
Sherr, C.J., G1 Phase Progression: Cycling on Cue, Cell, **79**: 551 (1994).

2.4 Figures

Figure 1. Schematic depiction of the phases of the cell cycle and the interactions of several key cell cycle regulatory proteins. Those proteins which have been implicated in cancer are shaded. This figure is adapted from Hunter and Pines 1994.

Figure 2. CHO K1 cells stimulated with insulin (2i) have a spindle shape morphology. CHO K1 cells stimulated with bFGF (2ii) have a round cell morphology and no cell-cell contacts. CHO K1:cycE cells (2iii) have the same morphology as CHO K1 bFGF-stimulated cells.

Figure 1


Growth Factors
Ras, Raf, Myc, Fos, Jun
+
Restriction Point
p16
p53
p21
Myc
+
G1 Phase
CDK 4,6
PCNA
Cyclin D
1, 2, & 3
pRb
E2F
p107
&E2F
Cyclin E
CDK 2
Transcription
of DNA
E2F
Mitosis
p
pRb
Cyclin
A & B
p
Cdc2
Cyclin A
CDK 2
p107
&E2F
G2 Phase
Cyclin B
p
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S Phase
Proteins implicated
in cancer
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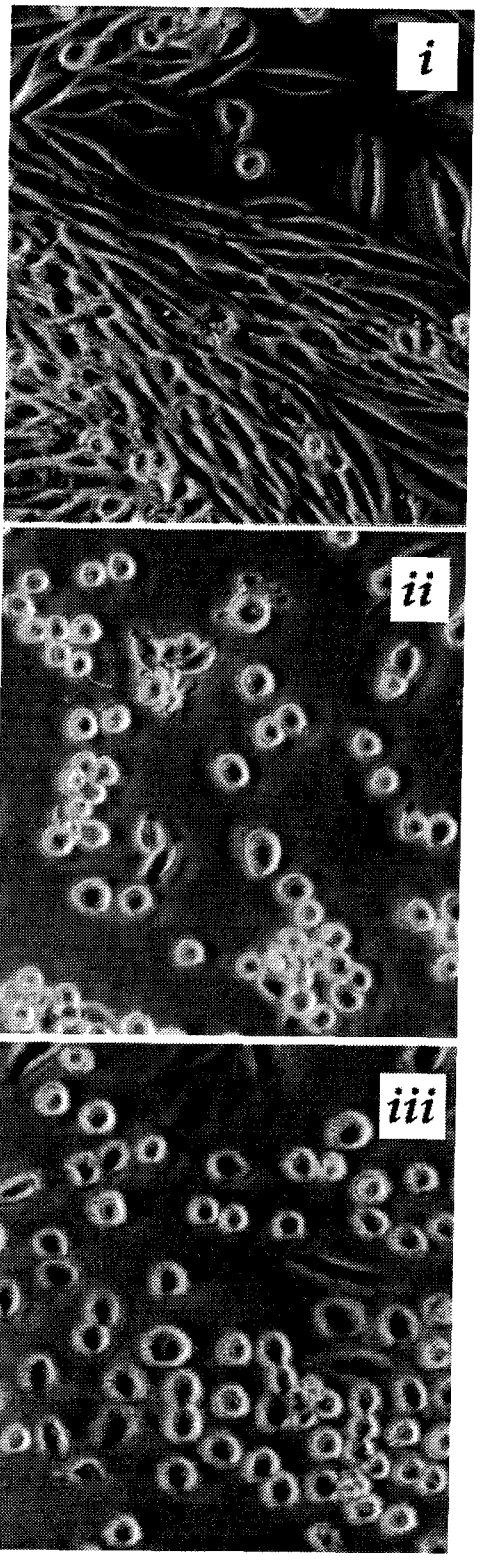
Mik1
Wee1
+
Cyclin H
CDK 7

Figure 2



CHAPTER THREE

Deregulated Expression of Cloned Human E2F-1 Enables Growth of Chinese Hamster Ovary Cells in Serum- and Protein-Free Media

Source: K. Lee, A. Guerini, and J. Bailey (1995).
The material in this chapter is being prepared for submission to Biotechnology and Bioengineering.

3.1 Abstract

E2F is a transcription factor involved in cell cycle regulation at the G1/S phase transition. Several proteins that are known to be intimately involved in DNA synthesis are regulated by the E2F transcription factor family. It is believed that the presence of free E2F in mammalian cells activates proliferation of cells through the restriction point at the G1/S phase transition of the cell cycle. Cloned human E2F-1 from Nalm 6 cells (a pre-B leukemia cell line) was subcloned into pRc/CMV and transfected into Chinese hamster ovary (CHO) cells. Ten stable transfectants isolated from CHO cells cultured under neomycin resistance selection pressure were isolated for further study. All of the clones expressed significantly higher amounts of E2F-1 than control cells as determined by Western analysis. Several clones were further studied by confocal microscopy and Southern analysis. Both of these studies also provide evidence for the expression of cloned E2F-1 in these cells. CHO K1:E2F-1 cells are able to proliferate on well-defined serum- and protein-free basal medium and have a prolonged S-phase compared to CHO K1 cells stimulated to grow by bFGF or by cyclin E overexpression. Thus, expression of E2F-1 may be an important means for bypassing the serum requirement of mammalian cell culture.

3.2 Introduction

The elimination of animal serum from Chinese hamster ovary (CHO) cell culture is a pressing challenge facing contemporary biopharmaceutical production (see Chapter 1). Metabolic engineering of cell cycle regulation provides a potential route for bypassing the cellular requirement for serum in cell culture by enabling growth on basal media. This route has been paved by recent advances in cell cycle and cancer biology, which have resulted in a clearer understanding of the basic mechanisms that control cell cycle progression. Much of the current research activity is focused on the E2F family of transcription factors which are known to be directly involved in the G1/S phase transition checkpoint (LaThangue 1994).

The E2F transcription factors are a heterodimeric family of proteins consisting of one member of the E2F (e.g., E2F-1) family and a corresponding member of the DP (e.g., DP-1) family. To date, four members of each of the E2F and DP families have been identified (Beijersbergeb et al. 1994, Dimri et al. 1994, J. Lees et al. 1993, Ivey-Hoyle et al. 1993). Of the subunits studied, only the expression of E2F-1 mRNA appears to be regulated during the cell cycle while all the others identified are constitutively expressed (LaThangue 1994). Human E2F-1 was cloned from Nalm 6 cells (Helin et al. 1992) and the complete cDNA sequence of 2517 base pairs is given in Figure 1 while the complete 437 base amino acid sequence derived from the cDNA is given in Figure 2. The gene product has a predicted size and isoelectric point of 47

kDa and 4.63 pH units, respectively; but E2F-1 migrates at approximately 50 kDa by SDS-PAGE (Helin et al. 1992) and 4.6 pH units by isoelectric focusing (see Chapter 4).

It was subsequently shown that the E2F transcription factor is a DNA binding protein that recognizes the duplicated sequence element: 5' TTT CGC GC within the E2 promoter (Nevins 1992). Furthermore, it has been shown that a single E2F site can confer regulation to a test promoter and that E2F binding sites are in the promoters of several key genes required for DNA synthesis including c-myc, dihydrofolate reductase, B-myb, cdc2, thymidine kinase, DNA polymerase α, cyclin A (Nevins 1992, Krek et al. 1993, LaThangue 1994) and E2F-1 itself (Neuman et al. 1994, Krek et al. 1994). Many of the E2F-site-regulated genes are transcribed in a periodic fashion during cell cycle progression, typically being induced during late G1 or S phase, a profile that parallels the appearance of transcriptionally active E2F-1 (LaThangue 1994).

These observations, in conjunction with the observations that E2F is complexed with the underphosphorylated form of the retinoblastoma gene product (pRb) and that E2F accumulates in its free form at late G1 phase, lead many to believe that the free form of E2F-1 is transcriptionally active and that an insufficient amount of free E2F-1 prevents progression into S phase (Fagan et al. 1994). If this is true, then the internal cellular signals which regulate the amount of free E2F-1, and hence control passage through the G1/S checkpoint (the "restriction point") of the cell cycle, can be bypassed by

deregulated expression of the E2F-1 gene product. Therefore, proliferation of mammalian cells can proceed on medium free from serum, provided the basal medium contains the necessary precursors for growth. These observations motivate E2F-1 overexpression in CHO cell culture with the goal of obtaining serum- and protein-free growth.

Other studies of the effects of E2F-1 expression also suggest a potential role for bypassing external cellular growth signals. It is observed that an increasing growth rate for a culture of cells correlates with increasing (normalized) levels of E2F-1 expression (see Chapter 4). Mathematical modelling of the interactions among some of the cell cycle regulatory molecules also suggests that increased expression of the active form of E2F can lead to proliferation in cells that are arrested because of low levels of active E2F (Hatzimanikatis et al. 1995b, Hatzimanikatis et al. 1995a). Moreover, Johnson et al. (1993) have demonstrated that E2F-1 overexpression can lead to a single round of S-phase progression in quiescent rat embryo fibroblast (REF-52) cells while Singh et al. (1994) showed that E2F-1 overexpression results in neoplastic transformation in rat embryo fibroblasts. Taken together, these results and observations raise the following question: Can the growth factor requirements in CHO cell culture be bypassed by engineering expression of cloned E2F-1?

3.3 Materials and Methods

Cell Culturing.

CHO K1 cells (Puck et al. 1958) were cultured on FMX-8 (F. Messi) media supplemented with 10% fetal calf serum (FCS, Life Technologies).

Vectors.

pBSKBP3B (see Figure 3) was a gift of K. Helin. pBSKBP3B is a plasmid of 4540 base pairs encoding the entire RBP3 cDNA (encodes E2F-1). This plasmid was digested with HindIII and XbaI, both of which cleave at unique sites resulting in fragments of 1622 and 2918 base pairs. The shorter of these fragments, containing the entire cDNA, was isolated in a 0.8% low melting agarose gel and was inserted into pRc/CMV, a 5542 base pair vector from Invitrogen (see Figure 4) which uses the enhancer/promoter sequences from the immediate early gene of human cytomegalovirus (CMV). pRc/CMV contains unique HindIII and XbaI sites in the polylinker. After ligation, the resulting vector, pRc/E2F, contained 7070 base pairs (see Figure 5) with the RBP3 cDNA (encoding for E2F-1) in the proper orientation downstream of the CMV promoter and just after the T7 promoter. This vector also contained a gene encoding neomycin resistance for antibiotic selection as well as a T3 promoter for culture in Escherichia coli. Escherichia coli DH5α was transformed with the vector and the resulting plasmid DNA was purified and used for mammalian cell transfection.

Creation of stable transfectants.

5μg of plasmid DNA were transfected into CHO K1 cells using DOTAP (N-[1-(2,3-Dioleoyloxy) propyl]-N,N,N-trimethyl-ammoniummethylsulfate, Boehringer) Detergent Delivery System according to manufacturer's instructions. Cells were initially maintained in FMX-8 media supplemented with 10% FCS. After five days, the cells were exposed to 400μg/mL neomycin in addition to the 10% FCS in order to select for transfected cells. Ten days after neomycin selection pressure was exerted, all CHO K1 control cells died. At this time, the surviving cells were diluted into two 24-well plates, one cell per well, and were cultured with conditioned media and 400μg/mL neomycin for continued selection pressure. The cells were maintained for 20 more days and fresh media and neomycin were added every three days. Finally, ten single clones were selected and preserved for further analysis. The E2F-1 overexpressing clones are designated CHO K1:E2F-1.

Western analysis.

Samples of the ten clones were grown and analyzed for E2F-1 expression by SDS-PAGE and Western blot. 1mg of protein from each clone (prepared as described in Materials and Methods, Chapter 4) in addition to CHO K1 cellular protein was loaded onto a 20cm slab gel. Bio-Rad SDS-PAGE broad range standards were used for reference and stained with Coomassie blue. The 12%T gel with 6%T stacking gel was run at 40mA for six hours and blotted to Immobilon P PVDF membrane (Millipore) for three

hours at 200mA. Western stain was performed as described in Materials and Methods, Chapter 4, and the Amersham ECL enhanced chemilluminescence kit was used for detection.

Fluorescence activated cell sorting.

One of the clones was grown in the presence of unsupplemented FMX-8 medium (which is serum- and protein-free) and isolated for FACS analysis of cell cycle phase distributions. The cellular DNA was stained with propidium iodide according to the method of Ormerod (Ormerod 1990, page 73). FACS analysis was courtesy of Dr. W. Minas, and data was analyzed with the aid of Multiplus Cell Cycle Analysis Software (Phoenix Flow Systems, San Diego, California) running on a PC.

Confocal microscopy.

Some of the clones were stained with E2F-1 antibody (Pharmingen catalog number 14971A) labelled with Texas Red in addition to fluorescein-conjugated phalloidin which binds to cellular actin. Confocal microscopy was performed by P. David and Prof. H. Eppenberger.

Southern Analysis.

Southern analysis of genomic DNA was performed with the digoxigenin (DIG) labelling kit from Boehringer according to manufacturer's instructions.

3.4 Results and Discussion

After transfection, ten stable clones were selected for further analysis. The total cellular extract from each of these clones was analyzed for the presence of E2F-1 by Western stain and compared with untransfected wild type CHO K1 cells. Figure 6 depicts the immunoreactive bands at 50 and 52 kDa with a more faint band at 51 kDa. It is known that E2F-1 migrates as a series of bands by SDS-PAGE due to heterogeneous phosphorylation (Peeper et al. 1995). All ten of the tested clones expressed more E2F-1 than the CHO K1 untransfected cells (lane 11). Faint bands of endogenous E2F-1 are visible in lane 11 on the original film. Clones number 2 and 5 (lanes 2 and 5) expressed the largest amount of cloned E2F-1 protein, but they exhibited slightly slower doubling times–30 hours for these clones, 24 hours for the others.

To further verify the production of E2F-1 in some of the clones, cells were stained for actin and E2F-1 and subject to confocal microscopy (Figures 7i and 7ii). The red dots are E2F-1 immunoreactive protein. It is clear from these figures that E2F-1 appears to be sublocalized. Although the images do not outline the nucleus, the size of the stained dots is far smaller than expected from an entire nucleus. It is likely that the observed E2F-1 is sublocalized in nucleosomes (H. Eppenberger, personal communication). Nucleosomes are highly-ordered structures containing DNA that is packed together, the packing being mediated by histone H1. It is known that

transcription of genes by RNA polymerase proceeds without disruption through nucleosome packing (Alberts et al. 1989, page 500). Further analysis of the subnuclear localization of E2F-1 might show that E2F regulated genes are topographically located in specific regions of the chromosome. Additionally, one expects some E2F-1 stain to localize at ribosomes where the E2F-1 mRNA is translated before being imported into the nucleus. Because of the nature of confocal microscopy, each image in Figure 7 represents only a cross-section of the cells. Thus, not all of the E2F-1 present in a (three-dimensional) cell can be visualized in a particular cross-section. Indeed, data from other cross-sections of these same cells depict E2F-1 protein in cells which do not stain for E2F-1 in the cross-sections seen in Figures 7i and 7ii.

We used Southern analysis to verify the stable integration of the E2F-1 cDNA into the genome of the host cell. Figure 8 depicts these results for clones 5 and 10. Lane 1 is CHO K1:E2F-1 (clone 5), lane 2 is clone 10, lane 3 is an untransfected control (a smile-shaped artifact is visible), and lane 4 is a positive control. Comparison with Figure 6 reveals that the expression level of a cloned protein does not necessarily have a direct relation with the number of copies of cDNA integrated into the host genome. This is a common feature of genetically engineered mammalian cell lines.

FACS analysis of these cells was done to estimate the length of the cell cycle subphases. E2F-1 stimulated CHO K1 cells are compared to cyclin E stimulated and bFGF stimulated CHO K1 cells, all growing under protein-

free conditions (see Table 1). The length of each subphase was calculated according to the homogeneous daughter model of Slater et al. 1977. The E2F-1 expressing cells spend the longest time in S phase. NIH3T3 cells stably expressing E2F-1 also show increased numbers of cells in S phase (Logan et al. 1994). An abundance of early S phase genes resulting from increased E2F-1 transcription could increase the average length of S phase. Interestingly, the length of the G1 phase is not shortened by E2F-1 upregulation which apparently contradicts the idea that free E2F-1 initiates the onset of S phase.

The most extensive testing of protein-free growth was done on clone number 10. These CHO K1:E2F-1 cells are capable of growth on FMX-8 media completely free from protein and serum (see Figure 9). These cells have a flat morphology in 0% serum which is similar to NIH3T3 cells constitutively expressing E2F-1 and grown in 0.5% serum (Logan et al. 1994). Figure 10 shows CHO K1 cells grown by stimulation by insulin, bFGF, and cyclin E expression, for comparison. CHO K1:E2F-1 cells grown in the presence of 400μg/mL neomycin and 10% FCS could be transferred directly into FMX-8 media alone; clones could also be split from lower percentages of FCS into 0% FCS (i.e., protein-free medium) or from 0% FCS into 0% FCS (i.e., protein-free into protein-free medium) at ratios of 1/5, 1/10, or 1/50. Furthermore, these cells could be transferred into FMX-8 containing varying amounts of neomycin with no observable effects on growth in a protein-free environment. These cells have a more elongated-cell morphology than typical CHO K1 cells grown in the presence of 10% FCS. However, this morphological observation may be a direct result of the transfection of these

cells. Further experiments on the other clones may reveal some insights into this morphological change.

The CHO K1:E2F-1 cells proliferated when plated at various seeding densities. In all cases tested, control CHO K1 cells invariably died. However, growth after three days in the presence of low serum (0.1%-1%) revealed drastically enhanced growth properties of the CHO K1:E2F-1 cells as compared to CHO K1 cells (see Figure 11). While the CHO K1:E2F-1 cells grew to very high cell densities on FMX-8 + 0.5% FCS (Figure 11 top), CHO K1 cells struggled to maintain viability (Figure 11 bottom).

These striking results complement the results obtained by Singh et al. (1994) with at least two important differences. First, the cell type used here has been extensively studied and serves as an important model system for recombinant production strains whereas REF-1 cells are arguably a better model for studies of the fundamental biochemistry of the cell cycle. Second, CHO K1:E2F-1 cells proliferate on basal medium without any additional growth factors, protein, or serum. The REF-1 cells could be maintained in the presence of 0.1% FCS, but not in a protein-free environment. The limitation inherent in serum-based studies, such as by Singh et al. 1994, makes it difficult to draw any conclusions about the effects of growth resulting from metabolic engineering. As discussed in Chapter 1, results obtained in the presence of serum are rife with ambiguity which underscores the need for removal of serum from cell culture systems.

Acknowledgements

The authors would like to thank Ellen Nollen and Wolfgang Minas for help with FACS analysis, Peter David and Hans Eppenberger for help with confocal microscopy, Cornelia Schwerdel for technical assistance, and Wolfgang Renner for technical assistance and useful discussions.

3.5 References

Alberts, B., Bray, D., Lewis, J., Raff, M., Roberts, K., and Watson, J.D., Molecular Biology of the Cell, 2 ed., Garland Publishing, Inc., New York, 1989.

Beijersbergen, R.L., Kerkhoven, R.M., Zhu, L.A., Carlee, L., Voorhoeve, P.M., and Bernards, R., E2F-4, A New Member of the E2F Gene Family, Has Oncogenic Activity and Associates with p107 In Vitro, Genes Dev., **8**: 2680 (1994).

Dimri, G.P., Hara, E., and Campisi, J., Regulation of Two E2F-Related Genes in Presenescent and Senescent Human Fibroblasts, J. Biol. Chem., **269**: 16180 (1994).

Fagan, R., Flint, K.J., and Jones, N., Phosphorylation of E2F-1 Modulates Its Interaction with the Retinoblastoma Gene Product and the Adenoviral E4 19 kDa Protein, Cell, **78**: 799 (1994).

Hatzimanikatis, V., Lee, K.H., and Bailey, J.E., manuscript in preparation (1995).

Hatzimanikatis, V., Lee, K.H., Renner, W.A., and Bailey, J.E., submitted (1995).

Helin, K., Lees, J.A., Vidal, M., Dyson, N., Harlow, E., and Fattaey, A., A cDNA Encoding a pRb-Binding Protein with Properties of the Transcription Factor E2F, Cell, **70**: 337 (1992).

Ivey-Hoyle, M., Conroy, R., Huber, H.E., Goodhart, P.J., Oliff, A., and Heimbrook, D.C., Cloning and Characterization of E2F-2, a Novel Protein with the Biochemical Properties of Transcription Factor E2F, Mol. Cell Biol., **13**: 7802 (1993).

Johnson, D.G., Schwartz, J.K., Cress, W.D., and Nevins, J.R., Expression of Transcription Factor E2F1 Induces Quiescent Cells to Enter S Phase, Nature, **365**: 349 (1993).

Krek, W., Ewen, M.E., Shirodkar, S., Arany, Z., Kaelin, W.G., and Livingston, D.M., Negative Regulation of the Growth-Promoting

Transcription Factor E2F-1 by a Stably Bound Cyclin A-Dependent Protein Kinase, Cell, **78**: 161 (1994).

Krek, W., Livingston, D.M., and Shirodkar, S., Binding to DNA and the Retinoblastoma Gene Product Promoted by Complex Formation of Different E2F Family Members, Science, **262**: 1557 (1993).

LaThangue, N.B., DRTF1/E2F: An Expanding Family of Heterodimeric Transcription Factors Implicated in Cell-Cycle Control, TIBS, **19**: 108 (1994).

Lees, J.A., Saito, M., Vidal, M., Valentine, M., Look, T., Harlow, E., Dyson, N., and Helin, K., The Retinoblastoma Protein Binds to a Family of E2F Transcription Factors, Mol. Cell Biol., **13**: 7813 (1993).

Logan, T.J., Jordan, K.L., and Hall, D., Altered Shape and Cell-Cycle Characteristics of Fibroblasts Expressing the E2F-1 Transcription Factor, Mol. Biol. Cell, **5**: 667 (1994).

Neuman, E., Flemington, E.K., Sellers, W.R., and Kaelin, W.G., Transcription of the E2F-1 Gene is Rendered Cell-Cycle Dependent by E2F DNA-Binding Sites Within Its Promoter, Mol. Cell Biol., **14**: 6607 (1994).

Nevins, J.R., E2F: A Link Between the Rb Tumor Suppressor Protein and Viral Oncoproteins, Science, **258**: 424 (1992).

Ormerod, M.G., Flow Cytometry: A Practical Approach, IRL Press, Oxford, 1990.

Peeper, D.S., Keblusek, P., Helin, K., Toebes, M., Vandereb, A.J., and Zantema, A., Phosphorylation of a Specific CDK Site in E2F-1 Affects Its Electrophoretic Mobility and Promotes pRb-Binding In Vitro, Oncogene, **10**: 39 (1995).

Puck, T.T., Cieciura, S.J., and Robinson, A., Genetics of Somatic Mammalian Cells. III. Long-Term Cultivation of Euploid Cells from Human and Animal Subjects, J. Exp. Med., **108**: 945-955 (1958).

Singh, P., Wong, S.H., and Hong, W., Overexpression of E2F-1 in Rat Embryo Fibroblasts Leads to Neoplastic Transformation, EMBO J., **13**: 3329 (1994).

Slater, M.L., Sharrow, S.O., and Gart, J.J., Cell Cycle of Saccharomyces cerevisiae in Populations Growing at Different Rates, Proc. Natl. Acad. Sci. USA, **74**,: 3850 (1977).

3.6 Tables

Table 1. The duration of the cell cycle subphases (G1, S, and G2-M) is given in hours (± 0.1 hours) for CHO K1 cells stimulated to grow by bFGF, cyclin E overexpression, and E2F-1 overexpression in a serum-free environment. The length of the cell cycle subphases was calculated based on the homogeneous daughter model of Slater et al. for the doubling time of the cells which was 24 hours in all cases.

	Duration of G1 (hours)	**Duration of S (hours)**	**Duration of G2-M (hours)**
CHO K1 + bFGF	13.9	5.3	4.8
CHO K1:cycE	14.4	6.9	2.7
CHO K1:E2F-1	13.9	8.8	1.2

3.7 Figures

Figure 1. The complete cDNA sequence of RBP3, the gene encoding E2F-1, as cloned from Nalm 6 cells by Helin et al.

Figure 2. The complete amino acid sequence of RBP3 (E2F-1) as determined from the cloned cDNA sequence.

Figure 3. Graphic depiction of plasmid pBSKBP3B (4540 base pairs) which contains the entire cDNA sequence of E2F-1, encodes for ampicillin resistance in Escherichia coli and has both T7 and T3 promoters. Note the unique restriction sites of HindIII and position 689 and XbaI at position 2311 which are used for subcloning.

Figure 4. Graphic depiction of plasmid pRc/CMV (5542 base pairs) from Invitrogen. The plasmid has a multicloning site downstream from the enhancer/promoter sequences from the immediate early gene of human cytomegalovirus (CMV) which results in constitutive expression of cloned gene products. Note the HindIII and XbaI restriction sites present in the multicloning site (positions 890 and 984, respectively).

Figure 5. Graphic depiction of plasmid pRc/E2F (7070 base pairs) which encodes for RBP3 (E2F-1) behind the CMV promoter.

Figure 6. SDS-PAGE analysis of CHO K1:E2F-1 clones. The figure depicts the anti-E2F-1 immunoreactive bands at 50 and 52 kDa (a fainter band at 51 kDa is also visible). The sizes were determined by comparison with Bio-Rad SDS-PAGE standards (data not shown). Lanes 1 to 10 are the CHO K1:E2F-1 clones with clone identification number equal to the lane number (from 1-10) and lane 11 (far right) is a CHO K1 untransfected cell line for control. All ten clones express far greater amounts of immunoreactive E2F-1 than the control. Faint bands of endogenous E2F-1 are visible in lane 11 on the original film.

Figure 7. Confocal microscopy image of CHO K1:E2F-1 cells. The red areas are E2F-1 immunoreactive protein and the green is anti-actin. The confocal microscopy is courtesy of P. David and Prof. H. Eppenberger. Figure 7i depicts clone number 10 (see Figure 6) and Figure 7ii depicts clone number 5 (see Figure 6).

Figure 8. Southern analysis of CHO K1:E2F-1 (clones 5 and 10). Lane 1 is clone 5, lane 2 is clone 10, lane 3 is an untransfected control, and lane 4 is a positive control. The original cDNA is 1622 base pairs in length. The probes were labelled with the DIG labelling kit from Boehringer. Clone 10 has more cDNA integrated than clone 5 although clone 5 expresses more of the protein (see Figure 6). This discrepancy is typical of cloned gene expression in mammalian cell culture systems. The dark patches (e.g., lane 1) and smiles (e.g., lane 3) are experimental artifacts which arise during detection.

Figure 9. Culture of CHO K1:E2F-1 cells growing on FMX-8 basal media without any additional protein or serum added.

Figure 10. CHO K1 cells stimulated with insulin (i) have a spindle shape morphology. CHO K1 cells stimulated with bFGF (ii) have a round cell morphology. CHO K1:cycE cells (iii) also have a round cell morphology.

Figure 11. A comparison of CHO K1:E2F-1 cells (top) and CHO K1 untransfected cells (bottom) grown in the presence of 0.5% serum after three days (no antibiotic selection pressure). Note the enhanced growth properties of the transfected cells as compared to the control cells.

Figure 1

```
1       ggaattccgtggccgggactttgcaggcagcggcggccgggggcggagcg
51      ggatcgagccctcgccgaggcctgccgccatgggcccgcgccgccgccgc
101     cgcctgtcacccgggccgcgcgggccgtgagcgtcatggccttggccggg
151     gcccctgcgggcggcccatgcgcgccggcgctggaggccctgctcggggc
201     cggcgcgctgcggctgctcgactcctcgcagatcgtcatcatctccgccg
251     cgcaggacgccagcgccccgccggctcccaccggccccgcggcgcccgcc
301     gccggcccctgcgaccctgacctgctgctcttcgccacaccgcaggcgcc
351     ccggcccacacccagtgcgccgcggcccgcgctcggccgcccgccggtga
401     agcggaggctggacctggaaactgaccatcagtacctggccgagagcagt
451     gggccagctcggggcagaggccgccatccaggaaaaggtgtgaaatcccc
501     gggggagaagtcacgctatgagacctcactgaatctgaccaccaagcgct
551     tcctggagctgctgagccactcggctgacggtgtcgtcgacctgaactgg
601     gctgccgaggtgctgaaggtgcagaagcggcgcatctatgacatcaccaa
651     cgtccttgagggcatccagctcattgccaagaagtccaagaaccacatcc
701     agtggctgggcagccacaccacagtgggcgtcggcggacggcttgagggg
751     ttgacccaggacctccgacagctgcaggagagcgagcagcagctggacca
801     cctgatgaatatctgtactacgcagctgcgcctgctctccgaggacactg
851     acagccagcgcctggcctacgtgacgtgtcaggaccttcgtagcattgca
901     gaccctgcagagcagatggttatggtgatcaaagcccctcctgagaccca
951     gctccaagccgtggactcttcggagaactttcagatctcccttaagagca
1001    aacaaggcccgatcgatgttttcctgtgccctgaggagaccgtaggtggg
1051    atcagccctgggaagaccccatcccaggaggtcacttctgaggaggagaa
1101    cagggccactgactctgccaccatagtgtcaccaccaccatcatctcccc
1151    cctcatccctcaccacagatcccagccagtctctactcagcctggagcaa
1201    gaaccgctgttgtcccggatgggcagcctgcgggctcccgtggacgagga
1251    ccgcctgtccccgctggtggcggccgactcgctcctggagcatgtgcggg
1301    aggacttctccggcctcctccctgaggagttcatcagcctttccccaccc
1351    cacgaggccctcgactaccacttcggcctcgaggagggcgagggcatcag
1401    agacctcttcgactgtgactttggggacctcacccccctggatttctgac
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1501    ccctggagccccctgtccctggccgtcctcccagcctgtttggaaacatt
1551    taatttatacccctctcctctgtctccagaagcttctagctctggggtct
1601    ggctaccgctaggaggctgagcaagccaggaagggaaggagtctgtgtgg
1651    tgtgtatgtgcatgcagcctacacccacacgtgtgtaccgggggtgaatg
1701    tgtgtgagcatgtgtgtgtgcatgtaccggggaatgaaggtgaacataca
1751    cctctgtgtgtgcactgcagacacgccccagtgtgtccacatgtgtgtgc
1801    atgagtccatctctgcgcgtgggggggctctaactgcactttcggccctt
1851    ttgctcgtggggtcccacaaggcccagggcagtgcctgctcccagaatct
1901    ggtgctctgaccaggccaggtggggaggctttggctggctgggcgtgtag
1951    gacggtgagagcacttctgtcttaaaggtttttctgattgaagctttaa
2001    tggagcgttatttatttatcgaggcctctttggtgagcctggggaatcag
2051    caaaaggggaggaggggtgtggggttgataccccaactccctctaccctt
2101    gagcaagggcaggggtccctgagctgttcttctgccccatactgaaggaa
2151    ctgaggcctgggtgatttatttattgggaaagtgagggagggagacagac
2201    tgactgacagccatgggtggtcagatggtggggtgggccctctccagggg
2251    gccagttcagggcccagctgccccccaggatggatatgagatgggagagg
2301    tgagtgggggaccttcactgatgtgggcaggaggggtggtgaaggcctcc
2351    cccagcccagaccctgtggtccctcctgcagtgtctgaagcgcctgcctc
2401    cccactgctctgccccaccctccaatctgcactttgatttgcttcctaac
2451    agctctgttccctcctgctttggttttaataaatattttgatgacgttaa
2501    aaaaaggaattcgatat
```
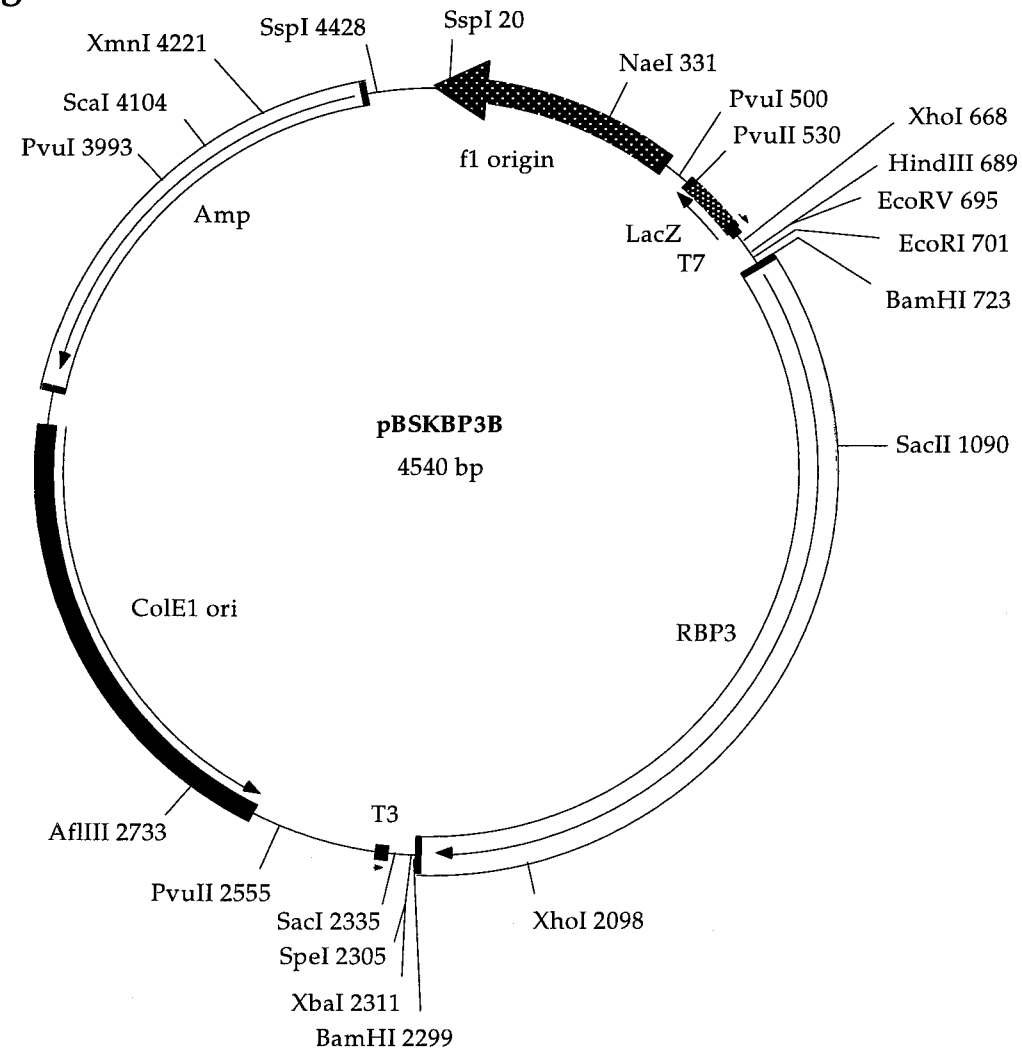

Figure 2

1	M	A	L	A	G	A	P	A	G	G
11	P	C	A	P	A	L	E	A	L	L
21	G	A	G	A	L	R	L	L	D	S
31	S	Q	I	V	I	I	S	A	A	Q
41	D	A	S	A	P	P	A	P	T	G
51	P	A	A	P	A	A	G	P	C	D
61	P	D	L	L	L	F	A	T	P	Q
71	A	P	R	P	T	P	S	A	P	R
81	P	A	L	G	R	P	P	V	K	R
91	R	L	D	L	E	T	D	H	Q	Y
101	L	A	E	S	S	G	P	A	R	G
111	R	G	R	H	P	G	K	G	V	K
121	S	P	G	E	K	S	R	Y	E	T
131	S	L	N	L	T	T	K	R	F	L
141	E	L	L	S	H	S	A	D	G	V
151	V	D	L	N	W	A	A	E	V	L
161	K	V	Q	K	R	R	I	Y	D	I
171	T	N	V	L	E	G	I	Q	L	I
181	A	K	K	S	K	N	H	I	Q	W
191	L	G	S	H	T	T	V	G	V	G
201	G	R	L	E	G	L	T	Q	D	L
211	R	Q	L	Q	E	S	E	Q	Q	L
221	D	H	L	M	N	I	C	T	T	Q
231	L	R	L	L	S	E	D	T	D	S
241	Q	R	L	A	Y	V	T	C	Q	D
251	L	R	S	I	A	D	P	A	E	Q
261	M	V	M	V	I	K	A	P	P	E
271	T	Q	L	Q	A	V	D	S	S	E
281	N	F	Q	I	S	L	K	S	K	Q
291	G	P	I	D	V	F	L	C	P	E
301	E	T	V	G	G	I	S	P	G	K
311	T	P	S	Q	E	V	T	S	E	E
321	E	N	R	A	T	D	S	A	T	I
331	V	S	P	P	P	S	S	P	P	S
341	S	L	T	T	D	P	S	Q	S	L
351	L	S	L	E	Q	E	P	L	L	S
361	R	M	G	S	L	R	A	P	V	D
371	E	D	R	L	S	P	L	V	A	A
381	D	S	L	L	E	H	V	R	E	D
391	F	S	G	L	L	P	E	E	F	I
401	S	L	S	P	P	H	E	A	L	D
411	Y	H	F	G	L	E	E	G	E	G
421	I	R	D	L	F	D	C	D	F	G
431	D	L	T	P	L	D	F	*		

Figure 3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XhoI 668
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EcoRV 695
Amp
LacZ
T7
EcoRI 701
BamHI 723
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SacII 1090
ColE1 ori
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XhoI 2098

Figure 4

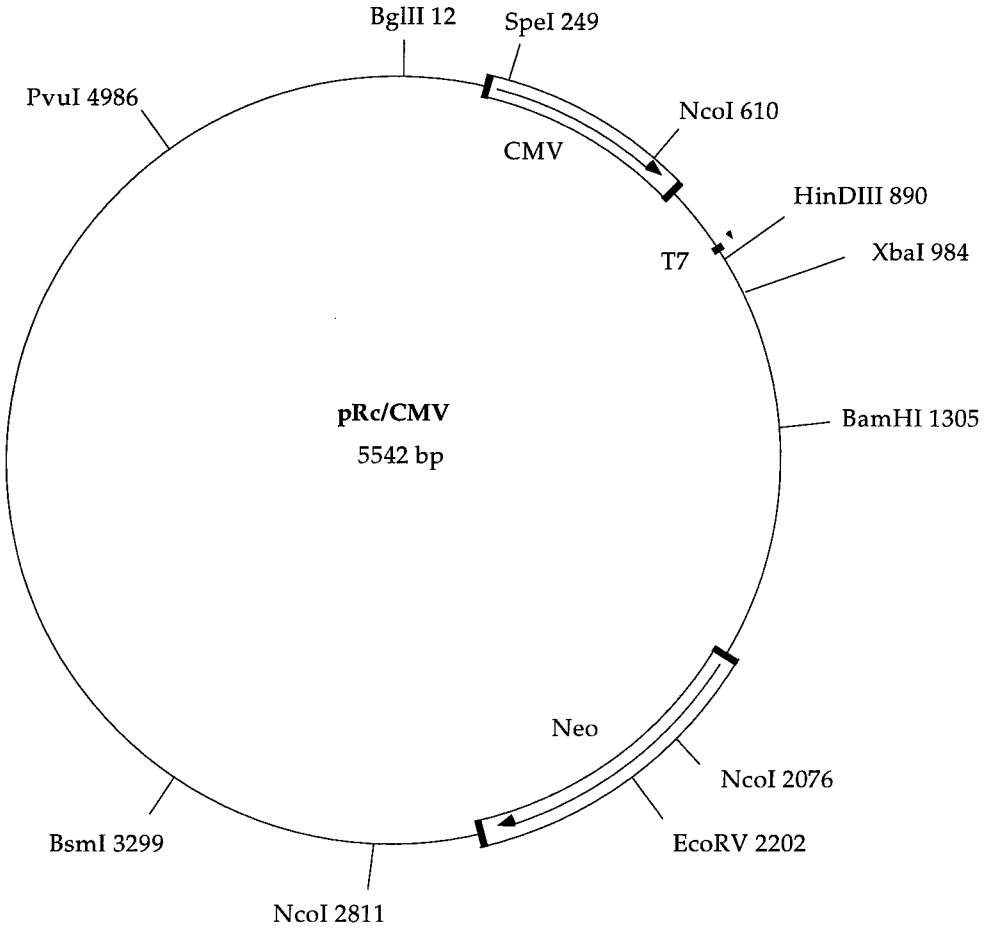
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Figure 5

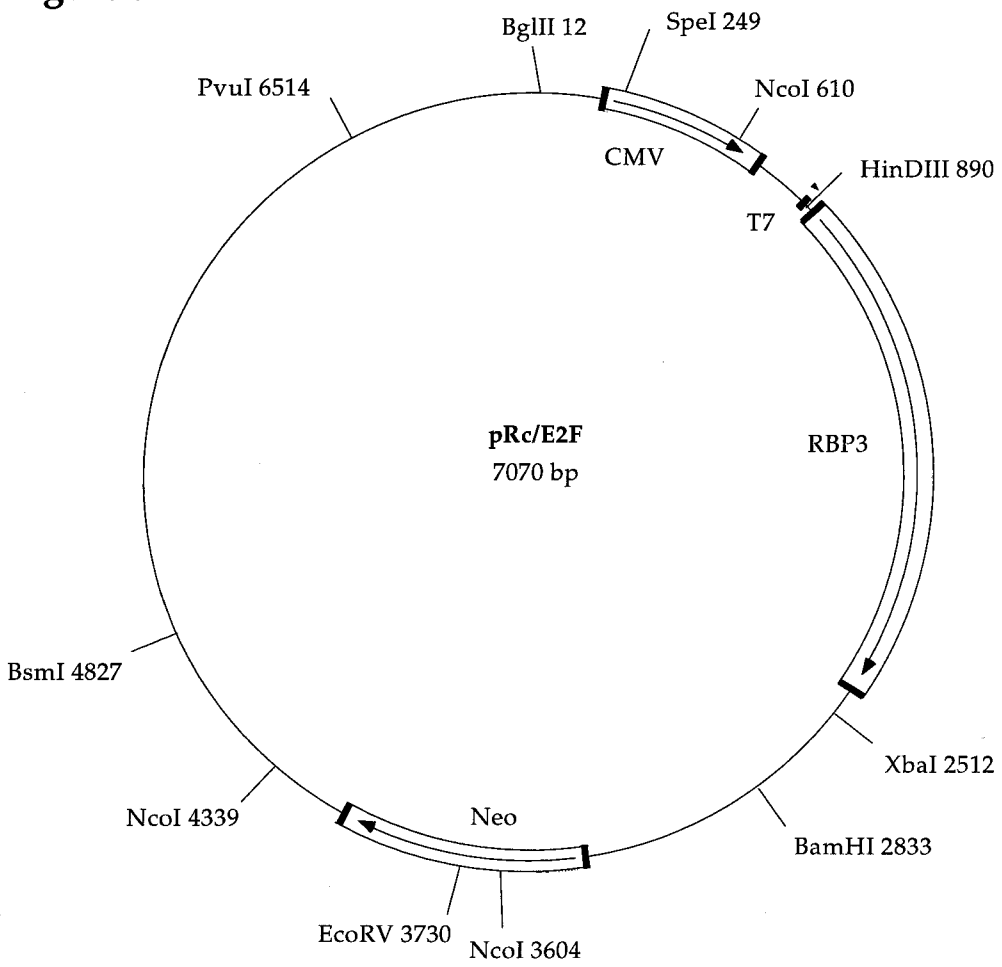
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Figure 6

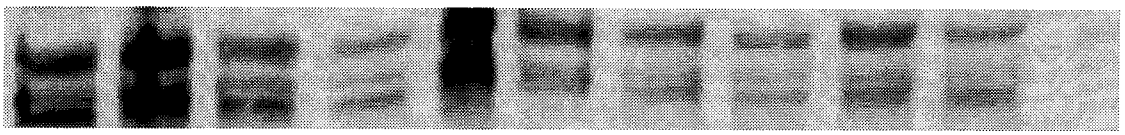

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Figure 7 i

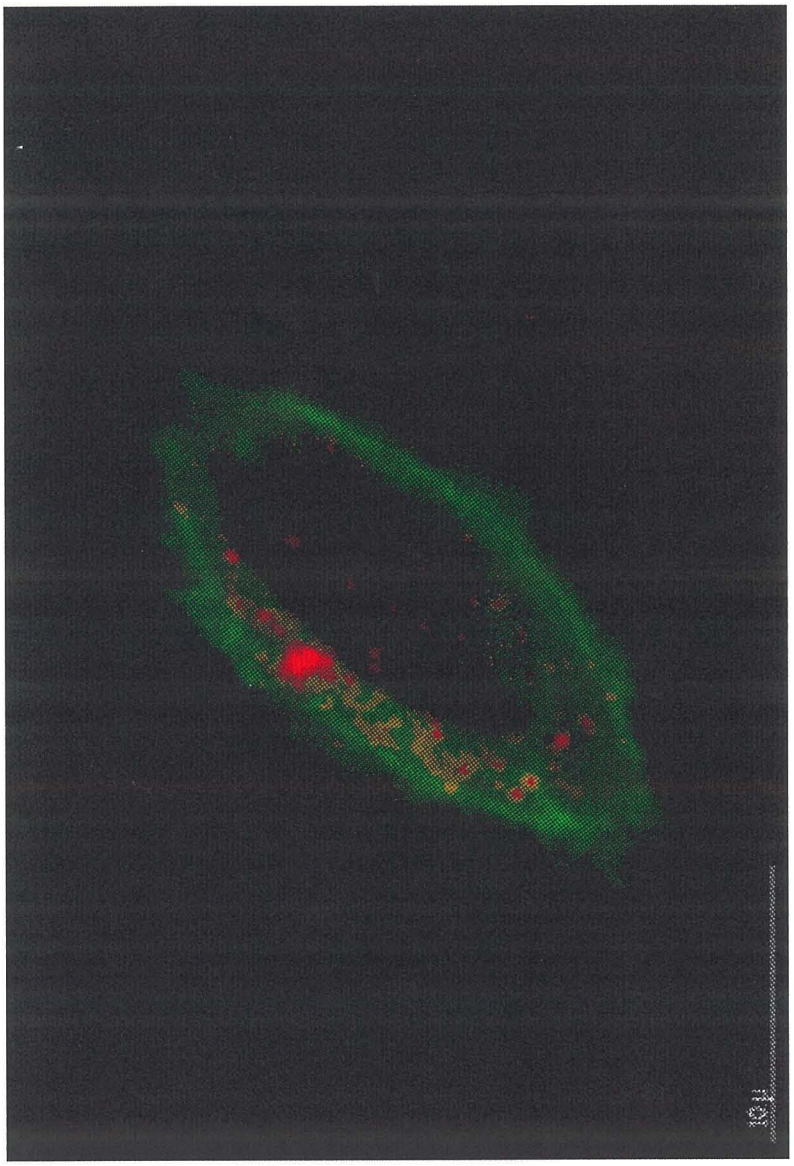


Figure 7 **ii**

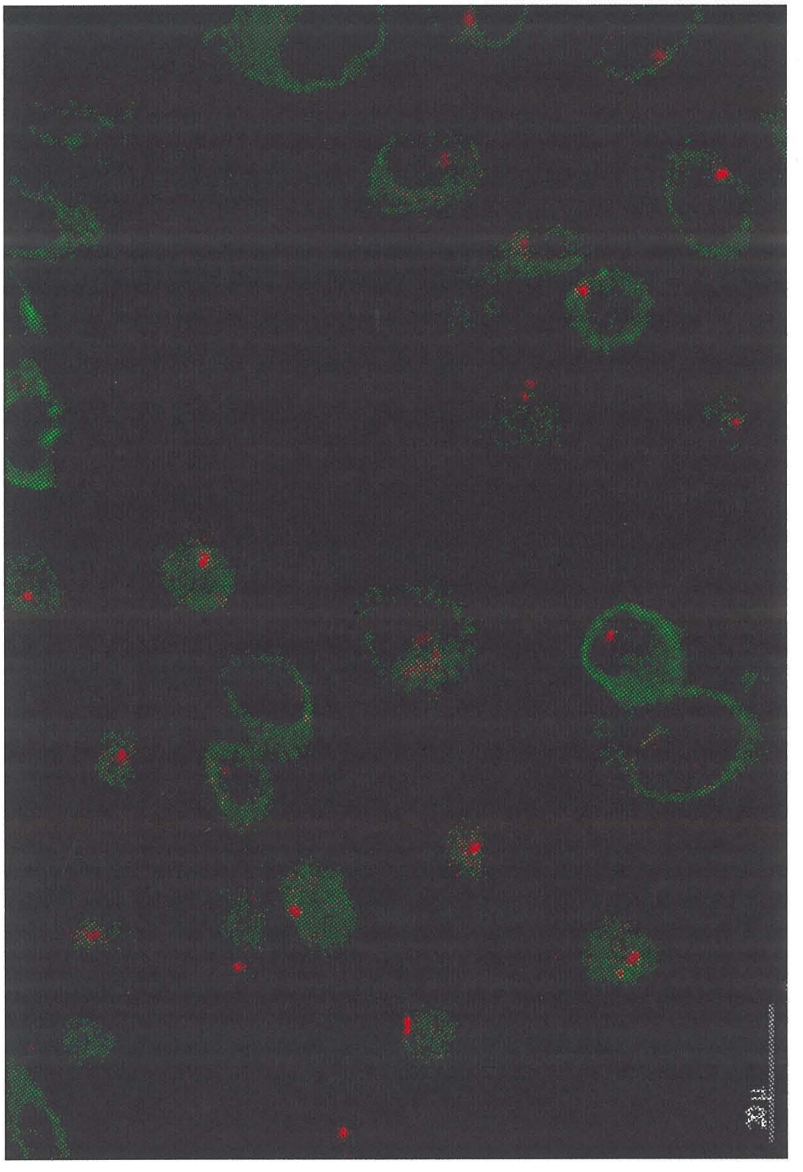


Figure 8

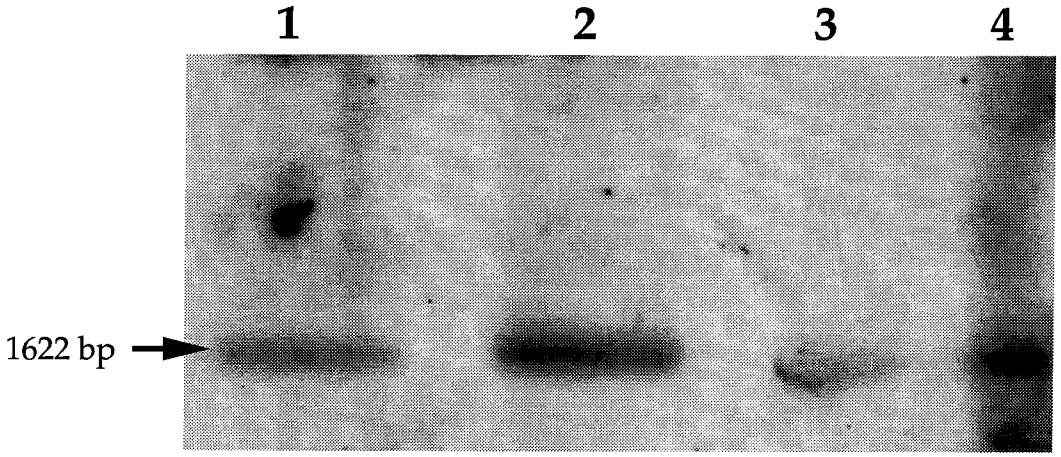


Figure 9

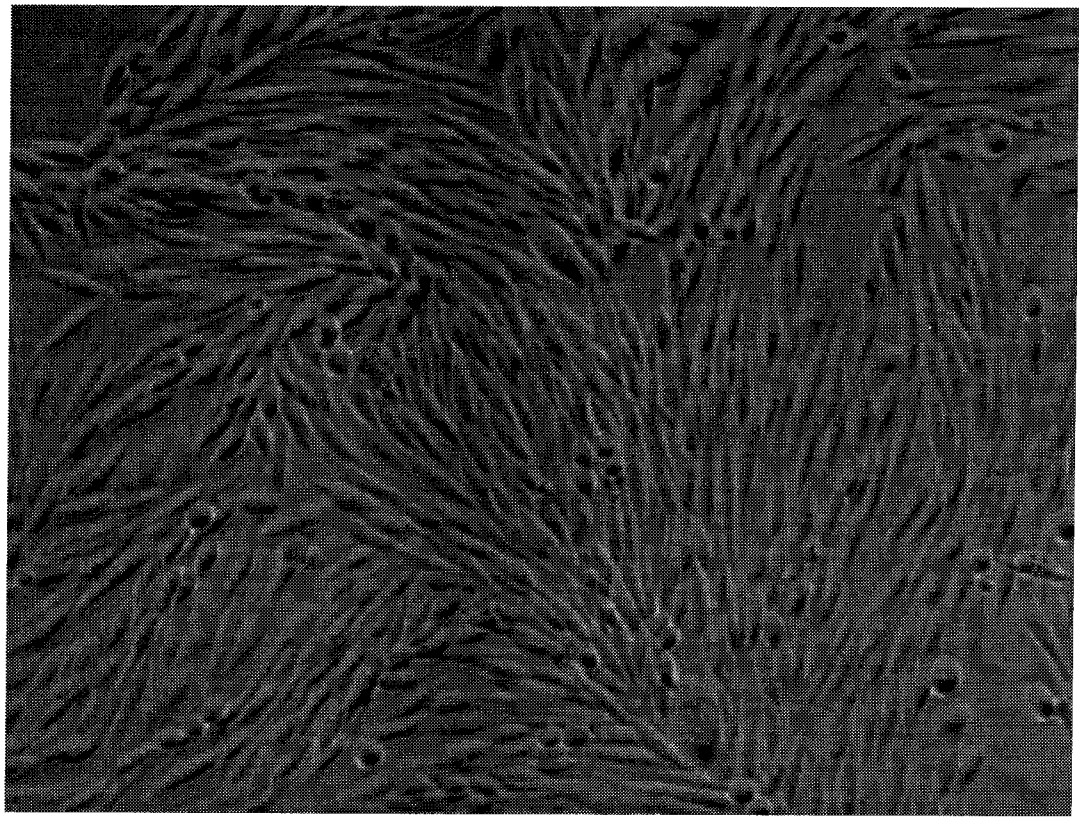


Figure 10

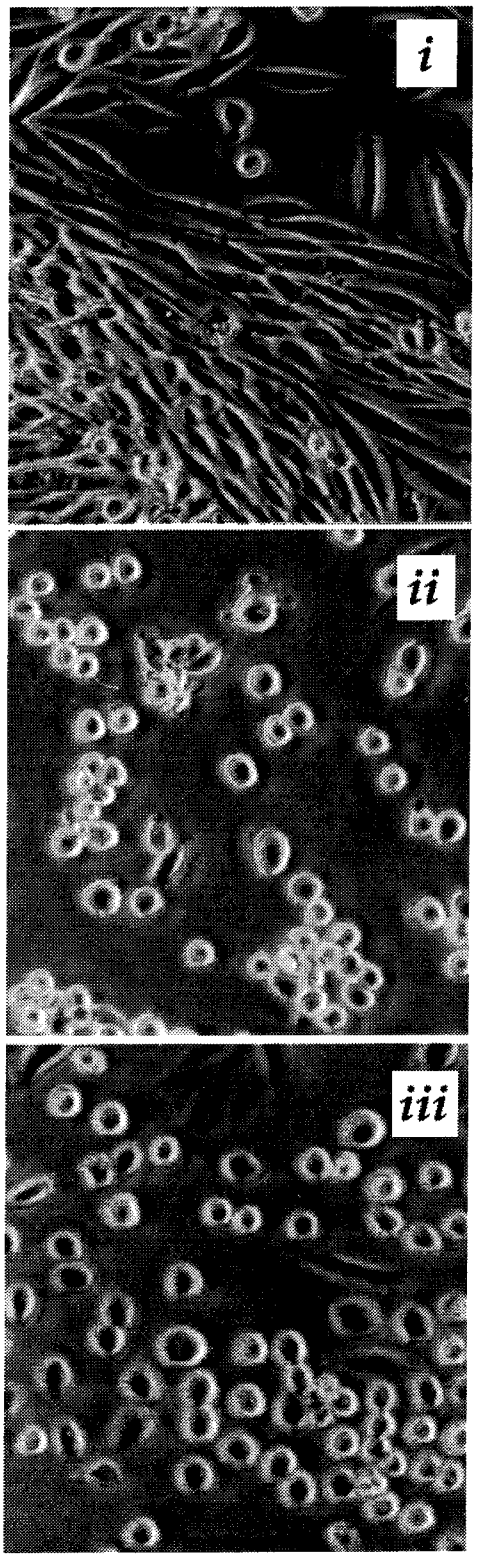
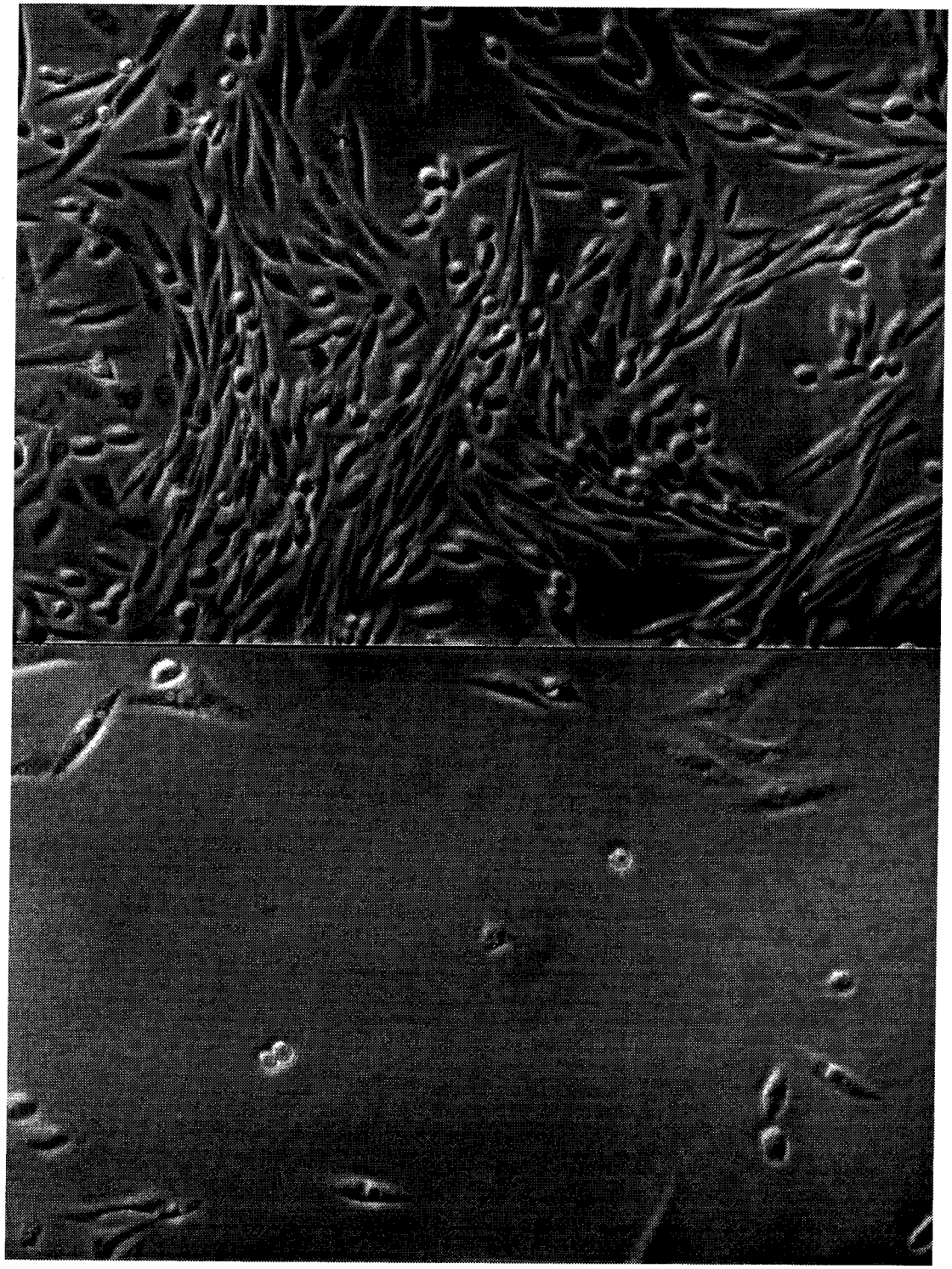


Figure 11



CHAPTER FOUR

Two-Dimensional Gel Electrophoresis Analysis of Chinese Hamster Ovary Cell Culture: Growth Stimulation

Source: K.H. Lee, M.G. Harrington, and J.E. Bailey (1995). The material in this chapter is being prepared for submission to Biotechnology and Bioengineering.

4.1 Abstract

Two-dimensional electrophoresis (2DE) of proteins was used to study the effects of various growth-promoting agents on Chinese hamster ovary (CHO) cell culture. 10% fetal calf serum (FCS), basic fibroblast growth factor (bFGF), insulin, cloned cyclin E, and cloned E2F-1 were used to stimulate proliferation of CHO cells on protein-free FMX-8 medium. The varying expression level of cyclin E in response to different mitogenic stimulation–higher for FCS-stimulated, lowest for insulin-stimulated growth–suggests an important role in cell cycle regulation. E2F-1 expression levels are highest for bFGF- and FCS-stimulated cultures, and deregulated expression of this protein enables proliferation on protein-free medium (see Chapter 3). 2D gel analysis of cloned E2F-1 overexpressing cells supports the functional efficacy of human E2F-1 in CHO cells and suggests several experiments for identifying E2F-regulated components. Gel-to-gel comparison of CHO protein patterns resulting from different combinations of growth-stimulation suggests that the presence of serum in cell culture activates the expression of a variety of proteins, many of which are not obligatory (in high levels) for proliferation.

4.2 Introduction

Currently, there is widespread interest in the study of the mammalian cell cycle (Heichman and Roberts 1994, Sherr 1994, Nurse 1994, Hunter and Pines 1994) particularly as it relates to cancer (Hartwell and Kastan 1994, Cantley et al. 1991, Marx 1994). Many of these studies focus on elucidation of the biochemical steps involved in deregulated growth (Ohtsubo and Roberts 1993, Singh et al. 1994). Such research relies heavily on interdisciplinary approaches including the use of molecular biology, genetics, medicine, and biochemistry. These studies begin with the identification of proteins involved in controlling these cellular mechanisms because metabolic engineering of particular regulatory molecules can bypass the need for serum in cell culture (Renner et al. 1995; Chapter 3). Therefore, an understanding of the basic biochemical mechanism which drives the mammalian cell cycle would provide an alternative route to engineering cells capable of growth on media free from growth factors.

Two-dimensional gel electrophoresis (2DE) of proteins (O'Farrell 1975) is an analytical method which can be used to study growth factor stimulation and possibly elucidate critical steps in the associated biochemical mechanism of cell proliferation control. This method is unique among contemporary analytical techniques in its ability to resolve complex mixtures of proteins into individual components. With two orders of magnitude (Harrington and Merril 1988) greater resolution than conventional protein analytical methods, typical 2D gels can separate

thousands of proteins into a unique pattern of "spots" based on the composition of the starting material. Therefore, this method potentially allows one to study the expression, synthesis, etc., of the entire cellular protein composition at a given moment for a defined biological system.

When 2D gel technology is coupled in series to downstream micropreparative technologies, such as peptide sequencing (Aebersold et al. 1987, Vandekerckhove et al. 1993) and/or mass spectrometry (Aebersold 1993), one has the power to characterize fully the protein changes that occur in response to a particular stimulus. Qualitative changes in the expression as well as post-translational modifications can be characterized by protein stain, radiolabel and double label techniques (Harrington et al. 1991b).

In an attempt to gain further insight into the expression of particular proteins, Chinese hamster ovary cells (CHO, Puck et al. 1958) were grown under different conditions. It is difficult to draw meaningful conclusions from cells cultured on varying percentages of serum despite the enhanced growth rates observed. Alternatively, in an attempt to utilize a particularly well-defined cell culture system, CHO cells were grown on the protein-free FMX-8 medium supplemented with a single, defined growth factor. A study of the expression levels of cell cycle regulatory proteins in response to various types of growth stimulation has proven to be an effective foundation for cloned gene expression resulting in desired phenotypes (Renner et al. 1995).

CHO K1 cells were stimulated to grow on protein-free FMX-8 medium (Dr. F. Messi, Cell Culture Technologies) by the addition of 10% serum, basic fibroblast growth factor (bFGF), insulin, transfection with a vector for human cyclin E expression (CHO K1:cycE), transfection with a vector for human E2F-1 expression (CHO K1:E2F-1), and a combination of cyclin E overexpression and basic fibroblast growth factor. While it is already known that expression of particular cloned cell cycle genes can lead to growth under low serum and protein-free conditions (Ohtsubo and Roberts 1993, Renner et al. 1995, Singh et al. 1994; Chapter 3), 2D gel analysis can identify alternative candidates as targets for metabolic engineering that may or may not be cell cycle regulatory molecules in order to achieve particular growth phenotypes. In particular, metabolic engineering of these other proteins or their associated pathways may lead to cell lines with characteristics particularly well suited for the production of a given recombinant protein in low serum, serum-free, or protein-free media. The expression of some important cell cycle regulatory proteins is studied and significant qualitative changes in the expression of proteins from CHO cell extract will be discussed.

4.3 Materials and Methods

Two-dimensional electrophoresis of proteins - general.

Although two-dimensional electrophoresis of proteins is finding increasing uses as an analytical technique, there are still several technical

issues which need to be addressed in order to run accurate, reproducible experiments. Recent attempts to overcome some of the technical challenges inherent in current 2D gel technology are discussed in Görg et al. 1988, Appendix 1, Appendix 2, and Simpson et al. 1992. Many of the "solutions" discussed in these studies are applied directly in this analysis both in practice as well as in principle.

Preparation of samples.

Cells were cultured in FMX-8 medium which is a protein-free basal medium for the culturing of Chinese hamster ovary (CHO) cells. The medium or the cells were supplemented with growth factors or cloned genes in order to obtain growth. CHO K1 cells were maintained in FMX-8 medium supplemented with 10% fetal calf serum (FCS) from Life Technologies. Basic fibroblast growth factor (bFGF) obtained from Life Technologies was supplemented at 20ng/mL for both CHO K1 and CHO K1:cycE cells; while insulin from Sigma was supplemented at 1.0µg/mL. CHO K1:cycE cells were a gift of W. Renner (Renner et al. 1995). CHO K1:E2F-1 cells were derived by stable transfection of CHO K1 cells with an expression vector containing the full cDNA for E2F-1 (Chapter 3).

Cells were counted and then harvested at between 40% and 80% confluence from T-125 flasks. The cells were dissociated from the culture flask with the aid of Sigma protein-free dissociation agent (catalog number C5914) and washed three times in phosphate-buffered saline solution (PBS) pH 7.4 before the pellet was sonified in a Branson 450 sonifier in a solution

of 10mM Tris-HCl pH 6.8, 2% 2-mercaptoethanol (BME), and 1% Nonidet P-40 (NP-40). The final volume for sonication was estimated based on the number of cells and adjusted to two million cells per 50µL. The 50µL aliquots of cells were sonified on ice at 100% output for 2 minutes to release cellular proteins and shear nucleic acids. The samples were then frozen at -80°C until use.

Prior to sample loading, the 50µL sample pellet was suspended in 133µL of 9M urea, 2% BME, 2% NP-40, and 0.8% BioLyte pH 3-10 carrier ampholytes (Bio-Rad). 10 - 15µg of total protein was loaded per gel. Subsequent to sample preparation the protein concentration was determined as described below.

Protein concentration assay.

The concentration of protein samples was obtained with the aid of the Bio-Rad Protein Concentration Assay Kit (catalog number 500-0002) which is a slightly modified version of the Bradford dye binding assay (Bradford 1976). Control samples containing serial dilutions of bovine serum albumin in 9M urea, 2% BME, 2% NP-40 and 0.8% BioLyte pH 3-10 were used as a standard. For each sample, the protein concentration was determined twice and the results were averaged. The concentration of samples was further verified by the Total Protein Assay performed on a Beckman Synchron CX5CE autoanalyzer as per manufacturer's instructions.

Isoelectric focusing.

Several immobilized pH gradients (IPGs) were tested for the separation of CHO K1 samples in order to determine the optimum first dimension separation for this particular sample type. After the defined pH gradient was chosen, it was used for all subsequent studies. For CHO cells the 18cm pH 3-10 linear IPG gradient (precast) from Pharmacia was used. The IPG combines exceptional reproducibility with ease of use and improved handling as compared to more traditional carrier ampholyte tube gels (for related discussions see Appendices 1 and 2). These IPG strips were rehydrated overnight in a solution containing 8M urea, 0.5% Nonidet P-40, 1.5% 3-([3-chol-amido-propyl)-dimethyl-ammonio]-1-propane sulfonate (CHAPS), 0.385% BioLyte pH 3-10 carrier ampholytes (Bio-Rad), 0.116% Servalyte pH 3-10 carrier ampholytes (Serva), and 0.2% DTT.

Electrodes from the Multiphor II unit (Pharmacia) were placed on top of filter paper presoaked in either 0.5mM NaOH (cathode) or 6mM H_3PO_4 (anode) and blotted dry before being placed on the IPG gel. Kerosene oil (Fluka) was used as heat transfer fluid between the cooling plate and the DryStrip tray and low viscosity paraffin oil (Merck) was used as heat transfer fluid between the tray and the alignment card. Samples were loaded and the paraffin oil was used to overlay the entire IPG gel and sample loading cups before the isoelectric focusing was started.

The gels were run for a total of 72,750 volt-hours at 15°C. The voltage was ramped linearly from 500V to 3500V during the first 3 hours and was

maintained at 3500V for a subsequent 17.5 hours using a MultiDrive XL power supply (Pharmacia).

Equilibration.

After completion of the isoelectric focusing step, gels were incubated first in a solution of 6M urea, 30% glycerol, 2% SDS, 2% DTT, and 0.05M Tris-HCl pH 6.8 for 15 minutes. This resolubilizes the peptides and reduces disulfide bonds (Bjellqvist et al. 1993). Free sulfhydryl groups were subsequently blocked by equilibration in 6M urea, 30% glycerol, 2% SDS, 2.5% iodoacetamide, 0.05M Tris-HCl pH 6.8 and a trace of Bromophenol blue for 5 minutes. Both equilibration steps were performed with gentle orbital shaking.

SDS-PAGE.

Second dimension 12% T gels were prepared as described elsewhere (Harrington et al. 1991a). These gels were overlayed with the IPG gel shortened by a total of 1.0 cm which was cut off from both ends of the gel. The SDS-PAGE was run at a constant 40mA per gel for 6 hours at 12°C.

Detection.

After completion of SDS-PAGE, the gels were either fixed and silver stained as described elsewhere (Harrington et al. 1991a) or blotted (Towbin et al. 1979) to polyvinylidene difluoride (Millipore catalog number IPVH20200) membranes at 200mA for 3 hours. Silver stained gels were scanned on a Molecular Dynamics Personal Densitomter running Image Quant 3.3

software. Data was obtained at 50μm resolution and 12 bit dynamic range. Blotted proteins were detected with colloidal gold stain (Bio-Rad catalog number 170-6527) and were further studied by Western analysis.

Western analysis.

The following antibodies were used in this study: anti-actin (Sigma catalog number A-2066), anti-pRB clone PMG3-245 (Pharmingen catalog number 14001A), anti-E2F-1 clone KH95/E2F (Pharmingen catalog number 14971A), anti-cyclin A clone BF683 (Pharmingen catalog number 14531A), anti-cyclin E clone HE12 (Pharmingen catalog number 14591A), anti-cyclin D1 clone G124-326 (Pharmingen catalog number 14561A), anti-mouse Ig, peroxidase-linked $F(ab')_2$ fragments from sheep (Amersham catalog number NA9310), and anti-rabbit IgG (whole molecule) peroxidase-linked from goat (Sigma catalog number A-8275).

Colloidal gold stained membranes were rinsed in phosphate-buffered saline solution with Tween 20 (PBST) pH 8.0 (Sigma catalog number P3563) and then blocked in 5% nonfat dry milk (Bio-Rad catalog number 170-6404) before incubation with the primary antibody for one hour at a 1:5000 dilution. The membrane was washed three times in PBST for a total of 15 minutes before incubation with the secondary antibody (one hour at 1:7500 dilution). The membrane was again washed three times in PBST for a total of 15 minutes before detection. Tris-buffered saline solution with Tween 20 (TBST) was found to be ineffective for reproducible identification of some cell cycle proteins. The Amersham ECL detection kit (catalog number

RPN2106) was used and the film (Hyperfilm, Amersham catalog number RPN2103) was developed manually. Western analysis by secondary antibody binding alone was performed for negative control.

Computer analysis.

Image Quant 3.3 (Molecular Dynamics) software running on a Compaq 486 DX2 33 MHz computer was used in conjunction with Melanie II (Bio-Rad) running on a Sun Sparc10 workstation and visual inspection to aid in identification of spots, gel analysis, and quantification of particular regions of interest. 8 bit data was further reduced in Adobe Photoshop 2.5.1 running on a Macintosh computer before being printed on a Lasertechnics 300D continuous tone printer interfaced to the 33MHz PC.

4.4 Results and Discussion

CHO 2D gel database.

Wildtype CHO K1 cells grown in the presence of 10% serum were used as the benchmark sample type for this entire analysis. Silver stained gels were first compared to previous published reports of 2D gel analysis of intracellular CHO proteins. Although these early reports used significantly different technologies for 2D gel analysis, some useful information was obtained. The location of HSP 90, HSC 70, HSP 60, and alpha tubulin and beta tubulin could be determined (Y.S. Lee et al. 1992b, Y.S. Lee et al. 1992a) in relation to the two spots which have the actin characteristic shape. The structural protein actin has been well studied by two-dimensional gel

electrophoresis (Garrels and Gibson 1976) and the characteristic shape of the actin spots is easily identified on many 2D gels. The specific location of actin on these 2D gels was verified by Western analysis. This analysis documented two spots which have the shape typical of beta and gamma actins, and at the appropriate molecular weight and isoelectric point. Based on this Western analysis, on the comparison to published reports, and on further Western analysis of particular cell cycle regulatory proteins, a basic 2D gel database of CHO proteins now exists (see Figure 1). The low expression levels of some of the proteins makes them difficult to identify on reproduced images.

The location of the identified proteins was standardized and further verified by comigration of CHO K1 proteins with 2D gel standards (Bio-Rad catalog number 161-0320) and with SDS-PAGE standards (Bio-Rad catalog number 161-0317). Several runs with standards were performed, both in gel and in parallel gels. The resulting average location was noted on a master gel and the corresponding isoelectric points and molecular weights are easily read from this gel. The data on migration of standards is also included in Figure 1.

Expression levels of cell cycle regulatory proteins.

Two-dimensional gel electrophoresis was used to analyze the simultaneous expression of several important cell cycle regulatory proteins. The data are normalized by the expression of actin which is assumed to be constant and serves as an internal standard for the amount of protein

loaded per gel. The average normalized expression levels of these proteins from multiple gels are listed in Table 1. From these data one can gain insight into possible mechanisms governing the interaction of these molecules.

There does not appear to be any important effect on cyclin D1 expression for the cases studied. In contrast, cyclin E upregulation was observed for CHO K1 cells grown in the presence of potent mitogens (FCS>bFGF>insulin). Although this general trend holds for E2F-1 expression in the mitogen-stimulated cases, the amount of E2F-1 observed in CHO K1:cycE cells remained relatively low for growth stimulated by cyclin E expression. This observation suggested that bFGF stimulation resulting in cyclin E upregulation may be a consequence of E2F-1 upregulation. If true, then the addition of more potent mitogens such as bFGF may lead to E2F-1 upregulation which may lead, in turn, to cyclin E upregulation. This rationale provided motivation to study the effect of E2F-1 on protein-free growth (Chapter 3). Indeed, deregulated expression of E2F-1 yielded higher levels of cyclin E as well as cyclin A. It is known that the promoter of cyclin A contains an E2F-1 binding site (Helin and Harlow 1993), but this has not been established for cyclin E. It has also been shown that a single E2F binding site can confer E1A regulation to a test promoter (Nevins 1992). If fully functional in this capacity, one would expect higher cyclin A levels in response to E2F-1 overexpression. Because of the nature of cell cycle regulation, it is not surprising that E2F binding sites could be found in the promoter of proteins known to interact directly with E2F in

control of the cell cycle such as cyclin A. Moreover, the autoregulation of E2F-1 (Neuman et al. 1994) accounts for the very large increase in expression also observed.

2D gel analysis of various growth conditions.

A detailed microchemical investigation of the particular changes that occur in expression of CHO cell proteins in cultures stimulated to grow by different means is beyond the scope of this work. However, cross-gel analysis between various samples can provide an important first step for the elucidation of parts of the known biochemical mechanism as well as lead to the identification of new genes and gene products which may also play important roles in the mammalian cell cycle.

Insulin-stimulated growth of CHO K1 cells was first compared to CHO K1 cells growing on 10% serum (Figure 2). The 2D gels revealed 16 upregulated spots in insulin-stimulated cells compared to 10% FCS-stimulated cells, while 47 spots were significantly downregulated. The larger number of downregulated spots with the removal of various serum components supports the hypothesis that the various growth factors and growth inhibitors in serum may have triggered the expression of several proteins involved in intracellular signal transduction cascades. The downregulation of 47 spots provides initial evidence for this.

CHO K1 cells grown on FMX-8 in the presence of bFGF seemed to show many more differences from serum-stimulated cells than did insulin-

stimulated cells. Although only 9 species were clearly expressed in higher quantities in bFGF-stimulated cells (but not serum-stimulated cells), 68 species did not appear in the bFGF case as compared to the serum case. There were 3 spots which were expressed in both the insulin- and bFGF-stimulated cells and not in serum-stimulated cells. The unknown number of growth factors and growth inhibitors acting at many different levels of control, which were present in the serum, suggests that these 3 proteins could be co-regulated by a growth inhibitor present in FCS which is not present in the absence of FCS. There are 21 proteins which were relatively highly expressed in serum-stimulated growth but not growth stimulated by insulin or bFGF. Here again the downregulation may be caused by the removal of serum components with corresponding downregulation of particular signal transduction cascades.

Figure 4 depicts the expression pattern of CHO K1 cells transfected with a constitutive expression vector encoding the gene for human cyclin E (CHO K1:cycE). When these cells are compared to CHO K1 cells grown in the presence of serum, 15 proteins are observed which are upregulated as compared to serum-stimulated cells and there are 40 proteins for which the converse is true. Further study of the 15 proteins may reveal if the upregulation is a cause or a consequence of either constitutive cyclin E expression or protein-free growth. Gene dosage experiments or inducible expression of cyclin E would be useful in determining which spots (up- or downregulated) are a direct consequence of cyclin E expression.

Figure 5 is the 2D gel pattern of CHO K1:cycE cells stimulated by growth on bFGF (cycE+bFGF). The markings on Figure 5 represent changes relative to the CHO K1:cycE cell line not stimulated by bFGF. There are 14 spots which appear to be upregulated in specific response to bFGF stimulation in the CHO K1:cycE cell line, and none of these correlate to upregulated spots for the CHO K1 case (K1:bFGF). Again, because of the nature of serum, this result is not surprising. A study of bFGF stimulation by 2D gels demands the removal of serum from all cases because serum probably stimulated many different signal transduction cascades including those stimulated by bFGF directly. Therefore, the set of spots upregulated in the bFGF-stimulated CHO K1:cycE cells probably represents a more accurate depiction of proteins actually upregulated in response to bFGF rather than as a consequence of the removal of serum. Consequently, no upregulation of spots caused specifically by bFGF can be seen in a comparison of bFGF- and serum- stimulation. There were 13 downregulated spots and only 1 of these appears to be downregulated when bFGF-stimulated CHO K1 cells are compared to 10% FCS-stimulated CHO K1 cells.

In Figure 6 the CHO K1:cycE bFGF-stimulated cells are compared to CHO K1 bFGF-stimulated cells. This comparison yielded observations on the effect of cyclin E overexpression independent of serum. 34 spots are upregulated while 8 spots appear to be downregulated. 4 of the upregulated species are also upregulated when CHO K1:cycE cells are compared to CHO K1 cells while only 1 of the downregulated proteins is present in the same comparison. It is likely that these 4 upregulated species have a relation to

cyclin E expression. Microchemical analysis of these 4 spots would provide further insight.

In Figure 7 the results from a comparison of a CHO K1 cell line constitutively expressing human E2F-1, CHO K1:E2F-1, versus CHO K1 cells grown in the presence of serum are given. 33 spots appear to be downregulated as compared to the wildtype. In contrast to the CHO K1:cycE strain which showed upregulation of some 15 species, deregulated expression of E2F-1 appears to stimulate the expression of 236 species. This is not a surprising result in light of the cellular function of E2F-1 as a transcription factor with regulated expression. This result provides further evidence for the significant regulatory effect of cloned human E2F-1 in CHO cells and suggests several more experiments which could help elucidate parts of the biochemical mechanisms controlling the mammalian cell cycle machinery. For example, E2F-1 upregulation resulting in higher expression of other proteins can lead to the identification of gene products which are transcriptionally activated by the E2F transcription factor. This is particularly useful in cell types for which a complete DNA sequence (i.e., E2F binding sites) is not established. More generally, 2DE can be an important tool for identifying changes in the protein patterns caused by stimulation by various growth factors. The gel analysis presented above involves serum and necessarily contains ambiguity. This analysis furthers the need for serum removal from studies of growth characteristics of mammalian cell culture systems.

With the aid of carefully designed experiments, one can begin to use two-dimensional electrophoresis of proteins to distill some elements of the pathways of mitogen stimulation. These pathways, from cell receptor to cell cycle regulatory proteins, probably have important roles not only in cell growth, but also in diseases of cell growth including cancer. Already, it has been shown that 2D gel analysis of CHO K1 cells grown under various conditions can corroborate existing theories of the biochemical mechanisms which govern cell cycle control and can serve as a foundation for further studies of cell cycle control.

4.5 References

Aebersold, R., Mass Spectrometry of Proteins and Peptides in Biotechnology, Curr. Opin. Biotech., **4**: 412 (1993).

Aebersold, R.H., Leavitt, J., Saavedra, R.A., Hood, L.E., and Kent, S.B.H., Internal Amino Acid Sequence Analysis of Proteins Separated by One- or Two-Dimensional Gel Electrophoresis after in situ Protease Digestion on Nitrocellulose, Proc. Natl. Acad. Sci. USA, **84**: 6970 (1987).

Bjellqvist, B., Pasquali, C., Ravier, F., Sanchez, J.C., and Hochstrasser, D., A Nonlinear Wide-Range Immobilized pH Gradient for Two-Dimensional Electrophoresis and Its Definition in a Relevant pH Scale, Electrophoresis, **14**: 1357 (1993).

Bradford, M.M., A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilizing the Principle of Protein-Dye Binding, Anal. Biochem., **72**: 248 (1976).

Cantley, L.C., Auger, K.R., Carpenter, C., Duckworth, B., Graziani, A., Kapeller, R., and Soltoff, S., Oncogenes and Signal Transduction, Cell, **64**: 281 (1991).

Garrels, J.I. and Gibson, W., Identification and Characterization of Multiple Forms of Actin, Cell, **9**: 793 (1976, part 2).

Görg, A., Postel, W., and Günther, S., The Current State of Two-Dimensional Electrophoresis with Immobilized pH Gradients, Electrophoresis, **9**: 531 (1988).

Harrington, M.G., Gudeman, D., Zewert, T., Yun, M., and Hood, L.E., Analytical and Micropreparative Two-Dimensional Electrophoresis of Proteins, Methods: A Companion to Methods in Enzymology, **3**: 98 (1991).

Harrington, M.G., Hood, L.E., and Puckett, C., Simultaneous Analysis of Phosphoproteins and Total Cellular Proteins from PC12 Cells, Methods: A Companion to Methods in Enzymology, **3**: 135 (1991).

Harrington, M.G. and Merril, C.R., Cerebrospinal Fluid Protein Analysis in Diseases of the Nervous System, J. Chrom., **429**: 345 (1988).

Hartwell, L.H. and Kastan, M.B., Cell Cycle Control and Cancer, Science, **266**: 1821 (1994).

Heichman, K.A. and Roberts, J.M., Rules to Replicate By, Cell, **79**: 557 (1994).

Helin, K. and Harlow, E., The Retinoblastoma Protein as a Transcriptional Repressor, Trends Cell. Biol., **3**: 43 (1993).

Hunter, T. and Pines, J., Cyclins and Cancer II: Cyclin D and CDK Inhibitors Come of Age, Cell, **79**: 573 (1994).

Lee, Y.J., Hou, Z.Z., Curetty, L., Armour, E.P., Al-Saadi, A., Bernstein, J., and Corry, P.M., Heat-Resistant Variants of the Chinese Hamster Ovary Cell: Alteration of Cellular Structure and Expression of Vimentin, J. Cell. Phys., **151**: 138 (1992).

Lee, Y.J., Hou, Z.Z., Curetty, L., and Borrelli, M.J., Development of Acute Thermotolerance in L929 Cells: Lack of HSP28 Synthesis and Phosphorylation, J. Cell. Phys., **152**: 118 (1992).

Marx, J., How Cells Cycle Toward Cancer, Science, **263**: 319 (1994).

Neuman, E., Flemington, E.K., Sellers, W.R., and Kaelin, W.G., Transcription of the E2F-1 Gene is Rendered Cell-Cycle Dependent by E2F DNA-Binding Sites Within Its Promoter, Mol. Cell Biol., **14**: 6607 (1994).

Nevins, J.R., E2F: A Link Between the Rb Tumor Suppressor Protein and Viral Oncoproteins, Science, **258**: 424 (1992).

Nurse, P., Ordering S Phase and M Phase in the Cell Cycle, Cell, **79**: 547 (1994).

O'Farrell, P.H., High Resolution Two-Dimensional Electrophoresis of Proteins, J. Biol. Chem., **250**: 4007 (1975).

Ohtsubo, M. and Roberts, J.M., Cyclin-Dependent Regulation of G1 in Mammalian Fibroblasts, Science, **259**: 1908 (1993).

Puck, T.T., Cieciura, S.J., and Robinson, A., Genetics of Somatic Mammalian Cells. III. Long-Term Cultivation of Euploid Cells from Human and Animal Subjects, J. Exp. Med., **108**: 945-955 (1958).

Renner, W., Lee, K.H., Hatzimanikatis, V., Bailey, J.E., and Eppenberger, H., Recombinant Cyclin E Expression Activates Proliferation and Obviates Surface Attachment of Chinese Hamster Ovary (CHO) Cells in Protein-Free Medium, submitted (1995).

Rodriguez, L.V., Gersten, D.M., Ramagli, L.S., and Johnston, D.A., Towards Stoichiometric Silver Staining of Proteins Resolved in Complex Two-Dimensional Electrophoresis Gels: Real-Time Analysis and Pattern Development, Electrophoresis, **14**: 628 (1993).

Sherr, C.J., G1 Phase Progression: Cycling on Cue, Cell, **79**: 551 (1994).

Simpson, R.J., Tsugita, A., Celis, J.E., Garrels, J.I., and Mewes, H.W., Workshop on Two-Dimensional Gel Protein Databases, Electrophoresis, **13**: 1055 (1992).

Singh, P., Wong, S.H., and Hong, W., Overexpression of E2F-1 in Rat Embryo Fibroblasts Leads to Neoplastic Transformation, EMBO J., **13**: 3329 (1994).

Towbin, H., Staehelin, T., and Gordon, J., Electrophoretic Transfer of Proteins from Polyacrylamide Gels to Nitrocellulose Sheets: Procedure and Some Applications, Proc. Natl. Acad. Sci. USA, **76**: 4350 (1979).

Vandekerckhove, J., Rider, M., Rasmussen, H.H., De Boeck, S., Puype, M., Van Damme, J., Gesser, B., and Celis, J., Routine Amino Acid Sequencing on 2D-Gel Separated Proteins: A Protein Elution and Concentration Gel System, Methods in Protein Sequence Analysis, K. Imahori and F. Sakiyama, ed., Plenum Press, New York 1993.

4.6 Tables

Normalized expression of five various cell cycle regulatory proteins as identified by two-dimensional gel electrophoresis. The values were obtained using ImageQuant 3.3 software. The integrated optical density of spots of interest were calculated and the local background was subtracted. The given values are normalized by the expression of beta and gamma actins on each gel and then multiplied by 1000. The error in measurement is ± 1.0. CHO K1 cells were stimulated to grow on FMX-8 media by the addition of: 10% FCS, bFGF, insulin, a constitutive expression vector for cyclin E, bFGF and cyclin E, or a constitutive expression vector for E2F-1. The sum of all identifiable cloned and endogenous isoforms is given as appropriate.

Growth	cyclinE	cyclinD1	cyclinA	pRb	E2F-1
10% FCS	**18**	**4.7**	**29.1**	**6.0**	**10.9**
bFGF	**14**	**2.6**	**103**	**17**	**10.1**
insulin	**6.1**	**1.8**	**34.5**	**1.4**	**2.2**
cycE	**35**	**3.7**	**72.5**	**15**	**1.7**
cycE+bFGF	**33**	**3.5**	**11.8**	**28**	**8**
E2F-1	**234**	**4.0**	**155**	**15**	**232**

4.7 Figures

Figure 1 depicts a 2D gel of CHO K1 cells grown in the presence of 10% fetal calf serum. Reference marks for isoelectric point and molecular weight are given as are various landmark proteins which have been identified by as described in the text. Some of the reference proteins are marked in later figures to aid in gel orientation. The low expression levels of some of the proteins makes them difficult to identify on reproduced images.

Figure 2 depicts a 2D gel of CHO K1 cells grown in the presence of insulin. Three landmark proteins are indicated. The green squares depict proteins which are downregulated, and the red circles depict proteins which are upregulated as compared to CHO K1 grown in 10% FCS.

Figure 3 depicts a 2D gel of CHO K1 cells grown in the presence of bFGF. Three landmark proteins are indicated. The green squares depict proteins which are downregulated, and the red circles depict proteins which are upregulated as compared to CHO K1 grown in 10% FCS.

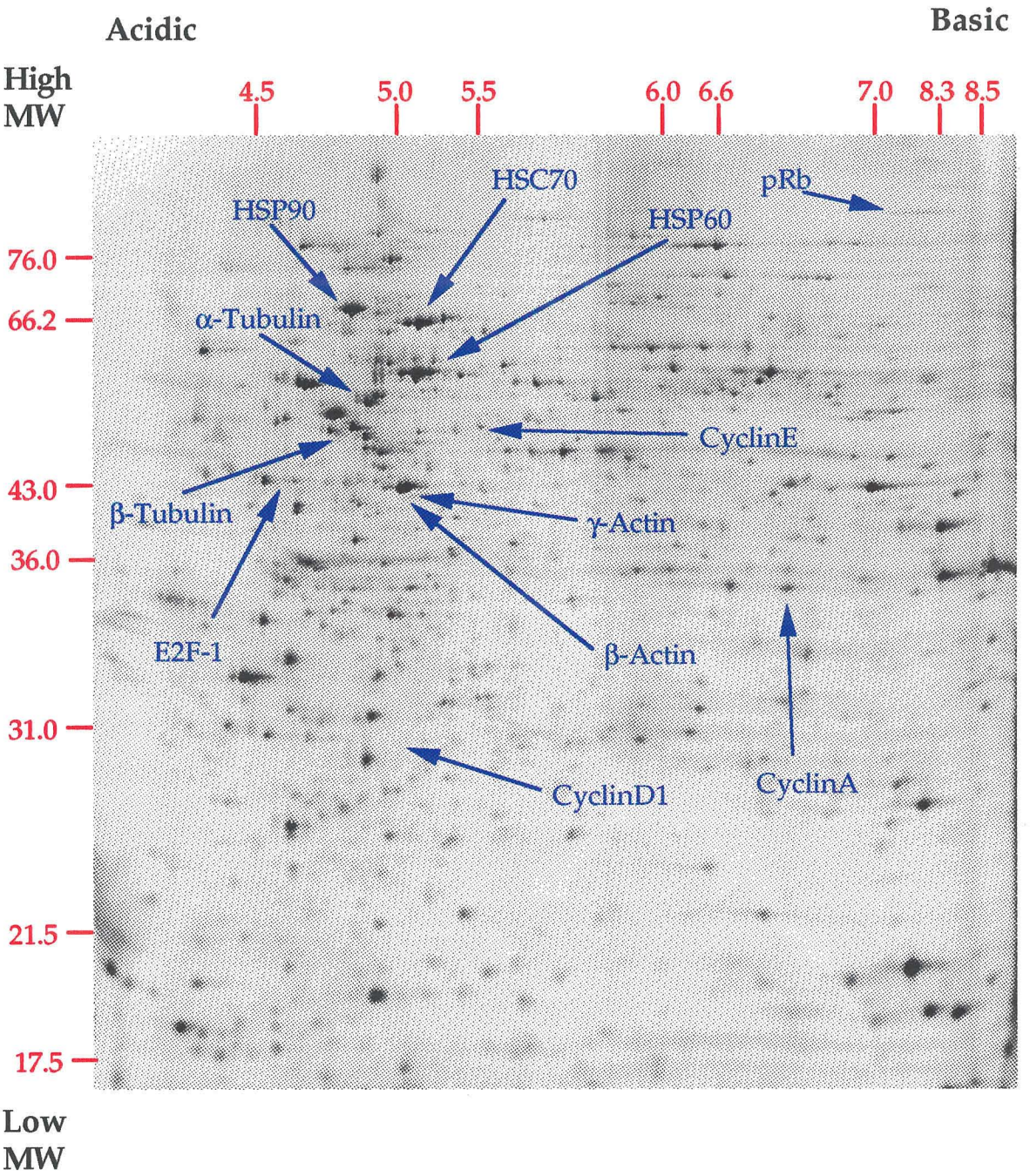
Figure 4 depicts a 2D gel of CHO K1:cycE cells grown on FMX-8 alone. Three landmark proteins are indicated. The green squares depict proteins which are downregulated, and the red circles depict proteins which are upregulated as compared to CHO K1 grown in 10% FCS.

Figure 5 depicts a 2D gel of CHO K1:cycE cells grown in the presence of bFGF. Three landmark proteins are indicated. The green squares depict proteins which are downregulated, and the red circles depict proteins which are upregulated as compared to CHO K1 grown in bFGF.

Figure 6 depicts a 2D gel of CHO K1:cycE cells grown in the presence of bFGF. Three landmark proteins are indicated. The green squares depict proteins which are downregulated, and the red circles depict proteins which are upregulated as compared to CHO K1:cycE cells grown on FMX-8 alone.

Figure 7 depicts a 2D gel of CHO K1:E2F-1 cells grown on FMX-8. Three landmark proteins are indicated. The green squares depict proteins which are downregulated, and the red circles depict a fraction of the proteins which are upregulated as compared to CHO K1 grown in 10% FCS.

Figure 1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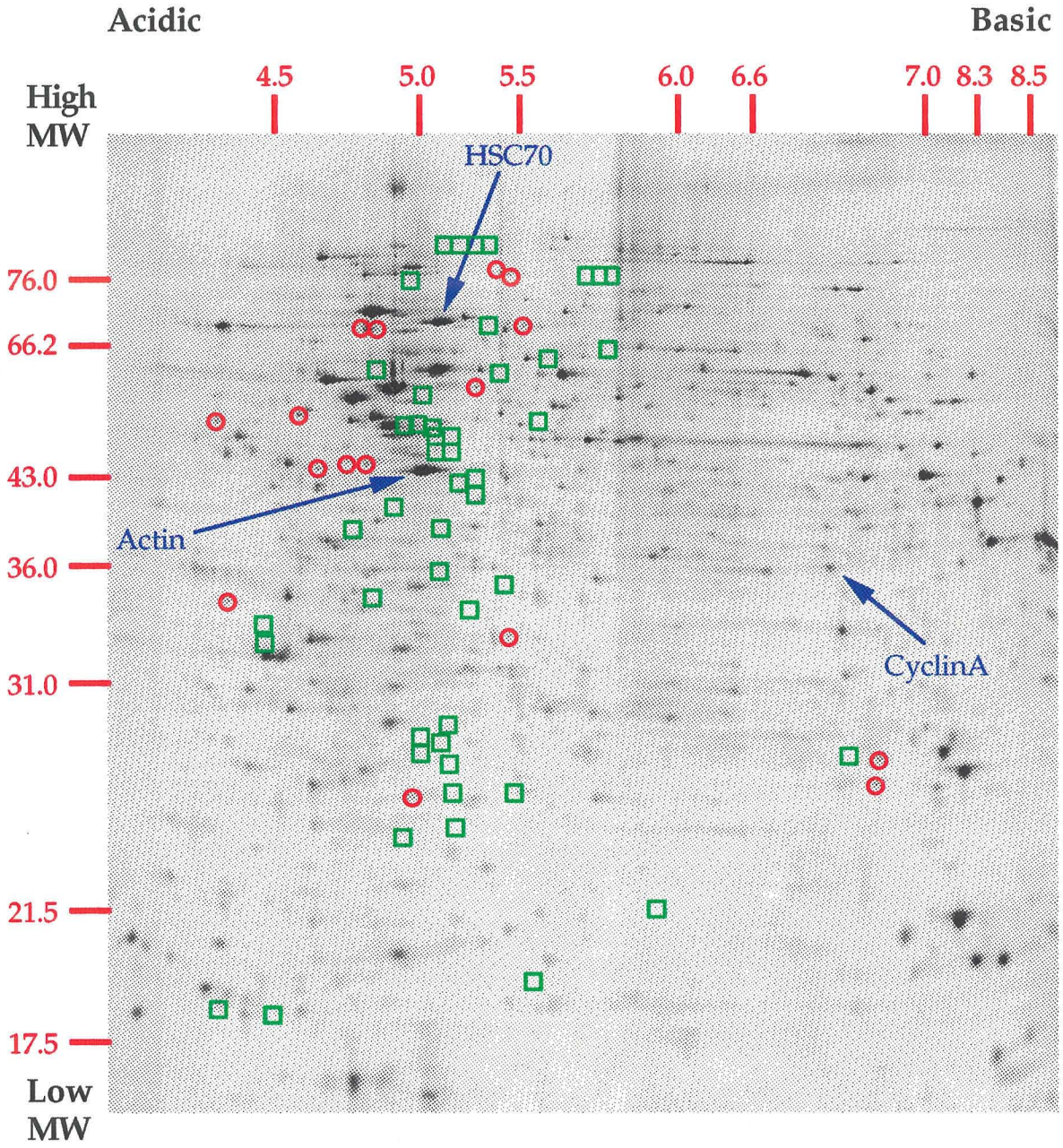
CyclinD1
CyclinA

Figure 2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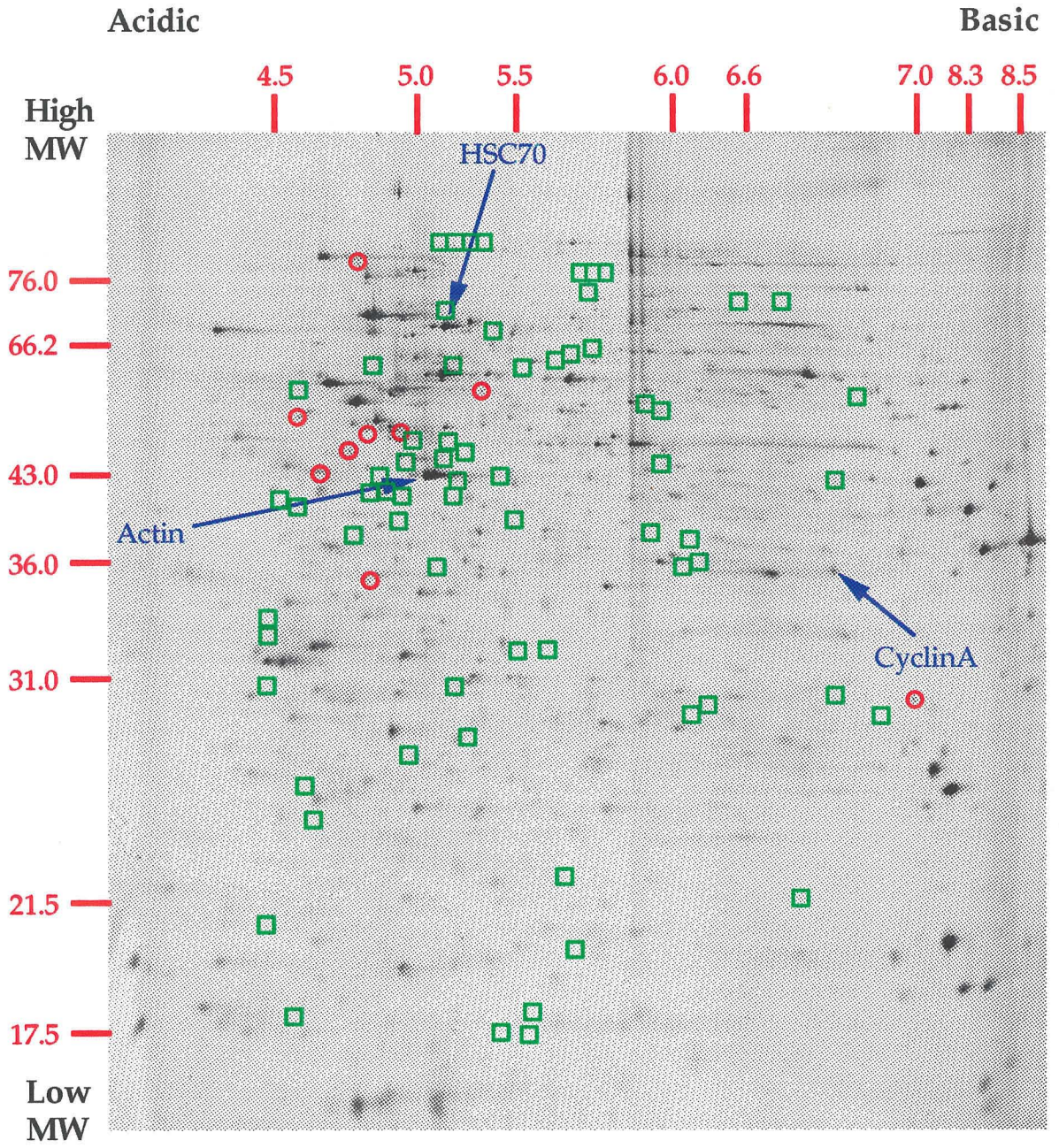
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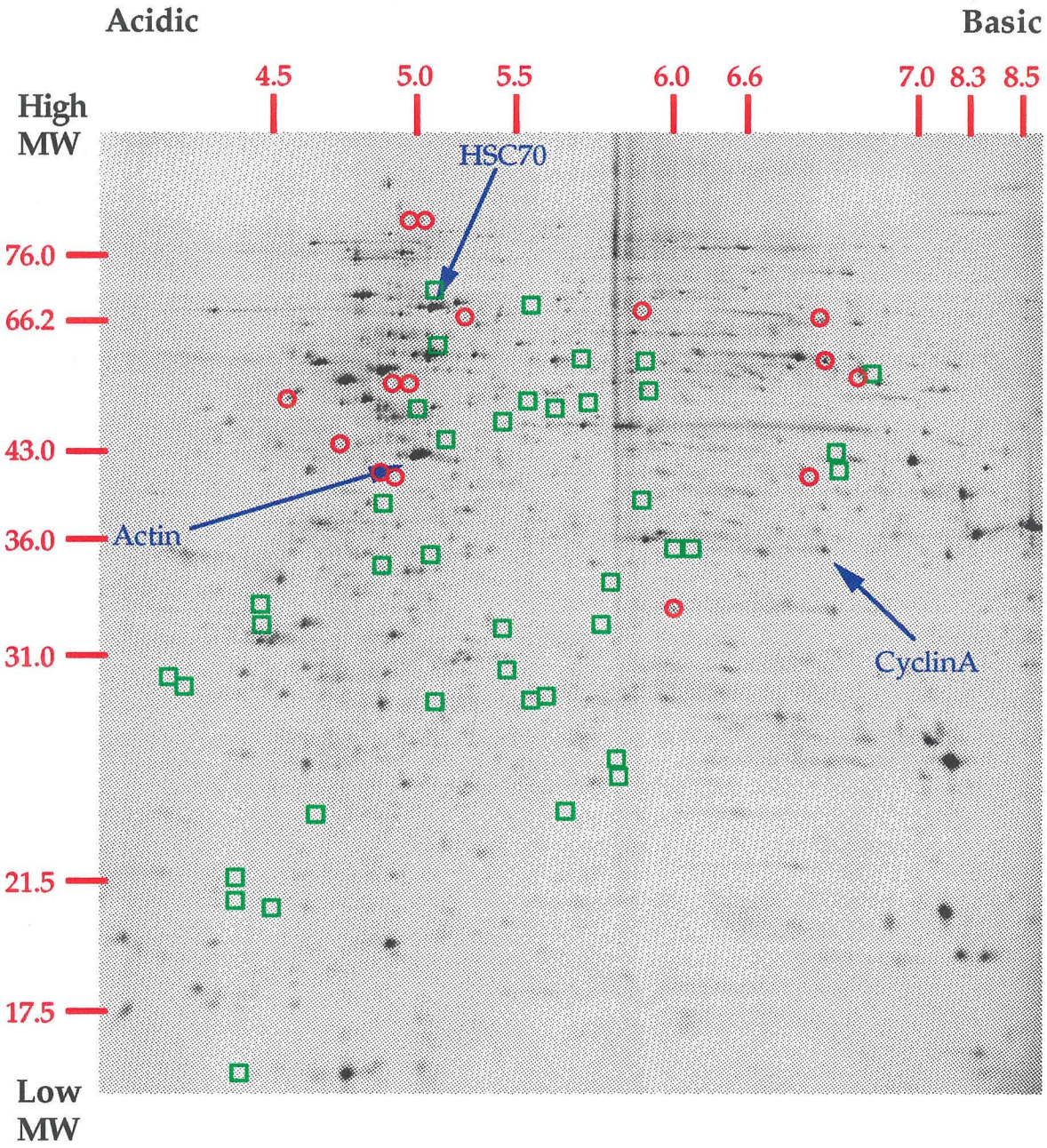
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Figure 5

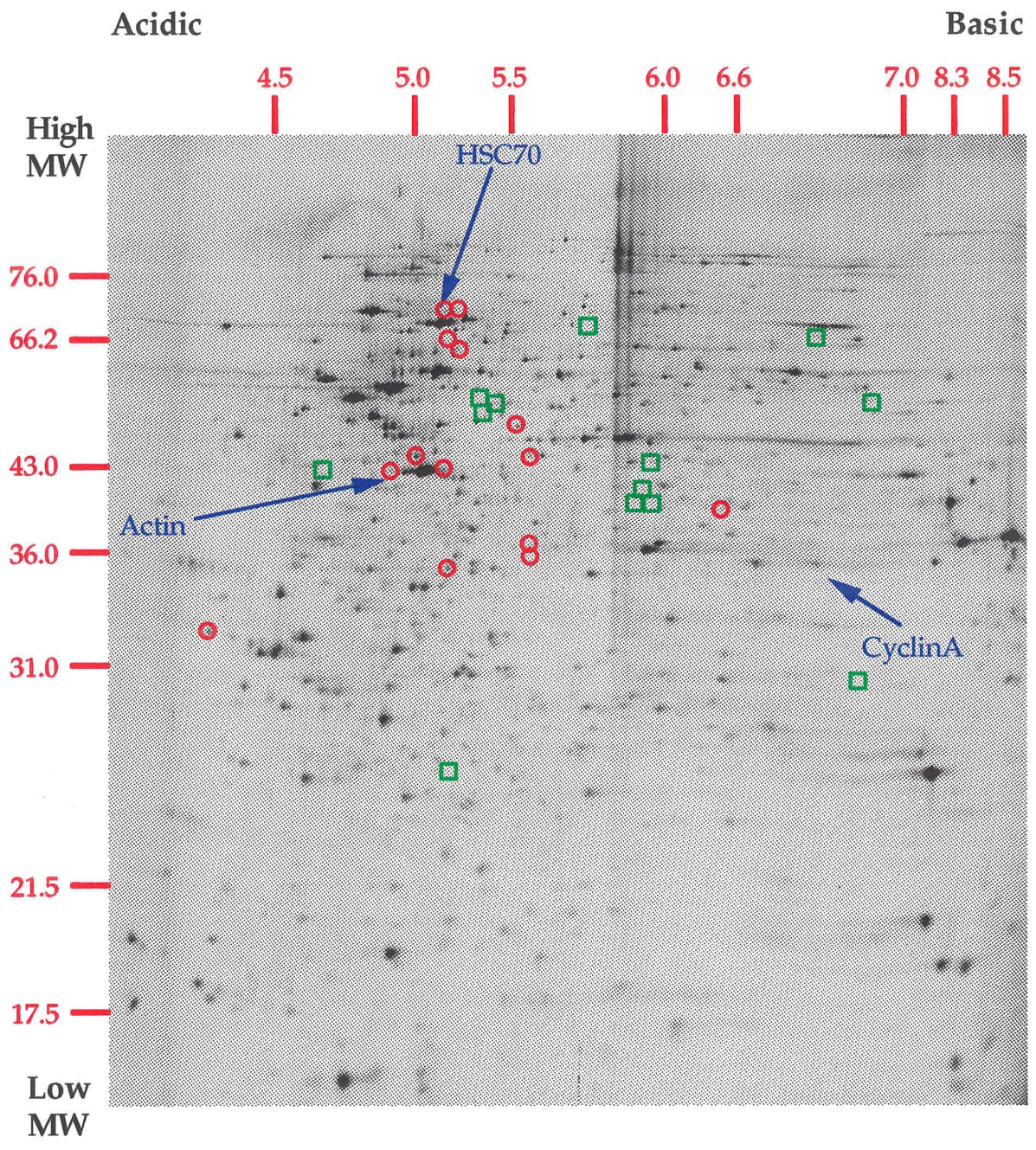
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Figure 6

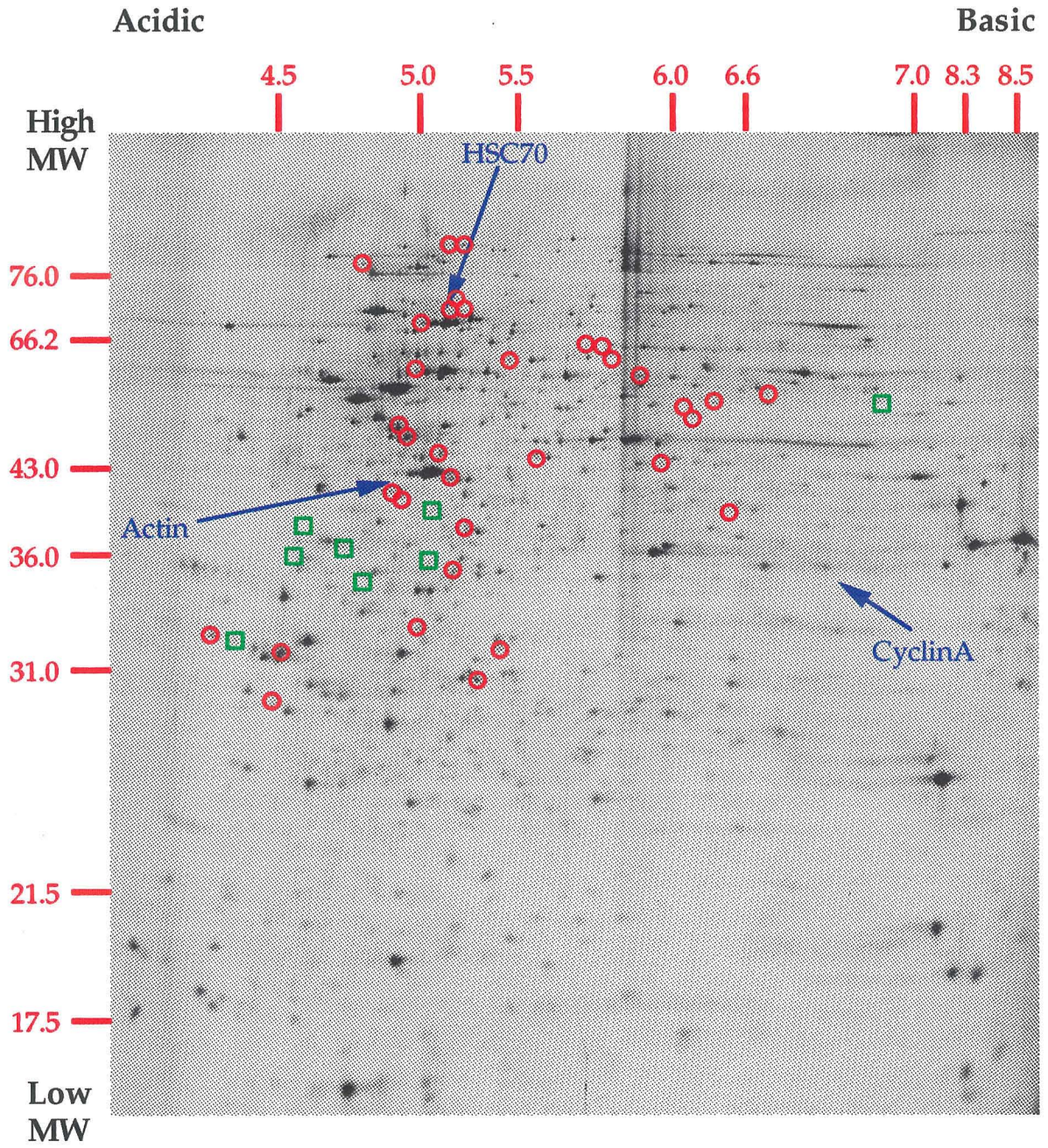
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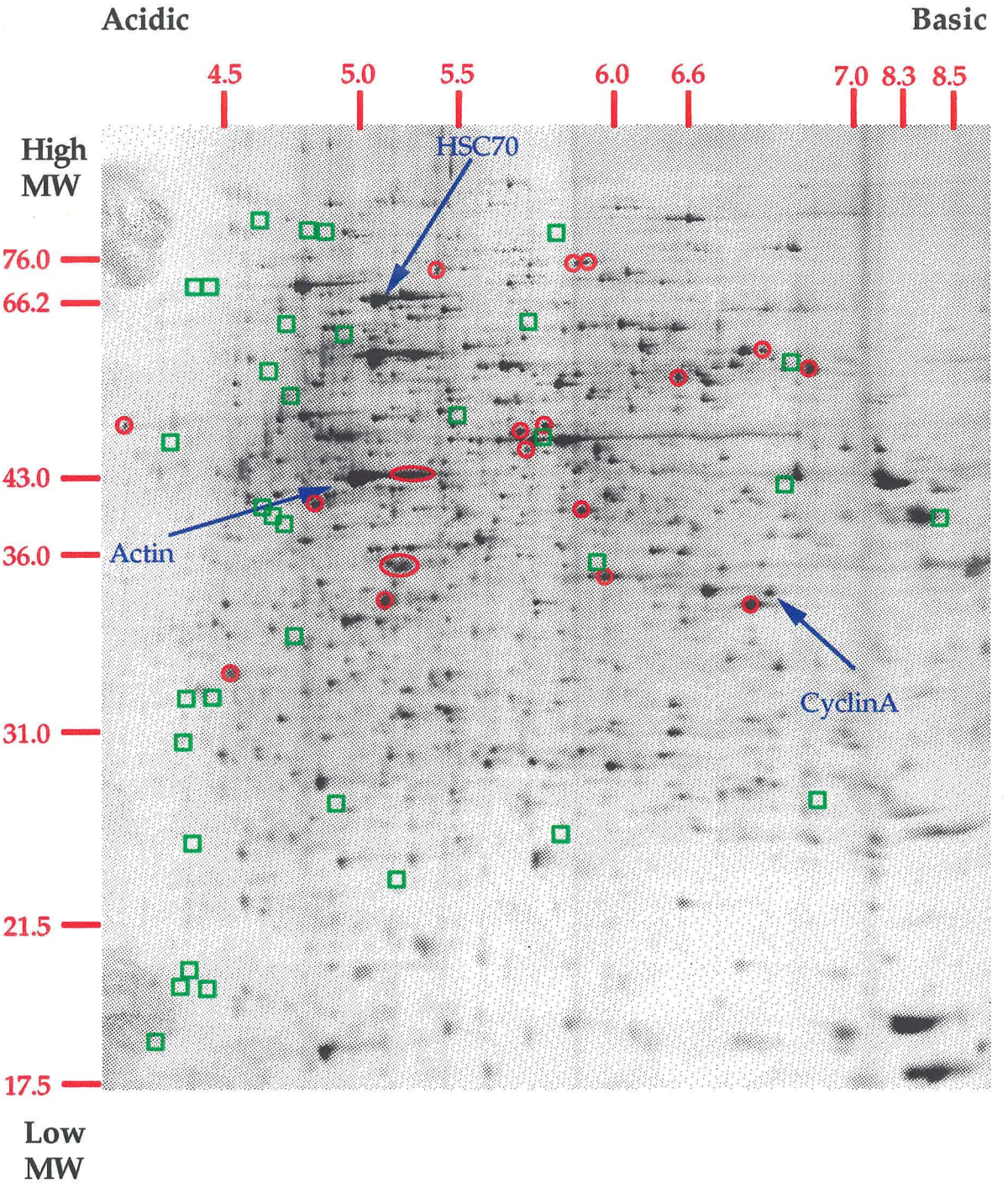
Figure 7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CHAPTER FIVE

Conclusions and Future Prospects

The experiments presented describe the rational manipulation of the metabolic pathways governing cell cycle control in order to engineer Chinese hamster ovary (CHO) cells capable of growth in a protein-free environment. It was found that the deregulated expression of E2F-1, a subunit of a transcription factor known to be involved in cell cycle control, activates the proliferation of CHO cells in a protein-free medium. Western analysis of ten stably transfected clones showed expression of the cloned protein. Confocal microscopy and Southern analysis also provided evidence for E2F-1 expression. Quantitative measurements of the E2F-1 expressing clones shows an increase of more than 21-fold in E2F-1 expression as compared to serum-stimulated CHO K1 cells. High levels of cyclin E and cyclin A are also detected in the transfected cells. A two-dimensional gel electrophoresis analysis of one of the E2F-1 expressing transfectants demonstrated increased expression of 236 spots as compared with growth stimulation by growth factors or serum. Such a large increase in protein expression was not observed in the other cases studied: serum-, bFGF-, insulin-, cyclin E-, and cyclin E-bFGF- stimulated growth. Most significantly, the CHO K1:E2F-1 cells could proliferate on basal medium in a serum- and protein-free environment.

The metabolic engineering approach used here is a powerful tool in biotechnology for the creation of new cell lines for improved biopharmaceutical production. Moreover, the tools of two-dimensional electrophoresis, when optimized, can provide important information about a particular system as well as provide potentially new avenues for the

discovery of gene products under transcription factor control. This is particularly important in organisms for which detailed genomic sequence data is still unavailable.

Perhaps more importantly, the experiments presented have provided a plethora of new problems for exploration. Subsequent investigations may lead to new information, clarification of current questions, and ultimately, new technology. Further studies of the effect of deregulated expression of cloned E2F-1 can begin with an analysis of the effect of gene dosage. Saturation of E2F-1 binding sites probably occurs at high levels of E2F-1 and the excess E2F-1 expression would thus decrease the overall growth capabilities of engineered cells. Gene dosage effects could be examined by futher analysis of the 10 clones - each expressing various amounts of E2F-1. A more rigorous analysis would involve inducible expression of the cloned gene. A tetracycline-inducible expression system for cyclin E has already been developed (Resnitzky et al. 1994) and a study of the detailed growth characteristics of cloned E2F-1 expressing cells compared to various induction levels may result in optimized growth characteristics. Building on other studies, one could create a metabolic switch (Chen et al. 1993) to activate proliferation under certain conditions and turn off proliferation while simultaneously inducing production (E. Nollen, personal communication). Even when optimized, the ultimate test of deregulated expression of E2F-1 is the engineering of cells with improved production capabilities. Therefore, the creation of production strains would be the next

important and logical study for E2F-1 overexpressing cells grown in serum- and protein-free media.

The intriguing results from the two-dimensional electrophoresis analysis of CHO K1 growth stimulation suggest further experiments. The most fundamental studies should begin with 2D gel databasing and involve microchemical analysis of several reference spots as well as spots which are up- or down-regulated because of a defined growth stimulation. Additional experiments will lead to the discovery of proteins which may be up- or down-regulated in response to E2F-1 overexpression. This is useful information since many of these proteins are likely to be involved in S phase onset or cell cycle control. Although E2F binding sites can be identified in the promoters of genes after the gene has been cloned, 2DE can provide important information for two reasons. First, the presence of E2F binding sites in promoters does not exclude further levels of control. Protein expression in response to various forms of control is best determined at the protein level rather than at the DNA sequence or even mRNA level. Second, despite the efforts of the Genome Initiative, DNA sequence data will not be readily available for many biological systems in the near future.

Carefully designed experiments can also help identify intracellular signal transduction patterns. For example, the cyclin E overexpressing cells grown in the absence of external growth factors should differ in the protein expression pattern of these cells grown in the presence of bFGF. The

upregulated and newly synthesized species may be proteins expressed in response to bFGF stimulation. A parallel experiment with E2F-1 overexpressing cells and bFGF stimulation would further support any data obtained on the bFGF signal transduction cascade by 2DE.

The above suggestions underscore the need for greater research activity in the areas of serum-independent growth, metabolic engineering, and two-dimensional electrophoresis. A marriage of these areas with existing technology and research areas will provide significantly improved methods for production of biopharmaceuticals and improved technology for the study of biological systems.

References

Chen, W., Kallio, P.T., and Bailey, J.E., Construction and Characterization of a Novel Cross-Regulation System for Regulating Cloned Gene Expression in Escherichia coli, Gene, **130**: 15 (1993).

Resnitzky, D., Gossen, M., Bujard, H., and Reed, S.I., Acceleration of the G1/S Phase Transition by Expression of Cyclins D1 and E with an Inducible System, Mol. Cell Biol., **14**: 1669 (1994).

APPENDIX ONE

Mechanical Precision in Two-Dimensional Electrophoresis Can Improve Protein Spot Positional Reproducibility

Source: Harrington, Lee, Yun, Zewert, Bailey, and Hood,
Appled and Theoretical Electrophoresis (1993) **3:** 347-353

A1.1 Abstract

Current methods for high resolution two-dimensional electrophoresis (2DE) of proteins are capable of separating over 5,000 protein spots in one procedure. Running and analyzing such 2DE gels requires skilled technical work. However, the variable reproducibility of spot positions means that, even under the best circumstances, one gel cannot be overlain directly on another for precise comparison. Therefore, new and improved technologies that enhance gel-to-gel reproducibility are required. To this end, we have designed and built a research instrument to test whether a precise mechanical device could improve the gel-to-gel reproducibility by reducing the amount of distortion and positional variation between the first and second dimension gels. Other causes of poor reproducibility, including sample type and preparation, gel matrices and running conditions were not varied in order to limit this study to the mechanical variations inherent in current 2DE systems. We found that the sample standard deviation of pooled data for measured protein spot-to-spot distances in the prototype device was 1.3 mm as compared to 4.3 mm in a conventional 2DE system. These improvements support the possibility that greater automation of the multistep 2DE process will enhance reproducibility. This approach seems justified in order to achieve significantly better matching between gels and between results from different laboratories.

A1.2 Introduction

High resolution separation of proteins by two-dimensional electrophoresis (2DE) (O'Farrell, 1975; Klose, 1975; Scheele, 1975) can provide a wealth of information on the content of the many different proteins in a sample, or on the post-translational modifications of individual proteins. Detailed protein atlases are being used in many medical (Harrington *et al.*, 1986) and biological studies (Garrels and Schubert, 1979) and primitive databases are now being constructed (see for examples Celis, 1991). Furthermore, amino acid sequence analysis of specific spots resulting from a 2DE separation can allow the determination of the primary structures of specific proteins as well as lead to the subsequent cloning of the genes associated with these proteins (Aebersold *et al.*, 1987).

An aspect of 2DE technology that requires improvement is the reproducibility of the procedure. For 2DE analysis, one requires reproducible positional identification of individual spots by their charge and mass coordinates and quantitation of their abundance after any one of several detection methods. Ideally, multiple gels produced from aliquots of the same protein sample should contain the same protein spot positions and quantities. Any two such gels overlain on top of each other should be exact duplicates for every spot. When a gel run under the same conditions with a slightly different protein content is overlain, the biochemically identical spots should precisely overlie their counterparts, while all differing proteins

should be immediately identified by their positional or quantitative change. While this is an obvious goal for users of this technology, it has not been realized. Studies of one small region of a 2DE gel are feasible with current technology, but inadequate reproducibility does limit the accuracy and capability of "global" 2DE analyses of the overall 2DE protein patterns.

It is difficult to define the minimum required, as well as the existing, levels of 2DE reproducibility because these levels depend on the particular protocol used and the associated experimental requirements. These may range from a one-time analysis of a small region of a few 2DE gels, to a comparison of detailed 2DE maps between many laboratories, over years. Examples of published measurements of reproducibility (e.g., Taylor *et al.*, 1983; Daufeldt and Harrison, 1984; Garrels and Franza, 1989; Harrington *et al.*, 1984; Kuick *et al.*, 1991; Miller and Merril, 1989; Gianazza *et al.*, 1986; Fosslien *et al.*, 1984) are specific to the authors' unique methods and define the variation in subsets of spots that are selected as the most reproducible (usually on the basis of being the most abundant) spots. In these examples, the number of the subset of spots analyzed per gel varies from 90 (Harrington *et al.*, 1984) to 1600 (Garrels and Franza, 1989). In one of the most detailed early reports (Taylor *et al.*, 1983), both the power of 2DE and its problems were illustrated: 16 identical samples were subjected to the same 2DE run; on one hand, 95% of the computer-analyzed spots registered within a size parameter smaller than the diameter of the spot, consistent with excellent utility for this subset of "well-behaved" spots; on the other hand, a relatively large discrepancy in total spots per gel was observed, ranging from 285-434,

reflecting the variation of low abundance proteins. These different studies provide appropriate validation for the level of global 2DE analysis performed under the conditions the investigators have chosen, but all have been forced to exclude the more variable, usually low abundance spots from 2DE gel analysis.

We have recently been forced to improve our reproducibility because of our interest in low abundance proteins among gels that contain 1,000-4,000 spots (Harrington *et al.*, 1992). Unfortunately, the low abundance spots are less reproducible. In these studies of nuclear regulatory proteins in the early development of the sea urchin embryo, optimal conditions allowed us to achieve a gel-to-gel positional matching capability of 99.5-99.7%, as judged by post-match editing. However, as we wish to compare these results with ongoing studies of embryonic development, this matching error may lead to as many as 20 mismatches per 4,000 spots in each gel-gel comparison, probably affecting the low abundance proteins of specific interest. This magnitude of mismatch in each gel-to-gel comparison would rapidly lead to an unacceptable cumulative error for multiple gels in our desired database studies. Therefore, we need to improve the reproducibility of our 2DE system. Less than two mismatches in the low abundance proteins in any gel comparison could feasibly be edited or tolerated; this would require a ten-fold improvement!

Before we can improve the level of reproducibility for 2DE gels, we must determine the major cause(s) of error. In the complex, multistep

protocol of 2DE gels, variability can occur in any of the multiple steps: sample type and manipulation, electrophoresis chemistry, physical and electrical conditions, detection and analysis methodology. Detailed review of the possibilities is beyond the scope of this paper, but we regard three aspects as of special interest since our last methods were described (Harrington *et al.*, 1991). First, the variability during isoelectric focusing (IEF) resulting from the unstable pH gradient with carrier ampholytes and the chemical variability between carrier ampholyte batches can be overcome with the use of immobilized pH gradient gels (Bjellqvist *et al.*, 1982), which have been progressively implemented in 2DE analyses (e.g., Görg *et al.*, 1988; Hughes *et al.*, 1992). However, there is still variability dependent on how the IPG gradient is formed. Secondly, there remains the positional variability of polyacrylamide gels when unsupported: they break, stretch, swell and contract, and partially dissolve on occasion. Some progress has been made in this area, most notably by the use of plastic-backed gels (in the Pharmacia/LKB "flat-bed" systems), a fiber support for IEF tube gels (Millipore), and a re-inforced "proprietary" stiffener in polyacrylamide gels (Millipore; Patton *et al.*, 1992). We believe there remains a need for more mechanically robust separation matrices, and we are investigating several candidates (Harrington *et al.*, 1994).

Finally, there are multiple opportunities for human errors during the handling stages of 2DE gel procedures. This aspect has received little attention in industry, but Shimadzu has recently introduced robotics to automate part of the 2DE process. Their instrument (TEP-1) performs the

central maneuvers involved in 2DE separations, with separation in an IEF tube gel, followed by robotic extrusion of the IEF gel onto the second (SDS-PAGE) dimension (Nokihara *et al.*, 1992). With perhaps different intentions and a different approach, we chose to determine whether precise mechanical control of the alignment of the IEF and SDS-PAGE gels, independent of the many other variables, could significantly improve positional reproducibility in 2DE gels. We also wanted to test our presumption that a 2DE gel system in which the gels themselves were not moved (our notion of an "all-in-one" 2DE gel) would significantly improve reproducibility. Here, we present experimental data to evaluate this notion: would precise mechanical control of each first and second dimension gel and their connections improve the reproducibility of spot-to-spot positions of 2DE gels?

A1.3 Materials and Methods

Protein Samples.

The protein samples used in all of the experiments were from a single collection of cerebrospinal fluid (CSF) that was concentrated six-fold by ultrafiltration in a Centricon-3 device (Amicon). For each gel, a 5 µL aliquot of concentrated CSF was mixed with 5 µL of a solution containing 9M urea, 2% beta mercaptoethanol and 2% carrier ampholytes (Bio-Rad, pH range 3-10).

Two-dimensional Electrophoresis.

2DE was performed in two different systems. There are many well-established 2DE gel system formats and, as one such example, we chose the system based largely on the Bio-Rad Protean II equipment, with our exact protocol, described recently (Harrington *et al.*, 1991). Briefly, the isoelectric focusing was performed in a 22 cm long glass tube, with a 1.4 mm internal diameter. The enclosed 4% polyacrylamide IEF gel was 16 centimeters long. Isoelectric focusing was performed for two hours at 200 volts, four hours at 500 volts and 13.25 hours at 800 volts, for a total of 13,000 volt-hours. These isoelectric focusing gels were immediately extruded and transferred to SDS-PAGE gels that consisted of a uniform 12% polyacrylamide gel, 1.5 mm thick, that incorporated a 30:0.8 ratio of acrylamide monomer:piperazine diacrylate crosslinker. The second dimension gels were run at constant current (40 mA per gel) for a total of six hours.

Prototype instrument.

A prototype gel box was built (Figures 1 and 2) to determine whether a system with the ability to precisely control the spatial relations of the two distinct gels (the isoelectric focusing and SDS-PAGE gels) might improve the reproducibility of protein separations from gel-to-gel. The dimensions of the gels in the prototype were the same as those in the Bio-Rad system.

The first dimensional unit was built using Plexiglas sheets that were bolted together, enclosing the IEF gel. As shown in Figure 1, the isoelectric focusing gel is polymerized in one of several different Plexiglas "cards" that

have a 16 centimeter long slit bounded at either end by a circular opening. The thickness of the "cards" and the width of the slit both varied from 0.8 mm to 1.4 mm, but for all experiments in this study, the 1.4 mm thick "card" with 1.4 mm slit were used. This "card" is clamped between two blocks of Plexiglas which form the lateral two walls of the slit. The resultant 160 mm x 1.4 mm x 1.4 mm cavity forms the boundary for the IEF gel. One of the outer blocks of Plexiglas has cylindrical openings [seen in Figure 1(ii) a & b] that provide direct access to the circular cavities in the "card." To pour the gel, the first dimension unit is bolted firmly together with part c) in Figure 1(ii) sandwiched between a) and e). The acrylamide/isoelectric focusing solution is poured into one of the circular wells, the apparatus is tilted to allow the solution to flow through to the other side, and the gel is thus polymerized in the tilted position. After polymerization, all gel material in the circular cavities is completely removed, leaving gel flush with the ends of the IEF slit. In preliminary experiments, the apparatus was tilted less so that the gel solution only partially filled the "card:" after polymerization, an unfilled cavity was left in the last 1 cm of the slit, in which a protein sample could later be loaded. Several experiments were performed to determine the best method for sample application, investigating loading either in the base of the circular well or in the short 1 cm region in the "card" slit. A colored protein standard (BioRad IEF standard) was visibly trapped against the wall of the circular cavity and never entered the gel when the sample was applied in this circular well. No such residual protein was visible outside the gel when the colored standard was directly loaded in the 1 cm slit in the "card." Thus, it was found that significantly more protein entered the IEF gel when the

sample was loaded directly into the 1 cm region of the slit in the "card," and this method was used in all subsequent experiments. Isoelectric focusing was performed by adding 10 mM sodium hydroxide and 6 mM phosphoric acid electrolyte solutions to the circular cavities in the apparatus, in which a platinum wire is located. This set-up is shown in Figure 2. The electrical conditions for isoelectric focusing were identical to those used in the conventional system, for a total of 13,000 volt-hours.

The second dimension in the prototype system is almost the same as the standard system and was achieved in a device (not shown) similar to the multiple gel casting unit of the Protean II System: this Plexiglas container was tall enough to contain the full 20 centimeter height of the glass plates and allowed for a removable Plexiglas lid to fit precisely on top of the glass plate surfaces. The same glass plates used in the Bio-Rad Protean II system were used, except the gels were poured between the space bounded by two 20 x 20 cm glass plates separated by 1.5 mm spacers. In the conventional Bio-Rad system, the gel is poured between a 16 and a 20 cm glass plate to a height of 15.5 cm and overlaid with water-saturated sec butanol. This creates a shallow trough, in which the first dimension gel is later inserted. In the prototype apparatus, the gel is polymerized all the way from the base to the top of the glass plates. Gel solution was poured in from a small opening at the base of the container, and a small air vent in the lid enabled the gel solution to be completely encased within the glass plates. After the acrylamide had polymerized, the lid was carefully removed and the glass plates, with gel polymerized flush with both ends, was clamped in a Plexiglas

cooling unit shown in Figures 1 and 2. The cooling unit has the same length as the glass plates. The glass plates containing the SDS-PAGE gel sit in a precise groove in both sides of the cooling unit, with less than 0.1 mm variation over the width of the gel. In this unit, circulating coolant (50:50 ratio of water: ethylene glycol) was allowed to flow on both sides of the glass plate, controlled at the same temperature as the buffer that was used to cool the Protean II Bio-Rad System (12°C).

After completion of the isoelectric focusing, the IEF "card" was removed by separating the Plexiglas boundary blocks from the first dimensional unit. The polyacrylamide gel remained attached to the two edges of the slit in the "card." This "card" containing the gel, was then applied to the alignment pins indicated at the ends of the second dimensional gel unit in Figures 1 and 2. These connections were specifically made so that all "cards" have the same alignment (within 0.1 mm) when they are compressed against the Plexiglas container of the second dimensional unit. This results in the IEF gel being aligned reproducibly and reliably in direct contact with the SDS-PAGE gel extremity. The total positional variation in this procedure is < 0.2 mm, versus the unmeasured, but clearly greater, variation in the conventional system with its extrusion process and distortion of the IEF gel and the human introduction of variation that occurs during the connection between first and second dimension processes.

Electrolyte in the prototype system is supplied from an agarose buffer block. Tris-glycine-SDS buffer blocks are formed in a Plexiglas closed casting unit (not shown) and are comprised of 2% agarose containing a two-fold increased concentration of tris-glycine-SDS as compared to the liquid electrolyte in the conventional Bio-Rad system. This quantity was determined experimentally as the minimum required to supply sufficient conductivity and SDS for the duration of the procedure. In order to run the SDS-PAGE gel, a tris-glycine-SDS agarose block is applied to the alignment pins in its Plexiglas container directly after the IEF gel card. Finally, a platinum electrode block is added on the outside of the same alignment pins, as shown in Figures 1 and 2. The platinum electrode spanned the entire length of the agarose block and it was mounted on a Plexiglas wedge that had numerous channels behind the wire that enabled gas and melting agarose to escape through a vent hole. As this bubbling process occurred during the electrophoresis, electrode tension on the agarose was maintained by tightening screw clamps 3-4 times during the procedure. At the other end of the SDS-PAGE gel, a tris-glycine-SDS buffer block and platinum block are applied to similar alignment pins. Running conditions were similar to the conventional Bio-Rad system with constant current (40 mA per gel) for eight instead of six hours, to enable the leading ions to reach the end of the longer gels (20 cm versus 15 cm).

At the end of the electrophoresis in both devices, gels were stained with ammoniacal silver as described previously (Harrington *et al.*, 1991).

Experimental Conditions.

After the initial experiments described above, we performed the following experiment, depicted in Figure 3. A common set of stock solutions for all steps in the procedure was made in quantity sufficient for six 2D gels to be performed, three each in both the conventional Bio-Rad system and in our prototype gel box. The only difference in chemicals, as described above, were the agarose buffer blocks used in the second dimension device in the prototype gel box. All first and second dimension gels were poured for each system simultaneously and gels were used within one week. Aliquots from the same CSF stock solution, prepared as described above, were run on each of the two different systems. A sample was run on each of the two systems on three successive days. At the end of each 2DE separation in both systems, the gel was removed from its glass container and fixed in 40% ethanol and 10% acetic acid for one hour followed by storage in 5% acetic acid, 5% ethanol. Silver staining was performed on all six gels simultaneously. The result of one silver stained sample (from the prototype instrument) is shown in Figure 4. Seven protein spots, selected because they spanned a variety of gel regions and were present in all gels, were used for measuring spot-to-spot distances. The four distances between these spots (A, B, C, and D in Figure 4) were measured on each of the wet gels on a light box, by placing a ruler between the centers of each spot pair. In order to reach a stable level of hydration for all gels, measurements were only made after the gels had been equilibrated in water for greater than 24 hours after staining.

A1.4 Results and Discussion

Table 1 shows the results of the measurements on the six gels, representing the distances illustrated in Figure 4.

The prototype 2DE instrument gave markedly improved results over the conventional 2DE system for the reproducibility of spot-to-spot distances. However, it should be noted that this study was not intended to be a definitive comparative study between the BioRad system and our prototype, but rather an investigation which uses a conventional system with which we have a lot of experience, as a reasonable benchmark for testing the principles of the design of the new device. The goal of building this device was to determine (independent of other known variables in 2DE gel reproducibility, such as sample type and preparation, the inherent chemical variations in the gels and in potential interactions between the sample and the gel) whether control of IEF gel distortion and the human positional variability in connecting the first to the second dimension gels would lead to more reproducible 2DE gels. These results strongly support the contention that these gel distortions and manipulations are significant contributory factors in 2DE gel positional reproducibility. The fact that our initial use with the prototype instrument has not yet been optimized (unlike the system with which we have had many years' experience) means that these preliminary results may be viewed as conservative. We can anticipate that with more refinement of the mechanical control of gel positions, 2DE positional reproducibility may be further improved.

At this stage, we can only estimate what the improved reproducibility shown with the prototype instrument will offer in terms of overall ability to match 2DE gels. We found about 20-40 protein spots were mismatched on our conventional BioRad system gels with 4,000 spots using new image analysis software (Solomon and Harrington, 1993) and minimal editing. The majority of these mismatches (about 75%) were clearly visible in the gel but had significant positional variation as the likely source of mismatch (MGH, unpublished results). The matching procedure allows for some variation from an absolute spot position (manuscript in preparation) in order to accommodate minor gel distortions. This degree of positional variation in the match process is relaxed in steady increments, under visual control of the results, until obvious mismatches are seen. Relaxation is based on a step-wise reduction in matching tolerance comparing the spot and its root mean square distance from five reference spots that have been selected on each gel at the start of the matching process. The positional improvement seen with this new instrument involves similar spot distances to those typical reference points selected on a 2DE gel, and should enable more correct matches to be made before relaxed parameters begin to miss or mismatch spots. We have not evaluated our new results with the new image analysis software, but this prediction is now testable.

We believe the detailed analysis of complex protein mixtures will have potential uses in medical diagnosis (Harrington *et al.*, 1986), biological studies (Garrels and Schubert, 1979) and genetic studies (Kuick *et al.*, 1991).

However, databases can only be constructed if the matching of 2DE gels is reliable. There are two approaches to ensuring such reliability. One is to subselect only the most reliable data from each 2DE gel and the other is to ensure maximum reproducibility between 2DE gels. Improved positional reproducibility is essential for the study of low abundance proteins, such as the nuclear regulatory proteins in our sea urchin embryo studies. The data from this prototype instrument shows that a more mechanically controlled 2DE gel device may improve the ability to build more complete databases. It does not seem surprising that an isoelectric focusing gel maintained in the "card" of this device can produce better positional results than from an isoelectric focusing tube gel that is extruded from a glass container by hydrostatic pressure and scooped with a spatula between glass plates, processes that clearly stretch and distort the gel.

One could anticipate further improvement if we incorporate IPG gels in this new instrument. Gianazza *et al.*, 1986, report a variability (standard deviations) of 1.18 mm in the IEF dimension and 0.9 mm in the SDS-PAGE dimension of 2D gels with IPG gels. These results are at the same level as those in our experiments, although their conditions (such as smaller gel size) limit direct comparisons. The authors interpret their excellent results as due to IPG stability and accurate gradient pouring of the IPG's. In relation to our experiments, we believe that by using these IPG techniques in a more mechanically optimal instrument, the reproducibility will be greater than from either approach by itself. This is directly testable.

We have now designed and are testing a more automated instrument, based both on the mechanical regulation principles of this prototype, as well as attempts to minimize moving parts. The intended goal of this next generation 2DE device is analagous to the added precision that HPLC instrumentation has provided for chromatography. This development has been justified for us by the improved 2DE data described in this paper.

Acknowledgment.

We are grateful for the technical help in the earlier part of these studies from Sean Latham. This work was supported by grants from the Beckman Institute Foundation to MGH, NSF STCDIR8809719 to LEH & MGH and Swiss National Science Foundation Priority Program Biotechnology 5002-037031 and NSF BCS8912824 to JEB. KHL was supported by an NSF graduate research fellowship.

A1.5 References

Aebersold, R. H., Leavitt, J., Saavedra, R. A., Hood, L. E., and Kent. S. B. H. (1987). Internal amino acid sequence analysis of proteins separated by one- or two-dimensional gel electrophoresis after *in situ* protease digestion on nitrocellulose. *Proc. Natl. Acad. Sci. USA,* **84**, 6970-6974.

Bjellquist, B., Ek, K., Righetti, P. G., Gianazza, E., Görg, A., Postel, W., and Westermeier, R. (1982). Isoelectric focusing in immobilized pH gradients - principles, methodology and some applications. *J. Biochem. Biophys. Meth.,* **6**, 317-339.

Celis, J. E., Editor, (1991) *Electrophoresis,* **12**, 765-994.

Daufeldt, J. A. and Harrison, H. H. (1984). Quality control and technical outcome of ISO-DALT two-dimensional electrophoresis in a clinical laboratory setting. *Clin. Chem.,* **30**, 1972-1980.

Fosslien, E., Prasad, R., and Stastny, J. (1984). Computer-aided standardization of two-dimensional electrophoresis: Improvement of biophysical description of proteins in relative versus absolute coordinate system. *Electrophoresis,* **5**, 102-109.

Garrels, J. I. and Franza, R. B., Jr. (1989). Transformation-sensitive and growth-related changes of protein synthesis in REF52 cells. *J. Biol. Chem.*, **264**, 5299-5312.

Garrels, J. I. and Schubert, D. (1979). Modulation of protein systhesis by nerve growth factor. *J. Biol. Chem.*, **254**, 7978-7985.

Gianazza, E., Astrua-Testori, S., Caccia, P., Giacon, P., Quaglia, L., and Righetti, P.G. (1986). On the reproducibility of band position in electrophoretic separations. *Electrophoresis*, **7**, 76-83.

Görg, A., Postel, W., and Gunther, S. (1988). Two-dimensional electrophoresis. *Electrophoresis*, **9**, 531-546.

Harrington, M. G., Coffman, J. A., Calzone, F. J., Hood, L. E., Britten, R. J., and Davidson, E. H. (1992). Complexity of sea urchin embryo nuclear proteins that contain basic domains. *Proc. Natl. Acad. Sci. USA*, **89**, 6252-6256.

Harrington, M. G., Gudeman, D., Zewert, T., Yun, M., and Hood, L. (1991). Analytical and Micro-preparative two-dimensional electrophoresis of proteins. *Methods:A Companion to Methods in Enzymology*, **3**, 98-108.

Harrington, M.G., Lee, K.H., Bailey, J.E., and Hood, L.E. (1994). Sponge-Like Electrophoresis Media: mechanically strong materials compatible

with organic solvents, polymer solutions and two-dimensional electrophoresis. *Electrophoresis*, **15**, 187-194.

Harrington, M. G., Merril, C. R., Asher, D. M., and Gajdusek, D. C. (1986). Abnormal proteins in the cerebrospinal fluid of patients with Cruetzfeldt-Jakob disease. *New England Journal of Medicine*, **315**, 279-283.

Harrington, M. G., Merril, C. R., Goldman, D., Xu, X-h., and McFarlin, D. E. (1984). Two-dimensional electrophoresis of cerebrospinal fluid proteins in multiple sclerosis and various neurological diseases. *Electrophoresis*, **5**, 236-245.

Hughes, G. J., Frutiger, S., Paquet, N., Ravier, F., Pasquali, C., Sanches, J.-C., James, R., Tissot, J.-D., Bjellqvist, G., and Hochstrasser, D. F. (1992). Plasma protein map: An update by microsequencing. *Electrophoresis*, **13**, 707-714.

Klose, J. (1975). Protein mapping by combined isoelectric focusing and electrophoresis in mouse tissues. A novel approach to testing for individual point mutations in mammals. *Human Genetik*, **26**, 231-243.

Kuick, R. D., Skolnick, M. M., Hanash, S. M., and Neel, J. V. (1991). A two-dimensional electrophoresis-related laboratory information processing system: Spot matching. *Electrophoresis*, **12**, 736-746.

Miller, M. J. and Merril, C. (1989). Strategies and techniques for testing the precision, reliability and reproducibility of computerized two-dimensional gel electrophoresis analysis systems. *Applied and Theoretical Electrophoresis,* **1**, 127-135.

Nokihara, K., Morita, N., and Kuriki T. (1992). Applications of an automated apparatus for two-dimensional electrophoresis, Model TEP-01, for microsequence analyses of proteins. *Electrophoresis,* **13**, 701-707.

O'Farrell, P.H. (1975). High resolution two-dimensional electrophoresis of proteins, *J. Biochem.,* **250**, 4007-4021.

Patton, W. F., Lopez, M. F., Barry, P., and Skea, W. M. (1992). A mechanically strong matrix for protein electrophoresis with enhanced silver staining properties. *BioTechniques,* **12**, 580-585.

Scheele, G.J., (1975). Two-dimensional gel analysis of soluble proteins. *Biochem.,* **250**, 5375-5385.

Skoog, D.A. (1985). Principles of Instrumental Analysis, 3rd Ed., Saunders College Publishing, New York, pp. 7-11.

Solomon, J.E. and Harrington, M. G. (1993). A robust, high-sensitivity algorithm for automated detection of proteins in two-dimensional electrophoresis gels. *CABIOS,* **9,** 133-139.

Taylor, J., Anderson, N. L., and Anderson, N. G. (1983). Numerical measure of two-dimensional gel resolution and positional reproducibility. *Electrophoresis,* **4**, 338-346.

A1.6 Tables

Table Legend: Measurement of protein spot-to-spot distances per gel, as indicated in Figure 4. The three gels run in the prototype instrument are identified as 1, 2, and 3; the gels run in the conventional system are identified as 4, 5, and 6. *The approximate sample standard deviation was calculated eliminating one degree of freedom for the small sample space [22].

Gel Identity	*Protein spot distances (mm)*			
	A	B	C	D
Prototype 1	44	42	38	111
Prototype 2	45	40	39	113
Prototype 3	46	43	40	114
Sample S.D.	1.0	1.5	1.0	1.5
Approximate sample standard deviation of pooled data*		1.3		
Conv. 4	36	39	30	110
Conv. 5	44	41	38	115
Conv. 6	39	32	31	107
Sample S.D.	4.0	4.7	4.4	4.0
Approximate sample standard deviation of pooled data*		4.3		

A1.7 Figures

1(i) Diagram (side view) of the prototype instrument. The IEF separation is performed in the gel card, which is shown here at the time of transfer to the second dimension container. The SDS-PAGE gel was enclosed in the gap shown between the top and bottom cooling plates. See text for further description.

1(ii) Diagram of the IEF prototype apparatus. a) & b), e) & f) are the top and bottom Plexiglas blocks that bound the gel card c) & d). As seen from above, a) is bolted on e), with c) sandwiched between them; when viewed from the side, b) is bolted on f), with d) in between. See text for more details.

2(i) A photograph of the prototype gel box used in these experiments (frontal view).

2(ii) A photograph of the prototype gel box used in these experiments (lateral view).

3. A schema for the experiment to compare the conventional, well-optimized 2DE system that we currently use and the new device. All of the necessary stock solutions were made and subsequently split into six equal parts; furthermore, the same sample preparation (concentrated human cerebrospinal fluid) was split into six equal parts. The samples

were run in each system on three consecutive days by the same person. All six resulting gels were silver stained together and the four spot-to-spot distances were then measured on each gel.

4. A silver-stained 2DE gel from the prototype gel box of human cerebrospinal fluid. Seven known proteins were selected to be used as guides for measuring four different spot-to-spot positional variations in these gels. The four distances (A, B, C & D) were recorded from each gel and are noted in the Table.

Figure 1 i.

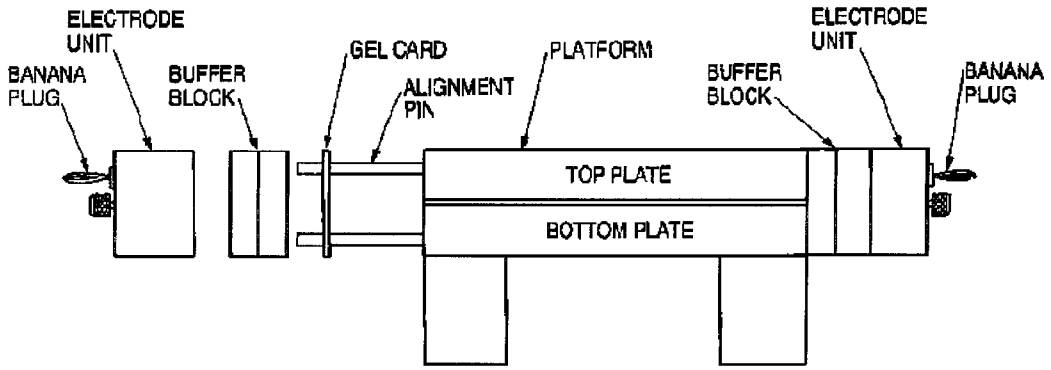

ELECTRODE UNIT
BANANA PLUG
BUFFER BLOCK
GEL CARD
ALIGNMENT PIN
PLATFORM
ELECTRODE UNIT
BUFFER BLOCK
BANANA PLUG
TOP PLATE
BOTTOM PLATE

Figure 1 **ii.**

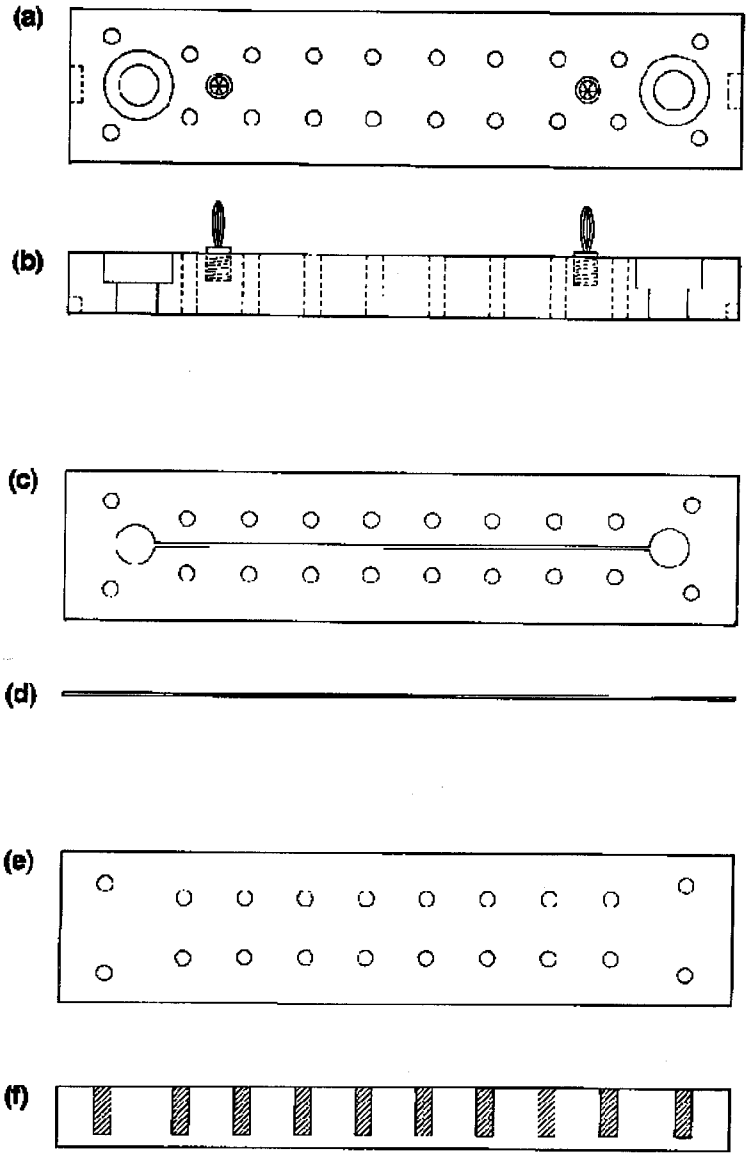
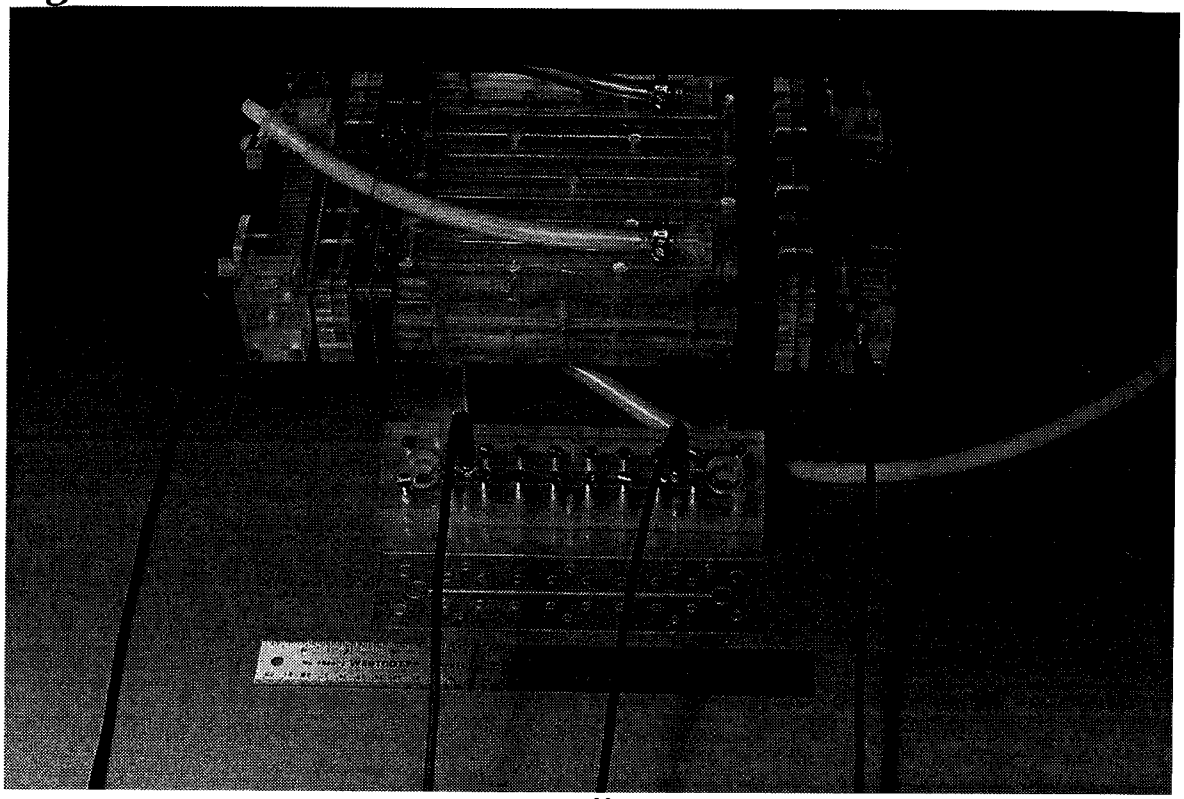


Figure 2

i.



ii.

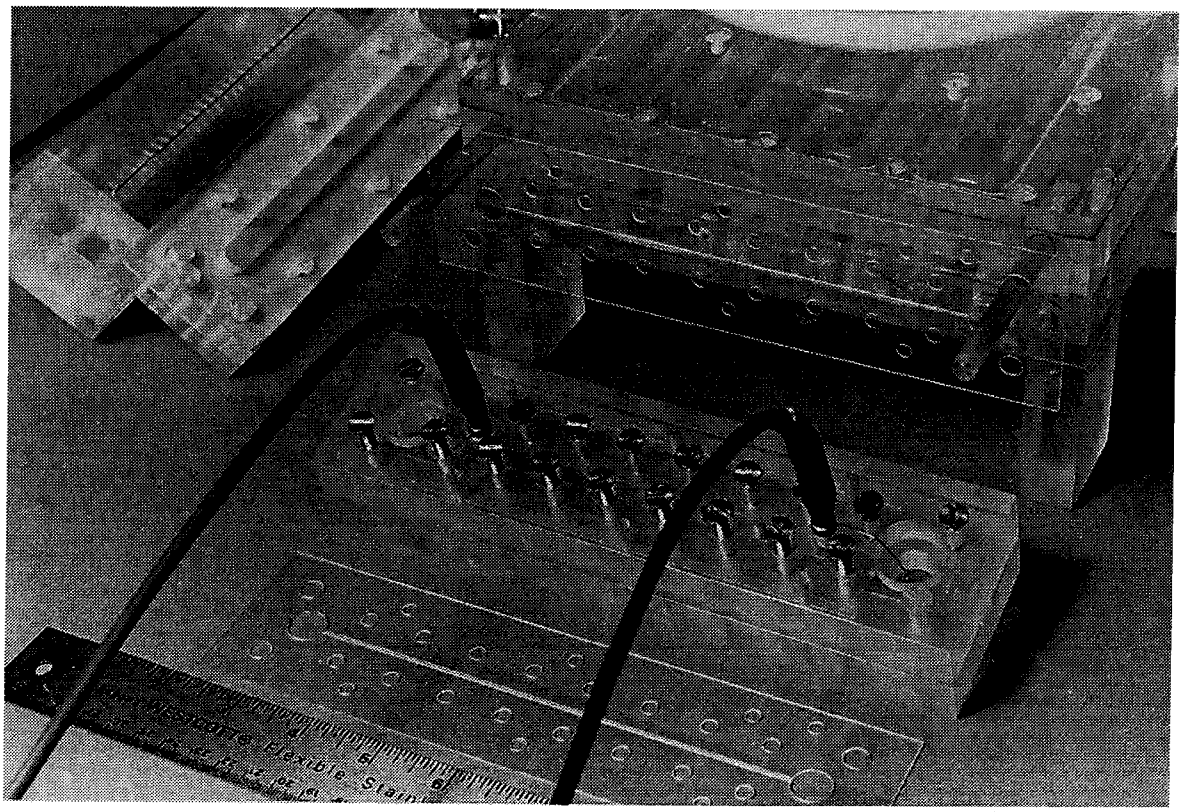


Figure 3

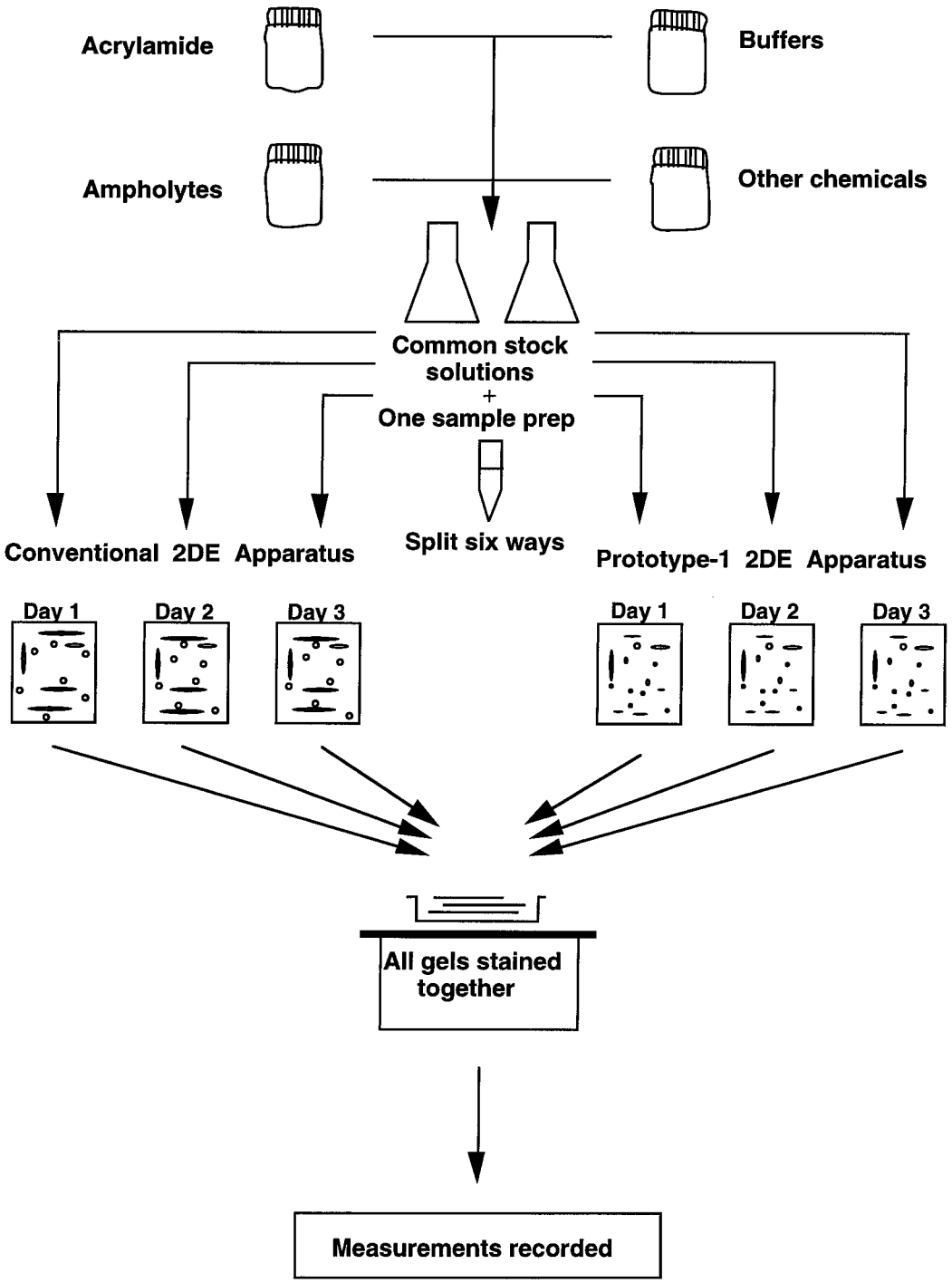
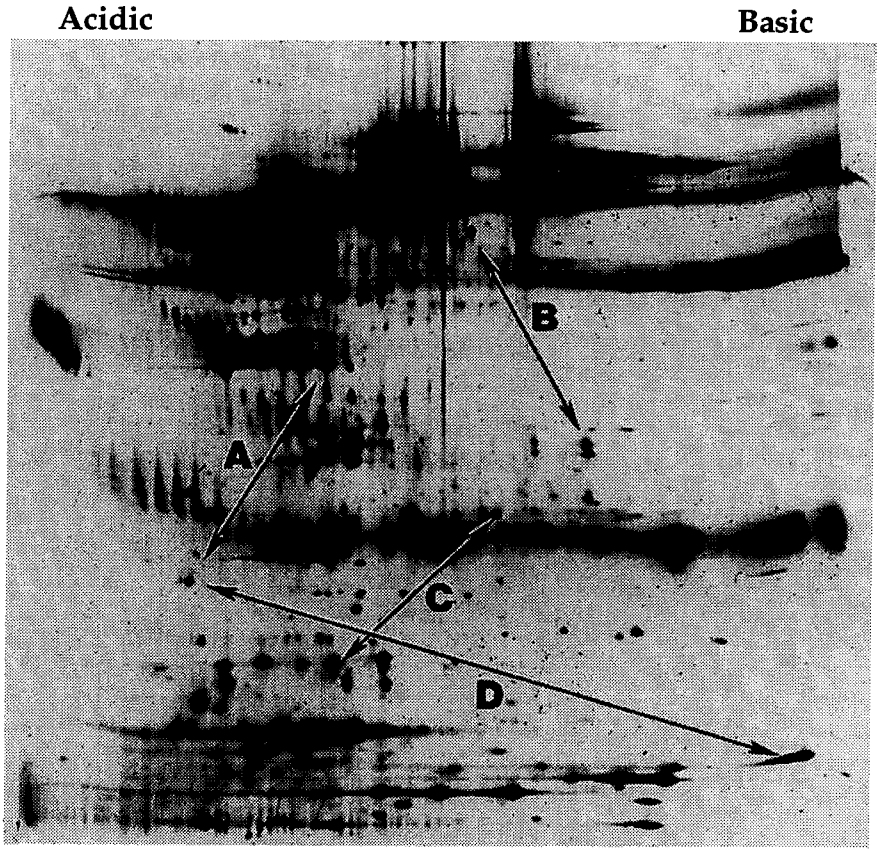

Acrylamide
Buffers
Ampholytes
Other chemicals
Common stock solutions
+
One sample prep
Split six ways
Conventional 2DE Apparatus
Prototype-1 2DE Apparatus
Day 1
Day 2
Day 3
Day 1
Day 2
Day 3
All gels stained together
Measurements recorded

Figure 4



APPENDIX TWO

Sponge-Like Electrophoresis Media: Mechanically Strong Materials Compatible With Organic Solvents, Polymer Solutions and Two-Dimensional Electrophoresis

Source: Harrington, Lee, Bailey, and Hood,

Electrophoresis (1994) **15**: 187-194

A2.1 Abstract

A new range of sponge-like media, called "electrophoresis sponges," are presented. They differ from electrophoresis gels primarily in that they are mechanically stronger, providing a permanent structure of directly measurable pore size dimensions. The new media have similarity to capillary electrophoresis in terms of pore size range, mechanically strong and directly definable walls and compatibility with polymer solutions. The sponges differ from capillary electrophoresis in that they provide large numbers of channels, with a corresponding high load capacity for simultaneous runs in multiple channels and they are compatible directly with multidimensional separations, such as high resolution two-dimensional electrophoresis. Furthermore, they can be molded (or cut) to any shape and retain that shape, they can be handled more easily than gels, they can be re-used if necessary, they can be distributed in the same format between labs easily and they can be stored indefinitely. Chemically, they can be hydrophilic or hydrophobic, with capability ranging from inert to reactive surfaces. Pore sizes can range from the sub-nanometer to 100 micron scale. Results with various hydrophobic sponges are reported for the carrier ampholyte-based isoelectric focusing of proteins. Broad and narrow pH gradients are established in the sponges that are more linear than those achieved with polyacrylamide gels. One- and two-dimensional electrophoresis of proteins has been achieved, for example, with high resolution of the charge isomers of haptoglobin beta chain, using sponge-based isoelectric focusing. Focusing is about three-fold faster in the tested

sponges than in equivalent polyacrylamide gels. This improved speed is probably related to the larger sponge pores. Moreover, both the quantity of sample entry of the hydrophobic protein zein and its resolution after isoelectric focusing in the electrophoresis sponges (in the presence of organic solvent) was superior to that achieved in polyacrylamide gels. Experimentation and applications of these electrophoresis sponges are still preliminary, but they appear to have potential as alternatives to the existing media used for electrophoresis.

A2.2 Introduction

Motivation.

Technical aspects are currently limiting two of our long-term research objectives. A two-dimensional electrophoresis (2-DE) database of transcription factors has been developed at the blastula stage of the early sea urchin embryo [1], but a lack of good positional reproducibility of protein spots in gel-to-gel comparisons of these very low abundance proteins is limiting detailed studies of different stages of the embryo. Secondly, we have a highly informative 2-DE database of human cerebrospinal fluid (CSF) proteins for both normal persons and patients with some diseases of the nervous system [2], but improved reproducibility and a more simplified system are essential for significant progress to be made in this area.

Experiments reveal three persistant technical problems: a) reproducibility from gel-to-gel is less than ideal, as manifested by variation in the position and quantity of proteins after separation; b) certain sub-classes of proteins are not well separated (especially some hydrophobic proteins and some glycoproteins which smear during attempts to separate, instead of resolving into discrete entities); c) 2DE is technically complex to perform, which severely limits the speed, throughput and reproducibility of experiments. The causes of these problems are multiple and often interdependent, as are potential solutions, but we believe that they stem

primarily from limits set by current instrumentation (not the topic of this paper) and electrophoresis media.

Recent attempts to improve the separation medium.

There are different types of electrophoresis with varying media requirements, but for high resolution 2DE of proteins, polyacrylamide has been used almost exclusively. There are alternatives to polyacrylamide that have been used for electrophoresis such as agarose [3], starch [4] and cellulose acetate [5]. However, none of them has found widespread use in 2DE.

One of the most important advances in electrophoresis media technology to date has been the development of immobilized pH gradients (IPG) [6,7]. These IPG gels greatly improve reproducibility. In carrier ampholyte isoelectric focusing, the components which form the pH gradient migrate to their respective positions as the current is applied [8,9] and are able to move during the course of IEF. Thus, the gradient is affected by factors such as diffusion, convection and evaporation. Immobilized pH gradients use a polyacrylamide gel in which the gradient-forming elements are covalently grafted to the polymer backbone; once formed, it is not subject to instabilities. The use of IPG gels requires a longer focusing time and a more laborious gel preparation procedure, but pre-cast gels are available commercially and they have many advantages over traditional carrier ampholyte gels. Among these advantages are increased resolution (especially when narrow pH gradients are used) and unlimited stability, as well as better run-to-run pH gradient reproducibility and increased sample

loading capacity. As a modification to the IPG technology, the use of carrier ampholytes have been combined with IPG to effect faster separations [10, 11]. It was found that this combination allows the use of a higher voltage and reduces the amount of protein adsorption to the gel surface. There are many reports of attempts to utilize IPG gels as the first-dimension in a 2DE separation [12, 13] in addition to their use as a precursor to N-terminal protein sequencing [14].

Duracryl [15] is a mechanically strong and elastic polyacrylamide-based medium for use in isoelectric focusing (IEF) and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) marketed by Millipore. Upon the addition of appropriate catalysts, Duracryl forms a "polymer-reinforced polyacrylamide." The main advantage that Duracryl possesses is improved mechanical strength. This should aid in overall reproducibility and handling, especially in the transfer of the first to the second dimension. MetaPhor agarose (FMC), a 'cousin' of Duracryl, is also 'polymer re-inforced' by hydroxyethylated agarose fragments.

The incorporation of stoichiometric positive and negative charges into polyacrylamide to regulate electroendosmosis has been discussed [e.g., 16], and Righetti and Macelloni [17] reported that electroendoosmosis can be eliminated by offsetting the fixed negative charges present in any polyacrylamide gel with stoichiometric amounts of tertiary or quaternary groups such as 3-dimethylaminopropylmethacrylamide. Electroendosmosis is one of the contributing factors in gel crumbling and pH gradient

instability. Therefore, materials where this phenomenon is reduced are superior to regular polyacrylamide gels.

The Hydrolink gels have been introduced to provide additional capabilities for electrophoresis, especially optimized for specific DNA separations [18]. Hydrolink gels are composed of varied crosslinkers, often more hydrophobic than standard crosslinkers, that are polymerized with acrylamide.

Hydrophobic protein IEF separations may be best achieved in organic solvent based systems rather than in the aqueous based, detergent aided systems typically used. Unfortunately, polyacrylamide dissolves in many organic solvents. Zewert and Harrington [19] have demonstrated the separation of zein, a hydrophobic protein, with organic solvents in a polyethylene glycol methacrylate 200 gel.

Various polymer solutions, including entangled polymers, are being investigated as media for capillary electrophoresis, and some demonstrate very promising resolving capabilities [e.g., 20, 21].

Electrophoresis sponges as electrophoresis media.

When considering new materials as electrophoresis media, there are many attributes/characteristics which may be desired. These include control over anticonvective and sieving properties (via pore size control), neutral or selectively modified surface charges, chemical inertness, mechanical

strength, reproducible pore structures of controllable pore size distribution, a wide range of pore sizes, long shelf lives, compatibility with various instrumentation, and cost.

We introduce the term electrophoresis sponges to distinguish from gels any porous polymer medium that is compatible with electrophoresis, that is stiffer than a gel and capable of being molded and then hold that shape. The range of chemical composition and method of production for these sponges is broad, but examples are given below. Although never used for electrophoresis, such sponge materials fulfill many of the "ideal" criteria described above and might result in improved functionality compared to current electrophoresis gels.

We have obtained (from Porex Technologies, 500 Bohannon Road, Fairburn, GA 30213-2828) a commercial supply of porous polyethylene, polyvinylidene difluoride (PVDF), polytetrafluoroethylene (PTFE) and polypropylene, made by a closed cell, scintering process of particles of the base polymer. PTFE has been used in a paste to improve mechanical strength in a polyacrylamide gel [22], but there have been no reports of attempts to utilize either porous polyethylene, PVDF, PTFE or polypropylene as the electrophoresis medium.

The purpose of this study is to examine the use of some representative electrophoresis sponge materials in IEF as the first dimension medium in a 2DE protocol. IEF was chosen for these initial studies because

the main pore size in these materials (25-150 microns) is more suitable to the non-sieving requirements of IEF than other types of electrophoresis. If successful, these mechanically strong electrophoresis sponges will warrant further investigation in other electrophoresis studies.

A2.3 Materials and Methods

Proteins and their preparation for electrophoresis.

Bovine serum albumin (BSA) and corn zein (#Z-3625) were purchased from Sigma, and soybean trypsinogen inhibitor was purchased from Boehringer Mannheim. Preliminary studies showed (predictably) that with the currently tested materials, we could not fix proteins for staining using conventional cross-linking with gluteraldehyde. Therefore, to visualize proteins we employed either naturally chromogenic, or radioactively or fluorescently labeled proteins, or we stained the proteins after their transfer into a polyacrylamide 2DE gel.

5-iodoacetamide fluorescein and tetramethyl rhodamine-5-iodoacetamide (Molecular Probes, Inc.) were dissolved in dimethyl sulfoxide at 2 mg/mL. They were conjugated overnight at a five-fold molar excess, to a solution of individual proteins dissolved in tris buffered saline (TBS) buffer pH 7.5 (at 10 mg/mL) + 0.2% dithiothreitol (DTT). The resulting labelled protein mixtures were purified by size exclusion chromatography on

Sephadex G25M columns (PD-10, Pharmacia-LKB) which were equilibrated with 3 volumes of TBS pH 7.5 buffer. The protein containing fractions were pooled and further purified and concentrated by ultrafiltration in Centricon-10 filters (Amicon, Inc.).

[^{35}S]-Methionine labelled proteins from 48 hour embryos of the sea urchin *Strongylocentrotus purpuratus* were a gift from C. Smith. Proteins were in solution in 8 M urea, 2% DTT and 2% carrier ampholytes (Bio-Rad 3-10) at a concentration of 8 mg/mL with 6.5 x 10^5 cpm/ 20 µL of TCA precipitable counts.

Human CSF, a gift from M. Yun, was from a 50-year-old male with ill-defined dementia, at a protein concentration of 80 mg/mL. For electrophoresis, 20-65 µL of CSF was mixed with 5 µL of a solution containing 8 M urea, 2% DTT and 2% carrier ampholytes (Bio-Rad 3-10) and used for each experiment.

Electrophoresis sponges: structure, composition, pore size distribution and scanning electron microscopy.

The chemical structures of the sponges used in these experiments are illustrated in Figure 1. The following samples of porous plastic sponges from the Porex Technologies inventory were used: extra fine polyethylene (average pore size - 27 µm, Porex #4920), fine polyethylene (average pore size - 69 µm, Porex #4900), medium polyethylene (average pore size - 119 µm, Porex #4903), coarse polyethylene (average pore size - 167 µm, Porex

#4913), polyvinylidene difluoride (average pore size - 22 μm, Porex #4732), polypropylene (Porex #4908) and polytetrafluoroethylene (#7750). Samples were provided as 12" x 12" x 1/16" sheets, except #7750 that was a 1" strip. Samples of 4920 and 4732 were analyzed with respect to elemental composition, pore size distribution, and by scanning electron microscopy. The elemental analysis was performed by Galbraith Laboratories, Knoxville, TN. The composition of the samples was consistent with the known structures (data not shown).

Pore size distribution measurements of extra fine polyethylene and PVDF were performed on a Micromeretics Mercury Intrusion Porisimeter at Porex Technologies. Pore size distribution measurements of these materials are shown in Figures 2 and 3.

A representative 50x magnification scanning electron micrograph (Porex Technologies) of the pore structure of fine polyethylene, after a solution of 0.5% 3-([3-chol-amido-propyl)-dimethyl-ammonio]-1-propane sulfonate (CHAPS) was wicked into the plastic and dried at 60 °C, is shown in Figure 4.

pH gradient measurements.

The pH gradients maintained by the carrier ampholytes in the plastic sponge strips or polyacrylamide tube gels were determined by sectioning the media into 15 pieces, each 1 cm long. These pieces were soaked in deionized water for one hour and the pH was measured on a Beckman Model pHI 31

pH meter. Measurements were recorded at 1 Vh, 1000 Vh, 2000 Vh, 3000 Vh, 5000 Vh, 13,000 Vh and 18,000 Vh. We performed the statistical analysis of the pH gradients on Microsoft Excel 4.0 and the square of the sample correlation coefficient (r^2) represents the proportion of the pH variability explained by a linear relation.

Isoelectric focusing in electrophoresis sponge materials.

Sheets (1/16" thickness) of porous plastic sponges were cut into 3/32" by 6" strips. The strips were soaked for two hours in a solution containing 9 M urea, 2% carrier ampholytes, 0.5% Nonidet-P40, 2% CHAPS, and water. This is the standard IEF carrier ampholyte/polyacrylamide solution as described elsewhere [23] with the change that H_2O was substituted for the acrylamide/crosslinker solution and no catalyst was added. 10 - 200 µg protein was placed onto the surface 2 cm from the basic end of the strips. The proteins wicked into the strips. The strips were run horizontally on a glass plate placed on top of an Ultra-Lum UV 365 nm transilluminator. The electrodes from a Pharmacia/LKB Multiphor II apparatus were attached to a second glass plate which was placed on top of the strips. Filter papers presoaked in 6 mM H_3PO_4 or 10 mM NaOH were used to mount the electrodes onto the plastic sponge. The second glass plate and the Multiphor II cover were used to reduce evaporation. The system was powered by a BioRad 3000Xi power supply. The strips were run at varying electrical conditions, but the optimum for IEF (with the least evaporation as the main limiting factor) was 500 V for 2 hours, 1000 V for 2 hours and 1250 V to a total of 5500 Vh or until excessive drying of the strips prevented further

migration from occurring. These times are about three-fold faster than that time to achieve similar separations in polyacrylamide gels. A tripod mounted 35 mm Pentax SF10 SLR camera with 50 mm Macro lens was used to record protein migration.

Measurement of dye-labelled proteins.

Photographs were taken every 15 to 20 minutes from the onset of voltage until focusing was achieved. The proteins were visible with the use of the UV transilluminator, and photographs were made from Kodak Ektar 25 speed film at an f-stop of 2.8 and either a 1 second or 1/2 second exposure time (depending on ambient lighting). Measurements of protein migration were made directly from the prints.

Radioactive quantitative analysis of protein transfers from electrophoresis sponge materials.

Radio-labelled proteins were visualized on a Molecular Dynamics Series 400 PhosphorImaging system utilizing ImageQuant software. The images were transferred to a SUN 4/260 workstation running GALtool software [24] and printed on a Lasertechnics Model 300D continuous tone printer. Measurements of the amount of protein present were made after isoelectric focusing in the electrophoresis sponge strips or polyacrylamide tube gels and then in the strips or gels after the proteins were electrotransferred onto a second dimension SDS-PAGE gel that was run for one hour (normally six hours) under standard conditions.

IEF in electrophoresis sponges with added polymers.

The following polymers were soaked into the pores of Porex coarse polyethylene (average pore size 165 μm): 1% dextran molecular weight 100,000 - 200,000 (Polysciences, Inc.), 1% hydroxyethyl cellulose (Polysciences, Inc.) in deionized water, 4% crosslinked polyacrylamide, and 1% uncrosslinked polyacrylamide molecular weight 700,000 - 1,000,000 (Polysciences, Inc.). These polymers were added to the carrier ampholyte solution and soaked into the sponge strips overnight. The solution was supplemented with 1% Nonidet P-40 to facilitate the penetration of polymer into the pores. The crosslinked acrylamide was polymerized and crosslinked into the pores in a horizontal chamber. The polymerization was initiated immediately prior to placing the sponges in the solution and continued overnight.

Organic solvent based isoelectric focusing.

Zein from corn was conjugated to 5-iodoacetamide fluorescein. The zein was purified by ultrafiltration in a Centricon-10 filter. The resulting protein was run in a 0, 10 or 50% dimethyl formamide/ 2% carrier ampholyte/ 1% Nonidet-P40 system in 4920 and 4732 to determine whether these matrices are compatible with organic solvent based separations.

2DE protocol.

Standard two-dimensional gel electrophoresis was performed as described in detail previously [23]. When the first dimension isoelectric

focusing was performed in PVDF or polyethylene sponges, this was achieved on a Pharmacia/LKB FBE-3000 apparatus (see Section 2.4) and the strips were transferred to a second dimension SDS-PAGE gel. The second dimension and silver staining protocols were identical to those described [23]. Stained gels were digitized on a Molecular Dynamics laser densitometer 300A, raw data was transferred to a Sun 4/260 running GALtool [24] and hard copy images were made on a Lasertechnics (Albuquerque) 300D continuous tone laser printer.

A2.4 Results and Discussion

pH gradients.

Carrier ampholyte-based pH gradients in polyacrylamide gels have been extensively studied (e.g., [25]) and this knowledge provides a basis for comparison of gradients in the electrophoresis sponge materials. pH gradients are formed in the sponges and they have a variety of pH distributions depending on both the running conditions and the type of material, as illustrated in Figures 5, 6 and 7.

One striking feature of the gradient in these materials is that they have greater linearity across their entirety than the polyacrylamide gel gradients, at most conditions studied. This is reflected in the linear

regression analysis. The r^2 results are given in the Figure 5-7 legends, with 0.99 for "extra fine" polyethylene and 0.97 for polyacrylamide, as examples. The more acute slope at the acidic and basic ends of the polyacrylamide gels is the region that is responsible for this lower linear regression value. This region of deviation from linearity at the polyacrylamide gel extremities is also a region of more variability over the different time points of study than the more uniform central region of the gel. This so-called anodic or cathodic drift has been described before, both in modelling studies and experimentally (e.g., [25]). Radioactive Ampholine progressively migrates into the electrolyte chambers until gels are devoid of carrier ampholytes [26]. Our results with the sponge materials indicate that the anodic and cathodic variation is dependent at least partly on the type of electrophoresis medium in addition to the electrophoresis or solution chemistry, as these components were kept constant in both sponge and gel gradient experiments.

The greater linearity of the sponge gradients allows us to predict that the protein separation based on this gradient may be more linear than that achieved in polyacrylamide gels, and that the less acute anodic and cathodic regions of the plastic media may provide more discrete protein separations at these extremities as compared to polyacrylamide. This will only be fully evaluated with more experimentation, but our current results (see below) are in keeping with this prediction. We did not observe higher variation in the pH gradients of the sponge media compared to polyacrylamide, which does display a more stable central gradient. However, much more extensive

studies are necessary to fully assess the strengths and weaknesses of the electrophoresis sponge media.

Another interesting feature is that the gradients in polyethylene and PVDF become flatter when IEF is run for progressively longer duration. Those gradients in polyethylene are relatively stable at the basic end, and they become less acute, with the change mainly occurring at the acidic end of the strip. The PVDF, on the other hand, has the more stable region at the acidic end, with the flattening of the gradient resulting from changes occurring mainly at the basic end of the strip. The gradient profiles in both of these materials are quite distinct from that in polyacrylamide, and each material can be predicted to have selective advantages. For instance, the greatest separation stability for acidic proteins might be with PVDF sponges, for basic proteins with polyethylene materials, for neutral proteins with polyacrylamide gels. For the broadest range survey studies, polyethylene sponge may be best. The results at this stage with proteins (see below) are preliminary, but are consistent with these predictions.

Another prediction from the pH graph data is that the window of optimal separation in the plastic sponges can vary from broad (4 pH units) to narrow (less than 1 pH unit) over the length of sponge, and across the entire pI range. In contrast to the focusing of basic proteins that can be well achieved with IPG-based polyacrylamide gels [6], polyacrylamide is less satisfactory for the basic range of proteins with carrier ampholytes. There are some good carrier-ampholyte-based separations of basic proteins [27, 28],

but difficulties still exist with this group, such as the immunoglobulin heavy chains [2]. Thus, the polyethylene sponge can be predicted to be a useful medium for IEF with carrier ampholytes in this pI range, and our results below are strongly supportive of this predicted advantage.

Isoelectric focusing in one-dimensional electrophoresis sponge materials.

We screened all of the various materials and pore sizes that are described in the Materials and Methods section 2.2, but as the best results were found with either the smallest pore size "extra fine" polyethylene or the PVDF media, only results with these materials in comparisons with polyacrylamide are presented in this paper. Fluorescently labelled proteins were observed to focus according to their pI, either by themselves or when mixed together and applied to the same sponge strip (results not shown). In addition, the sponge strips can be re-used after rinsing, with no observable change in performance (this was tested with three different proteins, on several occassions–data not shown).

The radioactively labeled sea urchin proteins focused and, when transferred out of either electrophoresis sponges or polyacrylamide gels, the residual radioactivity in the IEF media was <5% of the total protein applied (data not shown). This demonstrates that, with the proteins tested thus far, there is no serious problem of proteins sticking to the sponge during the transfer process of 2DE.

IEF was achieved with these same proteins when any of the added polymers described in Methods were used. The logic for using these added polymer solutions was to investigate whether they are compatible with the sponge materials (there do not appear to be any difficulties using them) and to investigate whether such entangled polymer solutions may enhance the separations achieved in a large (25μm) pore size sponge media. No distinct benefit was observed with any of these under the test conditions, but further experimentation is necessary. The advantage is that the sponge materials allow the incorporation of these polymers, whereas they cannot be added to polyacrylamide or agarose gels without complete change and disruption of the gel media.

The hydrophobic protein zein was focused under both aqueous and 50% dimethylformamide (DMF) conditions and the latter was significantly better than achieved in polyacrylamide gels (data not shown, but this is also reflected in the 2DE results of Figure 10, described later).

The wide range of IEF capabilities found in these electrophoresis sponge materials supports the possibility that this group of materials may be useful as new electrophoresis media. The speed for IEF in sponges is approximately three times faster than in the gels as tested, a rate that is consistent with the larger sponge pores than can be obtained with a polyacrylamide gel. More experiments will be required to further evaluate this approach. In terms of ease of use, however, there is absolutely no doubt that the electrophoresis sponge materials are already preferable to gels!

2D gels with the first dimension in electrophoresis sponge materials.

Figure 8 illustrates results from separations of cerebrospinal fluid (CSF) proteins in either polyacrylamide gels or porous polyethylene sponges for the first dimension, followed by a standard SDS-PAGE second dimension gel. We confirmed the identity of the four labelled proteins by immunostaining the blot from a gel run in parallel with the silver stained gel. These proteins are in the appropriate charge and mass positions. It is apparent that most proteins do not stick to this hydrophobic sponge under these circumstances, consistent with the radioactive studies reported above. The occasional smearing of hydrophilic CSF proteins from IEF to the SDS-PAGE (similar to what occurs in the transfer from a polyacrylamide IEF gel) was not influenced at all by the addition of organic solvent to the running buffer (data not shown) (organic solvent in the buffer significantly helps the transfer of hydrophobic proteins, as described below). While the results in the highly refined system using a polyacrylamide IEF gel are clearly more resolving, these early results in our experiments with the electrophoresis sponge materials demonstrate excellent resolution and recovery of CSF proteins. For instance, the individual haptoglobin beta chain isoforms, that differ only by additional sialic acid groups, are completely resolved (labelled C in Figure 8).

Consistent with the pH gradients described in section 3.1, Figure 8 reveals a broad pI distribution of proteins. The longer focusing time (18kvh) in sponge #4920 in Figure 7 produces a narrow gradient from pH 8-9 and we

investigated whether this could provide a sharp separation of the basic immunoglobulin heavy chain proteins that characteristically smear in carrier ampholyte based IEF. Figure 9 shows that sharp banding of the heavy chains was achieved in the middle of the 2DE gel using this polyethylene sponge IEF. These separations are similar to the results with IPG-based IEF, where a similar distribution of immunoglobulin heavy chain proteins can be seen on a 2DE gel (ref. [13], Figure 1). This approach illustrates the potential for specific pH range separations in carrier ampholyte-based IEF.

Hydrophobic proteins, such as zein, can be very difficult to separate without smearing during IEF. Furthermore, recovery of protein is also often poor. Several studies of zein proteins have produced very detailed IEF banding patterns (e.g., [29, 30]). Excellent IEF zein mapping has been obtained, but the results can still be very variable in comparison to more hydrophilic proteins (C. M. Wilson, personal communication). These are mainly agarose-based IEF with purified corn zeins that have been selectively ethanol extracted and prepared from individual strains. Commercial zein (as we are using) from a mixture of sources is not as clearly resolved into as many isoforms. We have made efforts to obtain a fair representative IEF "control" separation using polyacrylamide carrier ampholyte-based IEF and detergents. While our commercial zein "control" is not as good as the results published, we think it still provides a valid point of comparison for new approaches intended to better separate proteins that are known to be currently difficult to separate.

As mentioned above, zein was well-resolved in carrier ampholyte based IEF in electrophoresis sponge materials in the presence of organic solvents. We investigated how this might be used in 2DE studies. By transferring zein after IEF in a sponge to a normal SDS-PAGE gel, we obtained a large smear continually from the sponge into the PAGE gel. This poor transfer was completely overcome if we added organic solvent to the SDS-PAGE electrolyte. Figure 10 shows the separation of zein in a 2D gel, in DMF, when the IEF dimension was carrier ampholyte-based in a porous polyethylene sponge. The second dimension was a standard SDS-PAGE gel, except that the normal SDS-Tris-Glycine buffer included 30% DMF. The results in Figure 10 are superior to those with either polyacrylamide or polyethylene glycol methacrylate/acrylamide copolymer gels [31] with regard to both the quantity and the separation of the polymorphic species of zein. These results, although preliminary, are supportive of the notion [19] that more hydrophobic based systems for electrophoresis may be generally more successful for the separation of certain hydrophobic species than conventional polyacrylamide gel electrophoresis.

A2.5 Concluding Remarks

A new range of materials, called electrophoresis sponges, are presented. They differ from electrophoresis gels mainly in that they are mechanically stronger; this can provide non-deformable channels in the

media of directly measurable pore size and shape dimensions. Physically, they can be handled more easily than gels (i.e., they have greater mechanical reproducibility), and they can be re-used. Chemically, they can range between hydrophilic or hydrophobic, with inert to reactive surfaces. Pore sizes can range from the sub-nanometer to 100 micron scale. These larger pore sizes are not available with current gels.

There are other sponge-type materials which have been reported and which may also have potential as electrophoretic media. Bergbreiter and Kabza report that sulfonated polyethylene can be produced in varying hydrophobicities [32]. Desai and Hubbell created an interpenetrating network of poly(ethylene terephthalate) and poly(ethylene oxide) for biomedical applications [33]. Svec and Frechet [34] used a glycidyl methacrylate - ethylene dimethacrylate copolymer for use as a high-performance liquid chromatography separation medium. All of these materials may function as electrophoresis sponges with enhanced properties over polyacrylamide.

Preliminary results with various hydrophobic sponges are reported for the carrier ampholyte-based isoelectric focusing of proteins. Broad and narrow pH gradients are established in the sponges that are more linear than those achieved with polyacrylamide gels. One- and two-dimensional electrophoresis of proteins has been achieved, for example, with high resolution of the charge isomers of haptoglobin beta chain, using sponge-based isoelectric focusing. Focusing is about three-fold faster in the tested

sponges than in equivalent polyacrylamide gels (presumably due to the larger sponge pores). Moreover, both the quantity of sample entry of the hydrophobic protein zein and its resolution after isoelectric focusing in the electrophoresis sponges (in the presence of organic solvent) was superior to that achieved in polyacrylamide gels. These electrophoresis sponges have not yet overcome the technical limitations in our sea urchin or CSF protein studies (Section 1.1), but these media show potential as alternatives to the existing media used for electrophoresis. Relevent future investigations include studies with different pore sizes, different sponge chemistries and different types of electrophoresis.

Acknowledgements: This work was supported by grants from the Beckman Institute Foundation to MGH, NSF STCDIR8809719 to LEH & MGH and Swiss National Science Foundation Priority Program Biotechnology 5002-037031 and NSF BCS8912824 to JEB. KHL was supported by an NSF graduate research fellowship. We thank colleagues at Caltech for useful discussions, especially Miki and Jina Yun, Doug Packard, Tom Zewert and Bob Grubbs. We are grateful for the materials and cooperation from Haydee Puyo, Mark Bear and Mike Smith at Porex Technologies.

A2.6 References

1. Harrington, M.G., Coffman, J.A., Calzone, F.J., Hood, L.E., Britten, R.J., and Davidson, E.H., *Proc. Natl. Acad. Sci. USA* 1992, **89**, 6252-6256.

2. Yun, M., Wu, W., Hood, L., and Harrington, M.G., *Electrophoresis* 1992, **13**, 1002-1013.

3. Andrews, A.T., *Electrophoresis Theory*, 2nd Edit., Oxford University Press, Oxford 1986.

4. Gordon, A.H., in: Work, T.S. and Work, E. (Eds.), *Laboratory Techniques in Biochemistry and Molecular Biology*, Vol. 1, Part 1, North-Holland, Amsterdam 1975.

5. Lapin, A. and Feigl, W. A., *Electrophoresis* 1991, **12**, 472.

6. Bjellqvist, B., Righetti, P.G., Gianazza, E., Görg, A., Westermeier, R., and Postel, W. *J. Biochem. Biophys. Methods* 1982, **6**, 317.

7. Righetti, P.G. and Macelloni, C., *J. Biochem. Biophys. Methods* 1982, **5**, 1.

8. Svennson, H., *Acta Chemica Scandinavica* 1961, **15**, 325.

9. Svennson, H., *Acta Chemica Scandinavica* 1962, **16**, 456.

10. Fawcett, J. and Chrambach, A., *Protides Biol. Fluids* 1985, **33**, 439-442.

11. Altland, K. and Rossman, U., *Electrophoresis* 1985, **6**, 314.

12. Görg, A., Postel, W., and Günther, S., *Electrophoresis* 1988, **9**, 531.

13. Hughes, G.H., Frutiger, S., Paquet, N., Ravier, F., Pasquali, C., Sanchez, J.C., James, R., Tissot, J.D., Bjellqvist, B., and Hochstrasser, D.F., *Electrophoresis* 1992, **13**, 707-714.

14. Aebersold, R.H., Pipes, G., Hood, L.E., and Kent, S., *Electrophoresis* 1988, **9**, 520.

15. Patton, W.F., Lopez, M.F., Barry, P., and Skea, W.M., *BioTechniques* 1992, **12**, 580.

16. Hjelmeland, L., Allenmark, S., An der Lan, B., Jackiw, B., Nguyen, N., and Chrambach, A., *Electrophoresis* 1981, **2**, 82-90.

17. Righetti, P.G. and Macelloni, C., *J. Biochem. Biophys. Methods* 1982, **5**, 1.

18. Shorr, R. and Jain, T., US patent #5055517.

19. Zewert, T.E. and Harrington, M.G., *Electrophoresis* 1992, **13**, 824.

20. Chrambach, A. and Aldroubi, A., *Electrophoresis* 1993, **14**, 18-22.

21. Grossman, P. and Soane, D., *Biopolymers* 1991, **31**, 1221-1228.

22. Wilson, T.J., Haddad, L.C., and Hagen D.F., US patent # 5019232.

23. Harrington, M.G., Gudeman, D., Zewert, T., Yun, M., and Hood, L., *Methods: A Companion to Methods in Enzymology* 1991, **3**, 98.

24. Solomon, J.E. and Harrington, M.G., *CABIOS* 1993, **9**, 133-139.

25. Mosher, R.A., Saville, D.A., and Thormann, W., *The Dynamics of Electrophoresis*, VCH Publishers, New York 1992.

26. Bauman, G. and Chrambach, A., in: Righetti, P. (Ed.), *Progress in Isoelectric Focusing and Isotachophoresis*, Elsevier, Amsterdam 1975, pp. 13-23.

27. Burghes, A., Dunn, M., and Dubowitz, V., *Electrophoresis* 1982, **3**, 354-363.

28. Valkonen, K. and Piha, R., *Anal. Biochem.* 1980, **104**, 499-505.

29. Wilson, C., *Plant Physiol.* 1986, **82**, 196-202.

30. Wilson, C., *Cereal Chem.* 1988, **65**, 72-73.

31. Harrington, M.G. and Zewert, T.E., *Electrophoresis* 1994, **15**, 195-199.

32. Bergbreiter, D.E. and Kabza, K., *J. Am. Chem. Soc.* 1991, **113**, 1447.

33. Desai, N. and Hubbell, J.A., *Macromolecules* 1992, **25**, 225.

34. Svec, F. and Frechet, J., *Anal. Chem.* 1992, **64**, 820.

A2.7 Figures

Figure 1

The four different polymers used for electrophoresis are illustrated.

Figure 2

Pore size distribution for polyethylene #4920, obtained as described in Methods Section.

Figure 3

Pore size distribution for PVDF #4732, obtained as described in Methods Section.

Figure 4

Scanning electron photomicrograph of polyethylene #4900, as described in Methods Section.

Figure 5

The pH of carrier ampholyte based gradients in polyacrylamide plotted against the length of the gel, at different time-points. Results are the average of three experiments, obtained as described in Methods Section. The square of the sample correlation coefficient for the pH variability (r^2) is 0.97.

Figure 6

The pH of carrier ampholyte based gradients in PVDF #4732 plotted against the length of the sponge, at different time-points. Results are the average of three experiments, obtained as described in Methods Section. The square of the sample correlation coefficient for the pH variability (r^2) is 0.99.

Figure 7

The pH of carrier ampholyte based gradients in polyethylene #4920 plotted against the length of the sponge, at different time-points. Results are the average of three experiments, obtained as described in Methods Section. The square of the sample correlation coefficient for the pH variability (r^2) is 0.99.

Figure 8

Two silver stained 2DE gels of human CSF proteins, run as described in the Methods Section. The IEF in each gel was performed differently, but the SDS-PAGE separations were run in an identical manner as described in Methods. In the upper gel, IEF was performed in a conventional polyacrylamide gel for 18 kVh. In the lower gel, IEF was performed in a polyethylene electrophoresis sponge (Porex #4920) for 5 kVh, as described in Methods Sections. This pH gradient corresponds to the charge gradient in Figure 5. The four labelled proteins were identified as described in the Methods Section: A is Albumin; B is Alpha-1 antitrypsin; C is Haptoglobin beta chain; D is Apo-A1-lipoprotein.

Figure 9

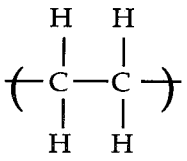
Region of the silver stained 2DE gel showing the well focused immunoglobulin heavy chain proteins from human CSF. The vertical artefact is a rip in the gel. The IEF was performed in a polyethylene electrophoresis sponge (#4920) for 18 kVh, the same conditions that generated the pH 7-8 range gradient in Figure 7. The SDS-PAGE was the same as for gels in Figure 8. Albumin was focused on the "acidic" edge of the gel (not shown) and the illustrated heavy chain proteins were in the center of the gel.

Figure 10

The localized region of the colloidal gold stained blot of 5 μg of zein that was separated in a 2DE gel. The IEF dimension was performed in a polyethylene electrophoresis sponge (Porex #4920) for 5 kVh in the presence of DMF and transferred to the SDS-PAGE gel in the presence of DMF, as described in the Methods Section.

Figure 1

Polymers



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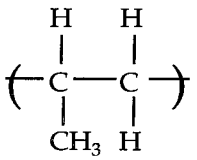

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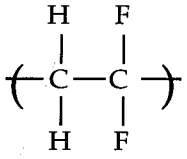

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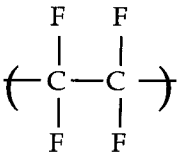
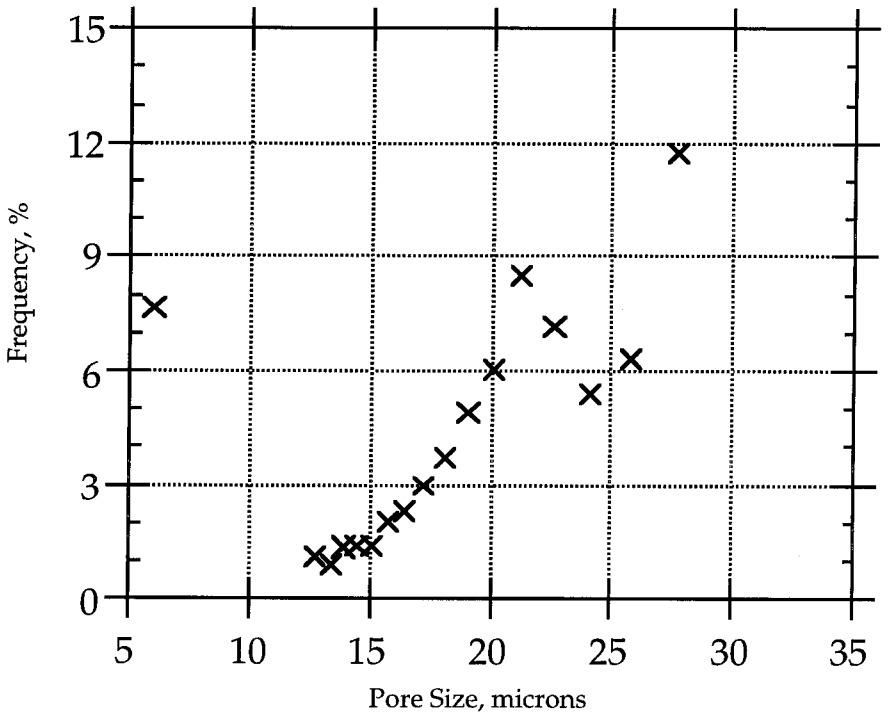


Polytetrafluoroethylene

Figure 2



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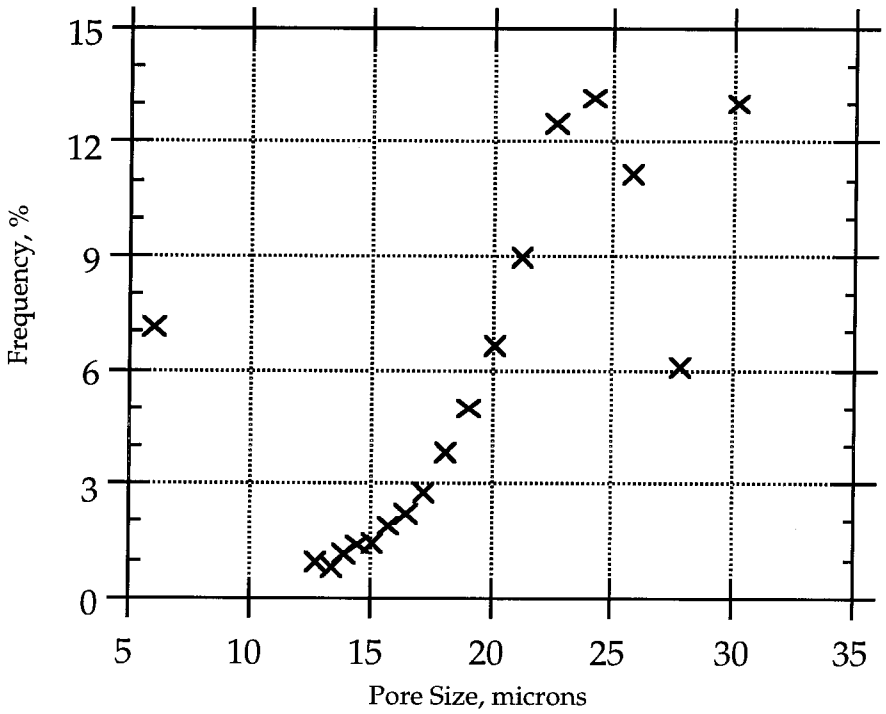

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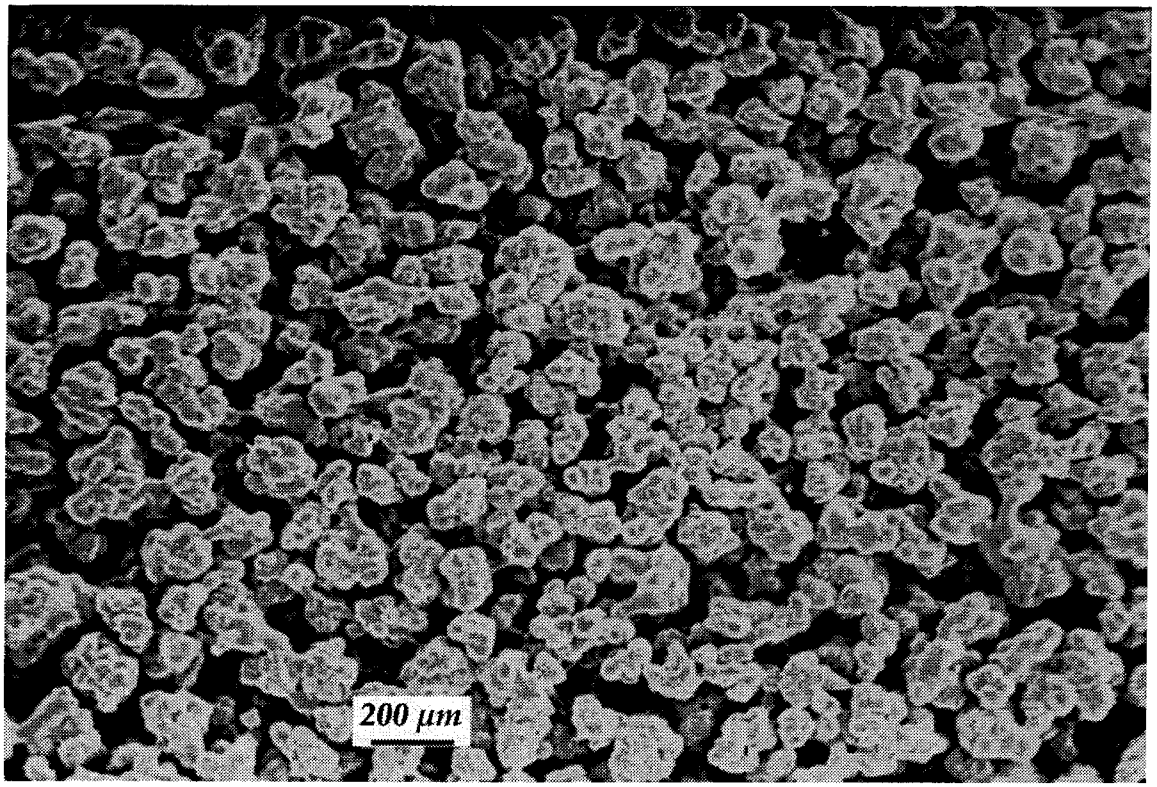

Figure 5

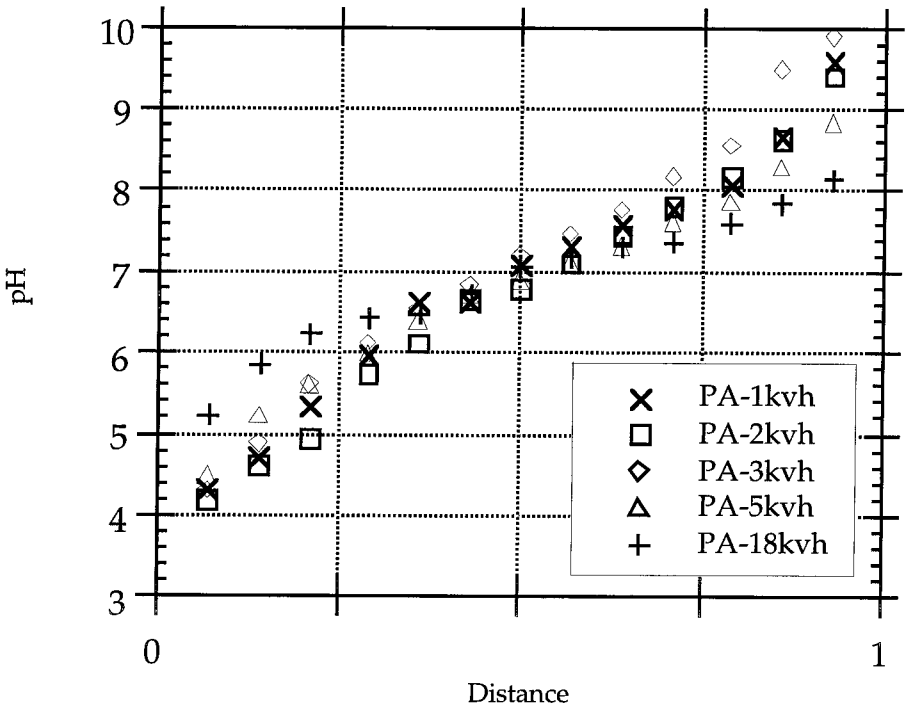
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Figure 6

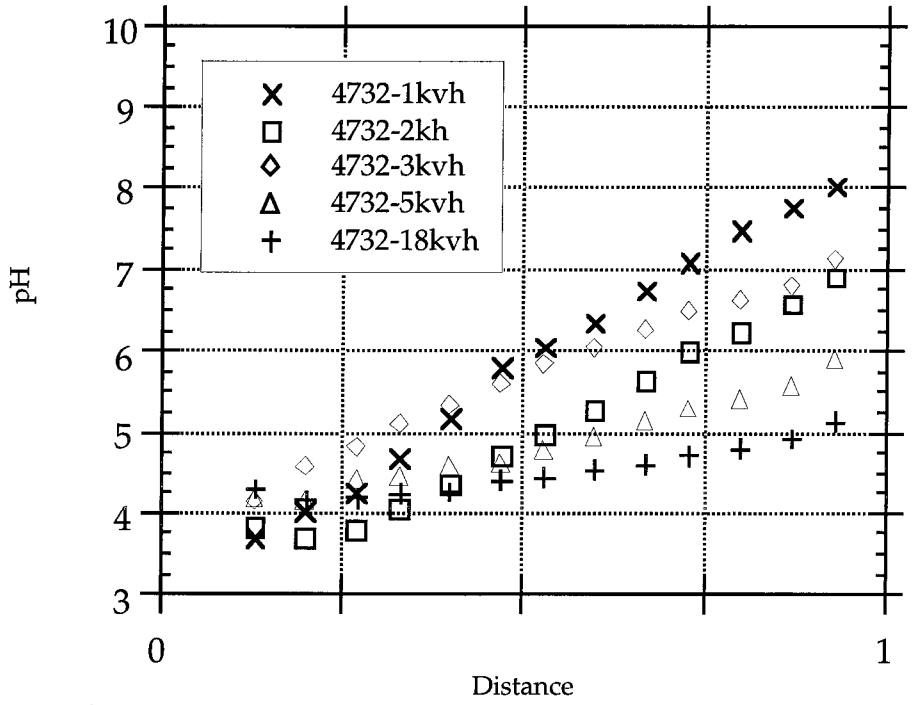
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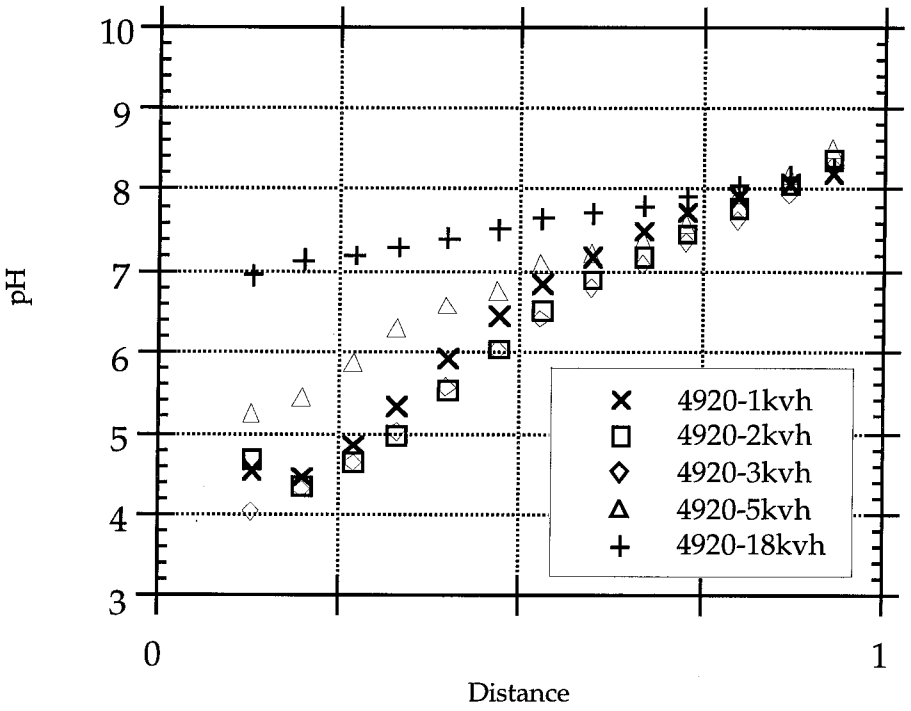
Figure 7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4920-18kvh

Figure 8

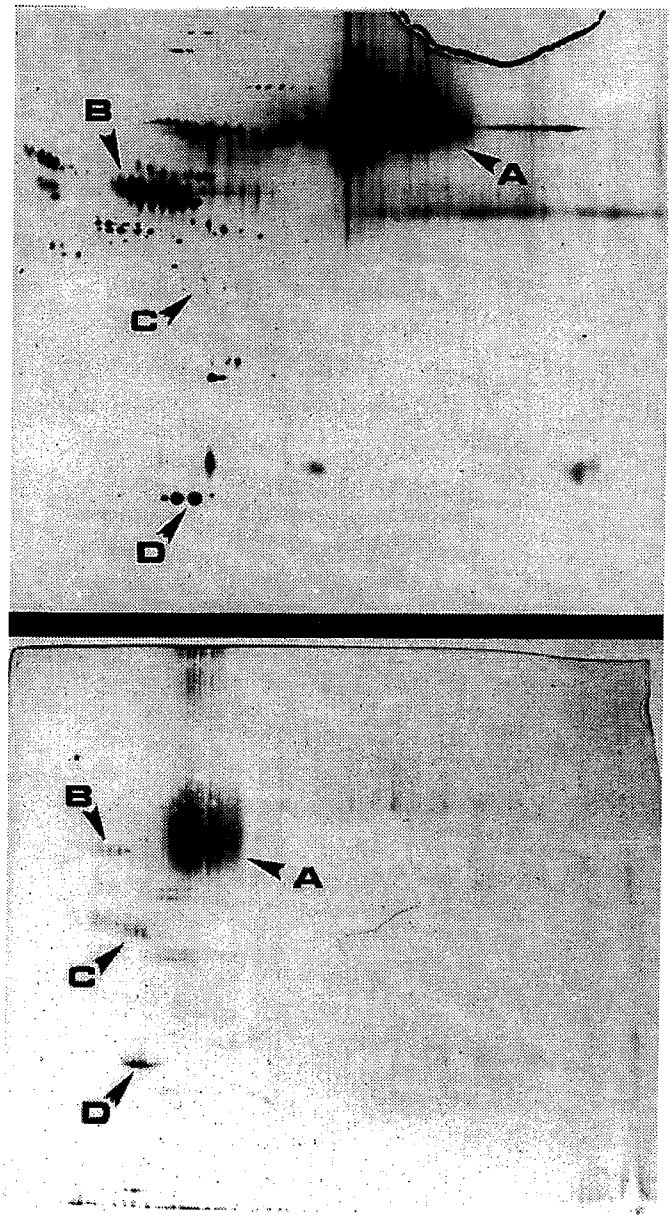

Figure 9

Figure 10