AD

GRANT NUMBER:  DAMD17-94-J-4411

TITLE: Development of Novel Peptide Inhibitors of the Estrogen Receptor

PRINCIPAL INVESTIGATOR: Richard J. Miksicek, Ph.D.

CONTRACTING ORGANIZATION: State University of New York
Stony Brook, New York 11794-3366

REPORT DATE: October 1995

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

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those of the author(s) and should not be construed as an official
Department of the Army position, policy or decision unless so
designated by other documentation.
This project aims to test the hypothesis that polypeptides designed to mimic the dimerization domain of the estrogen receptor are capable of serving as inhibitors of receptor function by virtue of their ability to interfere with the binding of receptor protein to DNA. During the first year of this award, peptides have been acquired and assays have been developed to initiate this project. Several candidate peptides have been identified that appear to specifically block the DNA-binding activity of the estrogen receptor as assessed using an in vitro gel mobility shift assay. These observations will be confirmed and extended during the final year of this project by assessing whether or not this inhibitory activity can be reproduced in a cell culture model system of estrogen receptor action. Due to a change in the institutional affiliation of the PI (Dr. R.J. Miksicek), requests have been filed to permit a change in the performance site for this project as part of a subcontracting arrangement. This request is currently pending with the US Army MRDC.
Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

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( ) In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (NIH Publication No. 86-23, Revised 1985).

( ) For the protection of human subjects, the investigator(s) have adhered to policies of applicable Federal Law 32 CFR 219 and 45 CFR 46.

( ) In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.
# Table of Contents

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Front Cover</td>
<td>1</td>
</tr>
<tr>
<td>SF 298 Report Documentation</td>
<td>2</td>
</tr>
<tr>
<td>Foreword</td>
<td>3</td>
</tr>
<tr>
<td>Table of Contents</td>
<td>4</td>
</tr>
<tr>
<td>Introduction</td>
<td>5</td>
</tr>
<tr>
<td>Experimental Methods</td>
<td>6</td>
</tr>
<tr>
<td>Results</td>
<td>7</td>
</tr>
<tr>
<td>Discussion and Conclusions</td>
<td>10</td>
</tr>
<tr>
<td>References</td>
<td>11</td>
</tr>
<tr>
<td>Appendix A... Letters of Appointment</td>
<td>12</td>
</tr>
<tr>
<td>Appendix B... Request for Subcontract</td>
<td>14</td>
</tr>
<tr>
<td>Appendix C... Description of Facilities</td>
<td>16</td>
</tr>
<tr>
<td>Appendix D... Synthetic Peptides Acquired</td>
<td>17</td>
</tr>
</tbody>
</table>
INTRODUCTION

Title: Development of Novel Peptide Inhibitors of the Estrogen Receptor
ID No.: DAMD1794J4411
PI: Richard J. Miksicek
Period: 09/30/94 through 09/29/96

This USAMRDC Breast Cancer Research IDEA Award was made to Richard J. Miksicek, who currently holds appointments as Adjunct Assistant Professor of Pharmacology at The State University of New York at Stony Brook (SUNY @ Stony Brook) and Assistant Professor of Physiology at Michigan State University (MSU). This annual report covers the first year of this award, through 09/29/95.

The aim of this project is to exploit what we have learned through the techniques of modern molecular biology about the functional organization of the estrogen receptor (ER) protein to develop a new class of ER antagonists with a novel mechanism of action. The general aim is to investigate the potential of disrupting subunit dimerization to block the transcriptional stimulatory activity of ER. Essentially all steroid receptor inhibitors developed to date represent steroid analogues that act as hormone antagonists by binding to the ligand-binding site of these proteins. However, instead of stimulating receptor activity as do the physiologically important steroids, these “antihormones” block receptor function through a mechanism that is not entirely understood. As an alternative to hormone antagonists, we are testing the possibility of using peptides to inhibit ER function. These peptides are designed to mimic the dimerization interface of ER and to interfere with subunit association, thereby preventing efficient DNA-binding by receptor. If this approach proves to be feasible, it would have several important advantages over classical hormone antagonists, since they should still be able to inhibit ER function in the face of stimulatory concentrations of estradiol. They should also have a different pattern of resistance than currently available antagonists and they are likely to display very different pharmacokinetic properties. Progress during year one of this award is described in greater detail, below.

During the first year of this award, the P.I. (Richard Miksicek) accepted a new position (effective 07/01/95) in the Department of Physiology at MSU, retaining an appointment as Adjunct Assistant Professor in the Department of Pharmacological Sciences of SUNY @ Stony Brook (letters of appointment included in appendix A). In conjunction with this change, permission was requested from the US Army MRDC to change the site of performance of this project from SUNY @ Stony Brook to MSU by establishing a new subgrant between these institutions (letter of request and a copy of the revised “Description of Facilities and Equipment” are included as appendix B and appendix C). This letter also requested that Dr. Sandra Haslam (Professor of Physiology and Director of the MSU Breast Cancer Program) be named as Principal Investigator for the MSU subgrant. A revised budget outlining an arrangement to subgrant unobligated funds from SUNY @ Stony Brook to MSU has been reviewed and approved by both institutions and has been submitted to the US Army MRDC for approval.

The original statement of work for this project included the following specific tasks:

Task 1, to identify the minimal region of the ER polypeptide able to inhibit the DNA-binding and transcriptional activity of intact ER when co-expressed in cultured cells (months 1-18)

a. to develop a series of plasmids for expressing progressively smaller portions of the ER open reading frame (months 1-6)
b. to perform gel mobility shift assays to ascertain which fragments of ER block DNA-binding when expressed together with intact ER (months 6-12)
c. to confirm that fragments of the ER open reading frame that block DNA-binding activity also function as dominant inhibitors of ER function in vivo (months 12-24)

Task 2, to identify short peptides modeled after the ER dimerization motif that act as inhibitors of DNA-binding and subunit association (months 1-24)

a. to obtain synthetic ER peptides (months 1-3)
b. to test the effect of synthetic peptides on the DNA-binding activity of ER using an in vitro gel mobility shift assay (months 3-12)
c. to characterize the inhibitory potency of active peptides and attempt to optimize the effect by testing additional overlapping peptides (months 6-24)
d. to perform feasibility experiments based on liposome-mediated peptide transfer to assess the ability of selected peptides to inhibit ER function in cultured cells (months 12-24)

EXPERIMENTAL METHODS

Construction of plasmids for efficient expression of ER in cultured mammalian cells.

We have developed a number of cDNA expression plasmids that contain various portions of the protein coding region of the human ER (hER) gene. Several of these plasmids were designed to express a family of variant hER cDNAs have been identified in breast tumors and tumor-derived cell lines (1). Derivatives expressing ERAE2, ERAE3, ERAE5, and ERAE7 (where the number refers to a deleted exon) are currently available and those expressing ERAE4 and ERAE6 will be completed by the end of 1995. These plasmids represent derivatives of pCMV4 (2) and pcDNA3 (InVitrogen Corp., San Diego, CA), which we have shown can support high levels of receptor expression in a variety of mammalian cell lines, including Cos7 (3). In addition to these hER splicing variants, we have a large number of engineered mutants of hER, including a series of carboxy-terminally truncated clones that remove progressively larger portions of hER beginning with domains reported to be important for activation of transcription (4) and subunit dimerization (5). Additional polypeptide expression plasmids will be constructed, as needed, by inserting short (60-180 bp) fragments of the hER cDNA into a vector designed in this laboratory that contains a consensus translation initiation motif, as described in the original application.

Transfection conditions for assessing inhibitory activity of expressed fragments of ER.

To measure the ability of hER polypeptides to interfere with activation of gene expression by intact receptor, transient co-transfection assays are being performed as previously described (6). Briefly, this assay utilizes a calcium phosphate-mediated transfection procedure to introduce plasmids harboring various ER cDNA expression clones into HeLa cells along with an estrogen-responsive reporter plasmid (pERE-TK-CAT). Each transfection experiment includes a plasmid expressing a portion of the hER open reading frame that is transcriptionally inactive on its own, but has the potential of inhibiting co-transfected wild-type receptor. The inhibitory potential of receptor fragments is assessed by measuring the activity of chloramphenicol acetyltransferase (CAT) enzyme produced in cultures of cells transfected with intact receptor alone (pCMV-ER), compared with transfections containing both intact hER and the various fragmentary hER expression clones. Levels of ER expression are being assessed where possible by western blot analysis using an ER-specific monoclonal antibody (Mab-17) that was previously developed in this laboratory (7). For ER polypeptides that retain the Mab-17 epitope or an intact ligand-binding domain, nuclear localization of these mutants is being assessed in individual control transfections either by indirect immunofluorescence analysis using Mab-17 (7) or by staining with fluorescent...
ER ligands (3). It is assumed that a correlation will exist between the in vivo potency of peptide inhibitors of ER, and their ability to accumulate in the nucleus.

Custom synthesis of estrogen receptor peptides.

We originally proposed testing the inhibitory potential of a limited number of polypeptides, focusing on the dimerization motif described by Lees et al. (5). Due, however, to recent advances in techniques for economical solid-phase synthesis of peptides, we have obtained a series of 40 synthetic hER peptides (Chyron Corp., La Jolla, CA), whose sequences are provided in Appendix E. Peptides # 24, 5, 42, 6, 10, and 14 overlap with the peptides proposed in the original application. Due to differences in amino acid content these peptides display a wide range of solubilities, but they all appear to be soluble in either deionized water or dimethylformamide. Dilutions (1000-fold) of each peptide stock are being tested for their ability to directly inhibit DNA-binding by hER, as described below.

In vitro gel mobility shift assay for analyzing inhibition of DNA-binding activity.

The DNA-binding activity of ER and its inhibition by synthetic peptides is being assessed using an in vitro gel mobility shift assay, essentially as described by Neff et al. (7). Briefly, ER is over expressed in Cos7 cells by transient transfection with pCMV-ER (Neff), a highly efficient expression plasmid containing a wild type hER cDNA (8). Whole cell extracts containing hER where prepared from these cells on the second day following transfection by sonicating the harvested cell pellet in a 0.4 M KCl extraction buffer containing, in addition, 20 mM HEPES (pH 7.4), 1 mM EDTA, 2 mM dithiothreitol, 10% glycerol, and 2.5 μg/ml each of antipain, aprotinin, chymostatin, and leupeptin. Following centrifugation for 10 min at 15,000 X g, aliquots of the clarified supernate (8 μl, containing 30-40 μg of protein) were preincubated for 15 min at room temperature in 20 μl of DNA-binding buffer [10 mM HEPES (pH 7.4), 1 mM MgCl2, 1 mM dithiothreitol, and 20% glycerol] also containing 1 μg tRNA, 1 μg poly(dl-dC), and 100 μg acetylated BSA. Reactions containing hER polypeptides included 2 μl of from a working dilution of the peptide stock, or approximately 20 pmol of peptide, assuming complete dissolution. Following preincubation of receptor-containing extracts in the presence or absence of the test peptide, approximately 20 fmol of peptide, assuming complete dissolution. Following preincubation of receptor-containing extracts in the presence or absence of the test peptide, approximately 1.2 fmol (40,000 cpm) of a 32P-labeled double-stranded ERE oligonucleotide (6) were added to each sample and incubation was continued for 30 min at room temperature, followed by an additional 30 min on ice. Samples were then loaded on a pre-electrophoresed non-denaturing 5% polyacrylamide gel that was run in 0.5 X TBE buffer at 280 volts for 2 hr. Gels were dried and exposed overnight for autoradiography. Inhibition of DNA-binding by ER in response to peptide addition was evidenced by a consistent decrease in the intensity of the slow-mobility hER/DNA complex. To confirm the specificity of this complex, control DNA-binding reactions were performed with equal amounts of extract from pCMV4 vector-transfected Cos7 cells and with Cos7/pCMV-ER extracts containing 1 μl of an ER-specific monoclonal antibody.

RESULTS

Specificity of DNA-binding by intact hER expressed in Cos7 cells.

A representative gel mobility shift assays is shown in Fig. 1, to demonstrate the specificity of this reaction. Intact receptor (wt hER) expressed in Cos7 cells is able to bind efficiently and with high specificity to an oligonucleotide containing a consensus estrogen response element (ERE, AGGTCACAGTGACCT) (Fig. 1, lanes 5-7), while extracts from Cos7 cells that were transfected with the pCMV4 expression vector alone (lanes 2-4) displayed only weak, nonspecific DNA-binding activity (indicated by an asterisk). The DNA-binding activity of wt hER (solid
(arrow) was enhanced by pretreatment of cells expressing the receptor with 1 nM estradiol (lane 6) and was further slowed in mobility by the addition of an ER-specific monoclonal antibody, Mab-17 (lane 7), to the binding reactions (hatched arrow). It should be noted that the Mab-17 antibody failed to stabilize or alter the mobility of the non-specific DNA binding displayed by endogenous Cos7 proteins (compare lanes 3 and 4), indicating that this complex appears not to contain immunologically reactive ER. The extracts used in these binding reactions representing control and pCMV-ER transfected Cos7 cells contained comparable amounts of protein as determined using the Lowry protein assay. Since it depends on the presence of hER and is intensified by conditions that are known to stabilize DNA binding by hER, the slow mobility band (indicated by a solid arrow) can be confidently assigned to represent the hER/DNA complex.

Screening synthetic hER peptides for their ability to interfere with DNA-binding by intact receptor.

To determine whether synthetic peptides with sequences derived from the hER polypeptide chain are able to interfere with the ability of this receptor to bind efficiently to DNA, we've initiated a series of gel mobility shift assays. Each assay includes control DNA-binding reactions performed with the ERE probe alone (lane 1), with protein from mock-transfected Cos7 cells (lane 2), and with protein from Cos7 cells transfected with the hER expression vector pCMV-ER (lanes 3-13). DNA-binding reactions including a single synthetic hER peptide (lanes 4-13) along with an equivalent amount of Cos7 extract were always compared with a control reaction lacking added peptide (lane 3). Representative results from two sets of experiments that have systematically screened through each of 40 synthetic peptides shown in Appendix D are given in Fig 2. As noted above, in the absence of added peptide, hER expressed in Cos7 cells is able to bind efficiently and with high specificity to an ERE-containing oligonucleotide (Fig. 2, lane 3). Our results suggest that hER suffers a dramatic reduction in DNA-binding activity in the presence of peptide P6, derived from the dimerization domain of hER (compare lane 7 with lane 3). Less dramatic inhibition of DNA-binding was caused by the addition of peptides P8 (lane 9) and P10 (lane 11). It is noteworthy that two of the peptides showing the greatest degree of inhibitory activity (P6 and P10) represent contiguous sequences in hER that correspond precisely to the dimerization motif identified by Parker and colleagues (5). Since each reaction included equivalent aliquots from a single hER-containing Cos7 cell extract, the intensity of the upper two hER-specific bands (indicated with solid arrows) should accurately reflect the amount of hER protein not complexed with peptide, that remains available for binding to DNA. It is also significant that a more rapidly migrating non-specific DNA complex (indicated with an asterisk) formed between an endogenous Cos7 cell protein (see lane 2) and the DNA probe shows little if any variation in intensity as a function of added peptide (lanes 2-13). Additional gel shift experiments with peptides P13 through P20 failed to identify other hER peptides that consistently
interfered with DNA-binding, although several peptides (P\textsubscript{16}, P\textsubscript{19}, P\textsubscript{37}, and P\textsubscript{38}) showed significant inhibitory activity in isolated experiments. A summary of the natural location of the peptides tested in Fig. 2 within the hER polypeptide is given in Fig. 3. Confirmation of these findings will require gel shifts such as those shown in Fig. 2 to be repeated with the entire series of synthetic hER peptides. There are also plans for the acquisition of a phosphoimage analyzer (BioRad Laboratories, Inc.) by this department that will permit the extent of DNA binding in this assay to be much more readily quantitated. Ultimately, titration experiments involving the strongest peptide inhibitors will be required to determine their relative inhibitory potency.

**Dominant inhibition of the function of hER in transfected cells by expressed hER polypeptides.**

A limited number of transfection experiments have been performed to test whether portions of the hER protein coding sequence are able to interfere with the transcriptional stimulatory activity of intact hER when they are co-expressed in cells. While it is too early to make any sweeping generalizations from these experiments, several cases have been noted where hER constructs that harbor an intact hormone binding domain possess dominant inhibitory activity, despite deletions elsewhere within the polypeptide chain. A good example of this is the repressive activity of the ERAE3 splicing variant (6), which suffers a loss of DNA-binding ability, but retains an intact hormone binding domain. We have recently observed similar behavior for pcDNA3-ER(DEF), a construct engineered to express only the hER hormone-binding domain (residues 256-595). One possibility for explaining these results is that the presence of an exposed dimerization motif (residues 497-518) in an otherwise transcriptionally inactive receptor is sufficient to confer trans-dominant inhibitory activity. Transfection experiments with additional hER constructs are in progress to test this hypothesis.

**Fig. 2.** Gel mobility shift assay showing inhibition of hER binding to DNA by selected synthetic hER peptides. Lane assignments are as described in the text. Peptide sequences are shown in Appendix E.

**Fig. 3.** Summary of the location of synthetic hER peptides used for the gel mobility shift assay shown in Fig. 2. DBD: DNA-binding domain, HBD: Hormone-binding domain.
DISCUSSION

During the first year of this award, significant progress has been made towards reaching the specific aims set out in our original application. In order to continue to pursue these research aims, a request for a change in the performance site of this project has been filed, necessitated by a change in the institutional affiliation of the PI. This is to be effected by a subcontracting arrangement under terms that have been agreed upon by the two institutions involved (The Research Foundation of SUNY and Michigan State University). A request for approval of this arrangement is pending with the US Army MRDC.

The reagents necessary for completing this project have been assembled and assays to test for the inhibitory activity of synthetic and expressed hER peptides have been developed. We have strong preliminary evidence that at least two peptides (P₆ and P₁₀), and possible more, derived from the open reading frame of the hER protein appear to be capable of interfering with the DNA-binding activity of this protein. If additional gel shift experiments confirm this finding, it becomes less important to continue to employ the transient transfection assay to identify expressed hER peptides with in vivo dominant inhibitory activity (Task 1C). In this case, priority will be shifted away from this series of experiments. Instead, emphasis will be placed on using the in vitro gel mobility shift assay to define the shortest possible peptides and the optimal peptide sequence that exhibits inhibitory activity (Task 2C). Additionally, a higher priority will then be placed on testing these peptides for inhibitory activity in vivo using liposome-mediated peptide transfer (Task 2D).

In summary, we can report significant progress towards our goal of identifying short peptides, derived from the sequence of the hER protein, that are able to interfere with the DNA-binding activity of this receptor. The underlying justification for this work continues to be the development of a novel class of steroid receptor inhibitors that may someday prove to be useful as therapeutic agents.

Publications During Current Award Year:


REFERENCES


Appendix A: Letters of Appointment for PI

Michigan State University

Name: Miksicek, Richard J.

Rank: Assistant Professor

Title: Academic Year

Appointment Status: Tenure System

Primary Department: Human Medicine

Effective Date: August 16, 1995

Annual Salary Rate: $52,000

End Date (if temporary): August 15, 1999

Previous University Experience:
- SUNY-Stony Brook, Assistant Professor, 7 years
- Inst. Cell and Tumor Biology, Heidelberg, Germany, Visiting Scientist, 5 years

Other relevant experience:

Degrees earned:
- Ph.D., Biochemistry, Univ. of Minnesota, 1982

Current Licenses:

United States Citizen: Yes

Countries of Citizenship:

Any relative employed by MSU: No

If yes, give Name and Relationship:

Rank/Title:

Department/College:

Salary in each unit:

100%

Account Number:

11-2871

Salary:

Adm. by Dept/College:

Cancer Ctr./Hum.Med.

COMMENTS:

For Postdoctoral Fellow:

Faculty sponsor certifies that the academic and/or professional qualifications of the appointee identified above have been reviewed and recommends the appointee for postdoctoral appointment.

Recommendation by:

Primary Chairperson/Director Signature: 6/1995

Second Chairperson/Director Signature: Date

Primary Dean/Adm. Head Signature: Date

Second Dean/Adm. Head Signature: 6-28-95

Budget Office Signature: Date

Financial Services Fiscal/Departmental Initiative:

* There is no tenure in administrative assignments.

** If probationary period is other than four years for an Associate Professor, prior approval from Provost and President is required.
June 15, 1995

Richard Miksicek, Ph.D.
14 Paul Street
Port Jefferson Station, NY 11776

Dear Dr. Miksicek:

On behalf of President Kenny, I am writing to confirm the end of
your temporary salaried appointment as Research Assistant Professor
in the Department of Pharmacological Sciences in the School of

In view of your desire to continue your affiliation with the State
University of New York at Stony Brook, effective July 1, 1995, I am
pleased to grant you a non-salaried appointment as Research
Assistant Professor as a member of the voluntary faculty in the in
the School of Medicine. This appointment is at the pleasure of the
President and subject to the Policies of the Board of Trustees
governing such appointments.

If you wish to accept this appointment, please sign and date the
endorsement on the enclosed copy of this letter and return that
copy to me by July 15, 1995.

Sincerely,

J. H. Oaks
Vice President for Health Sciences

JHO:ha

cc: Dr. B. Hool
    Dr. N. N. Abumrad
    Dr. A. P. Grollman

ACCEPTED:

DATED: June 29, 1995
Appendix B: Letter Requesting Approval for Subcontract Agreement

MICHIGAN STATE UNIVERSITY

June 13, 1995

Mr. Mike Younkins
Grants and Contracts Officer
US Army Medical Research and Development Command
Fort Detrick, MD 21702-5012

re: Breast Cancer Research Award
PI: Richard J. Miksicek
Title: Development of Novel Peptide Inhibitors of the Estrogen Receptor
Award Period: 10/94-09/96
Code: A125
ID: DAMD-17-12J-44J1

Dear Mr. Younkins:

In relation to the prior request of Dr. Richard Miksicek (in a letter to you dated May 22, 1995), arrangements are being made as of July 1, 1995, to move the above referenced US Army MRDC Breast Cancer Research Project to the Department of Physiology, Michigan State University. In consultation with Dr. Miksicek and the grants and contracts officers of our respective institutions, I will be assuming the role of Principal Investigator on the balance of the research award that is to be subcontracted to Michigan State University. I currently hold an appointment as Professor of Physiology as well as Director of the Breast Cancer Program of the Michigan State University Cancer Center. My qualifications to assume the administrative and supervisory role on this project include the prior and current direction of a number of federally sponsored research projects in the area of hormonal control of the development and physiology of the breast. In addition, Dr. Miksicek will maintain a close supervisory role during the duration of the project period.

With this letter I am requesting that the Office of Contracts and Grants Administration of Michigan State University be permitted to initiate negotiations with the Research Foundation of SUNY to subcontract the unobligated balance of this research contract to Michigan State University for the duration of the award period (07/01/95 through 09/30/98). All terms of the parent contract, if acceptable to Michigan State University, including the specific aims, the award period, and the total award amount will remain unchanged, although some rebudgeting of funds between categories will occur as part of the subcontract. A revised budget is being prepared in consultation with the grants and contracts administrators of the respective institutions. This budget will be submitted to you for approval as soon as it is available.
Mr. Younkins
June 13, 1995
Page 2

The names and phone numbers of the administrators at Stony Brook and MSU are as follows:

Michigan State University:
Suzanne Morgan
Contract and Grant Administration
Michigan State University
East Lansing, MI 48824
Phone: (517)-353-7297
FAX: (517)-353-9812
e-mail: CGA06@msu.edu

SUNY at Stony Brook:
Barbara Harris, Associate for Sponsored Research
The Research Foundation of SUNY
Office of Research Services
State University of New York at Stony Brook
Stony Brook, NY 11794-3366
Phone: (516)-632-9028
FAX: (516)-632-6963
e-mail: bharris@ors.ors.sunysb.edu

I would greatly appreciate a letter from you providing authorization to proceed with this arrangement. Also please appraise me of any certifications or approvals that will be required by the US Army MRDC from myself or the Grants and Contracts Office of Michigan State University in conjunction with the subcontract. Thank you for your prompt attention to this matter.

Sincerely yours,

Sandra Haslam, Ph.D.
Professor of Physiology

Approved:

William S. Spielman, Ph.D.
Professor and Chairperson

Daniel Evon
Assistant Director
Contract and Grant Administration

SHjr
Appendix C: Revised Description of Facilities and Equipment

Permission has been requested to move the performance site of this project as of 07/01/95 from SUNY at Stony Brook to the Department of Physiology at Michigan State University (MSU). To effect this move, we are proposing that the parent award will remain with the Research Foundation of the State University of New York and, as agreed upon by both institutions, unobligated costs will be subcontracted to MSU.

The laboratory assigned to Dr. Miksicek at MSU occupies 850 square feet of space on the 2nd floor of Giltner Hall on the MSU campus in East Lansing, MI. It is fully equipped for the proposed biochemical and molecular biological research. The laboratory contains a separate area for tissue culture work. Cold room and darkroom facilities are available on site.

Items of equipment available for exclusive use of Dr. Miksicek and his associates include a Pharmacia Ultrospec II spectrophotometer, electrophoresis chambers and power supplies, standard and low temperature freezers, refrigerated centrifuges, microcentrifuges, a Savant Speed Vac, Savant thermal cycler, balances, a pH meter, and a Millipore water purification system. A designated cell culture room adjacent to the research lab houses a laminar flow hood, a humidified CO₂ incubator, a Nikon inverted microscope, and a table top centrifuge. The following equipment is shared with other departmental faculty located in Giltner Hall: ultracentrifuges, liquid scintillation counters, a spectrofluorimeter, a Perkin-Elmer thermal cycler, low temperature freezers, a Zeiss photomicroscope equipped for epifluorescence illumination, dark room and X-ray film developing equipment.

Personnel have access to personal computers which include an ethernet connection to internet. Programs for DNA sequence analysis are available as a GCG software package. All investigators involved with this research have access to relevant databases, literature retrieval (Medline) services, and computer networks linking them to other universities and research institutions. Office space (100 sq ft) is assigned to the Co-Principal Investigator immediately adjacent to the laboratory space. Individual desks have been made available to all research personal adjacent to their work areas. Two personal computers for word processing and data management are present for the sole use of this research staff.

Access to custom oligonucleotide synthesis is available through the Depts. of Microbiology and Biochemistry on a cost-for-service basis. The university operates machine and electronic shops that are available to faculty.

Arrangements for disposal and storage of radioactive wastes are provided as an institutional service by the university. Dr. Miksicek is fully licensed and registered to use radioisotopes on the MSU campus.
Appendix D: Sequences of Synthetic Peptides Obtained for this Project

*note, these peptides are numbered 3-42 based on increasing size (peptides 1 and 2 were synthesis controls), but they have been ordered by their position within the protein sequence or ER, beginning at the N-terminus.

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