STUDIES ON INSULIN RECEPTOR IN X. LAEVIS OOCYTES

STUDIES ON INSULIN RECEPTOR IN *XENOPUS LAEVIS* OOCYTES

By

AIKATERINI VASSILAKOS, B.Sc.

A Thesis

Submitted to the School of Graduate Studies

in Partial Fulfilment of the Requirements

for the Degree

Master of Science

McMaster University

MASTER OF SCIENCE (1993) McMASTER UNIVERSITY
(Biochemistry) Hamilton, Ontario

TITLE: Studies on Insulin Receptor in *Xenopus laevis* Oocytes.

AUTHOR: Aikaterini Vassilakos, B.Sc. (McMaster University)

SUPERVISORS: Assoc. Professor D. W. Andrews
Professor V.S. Ananthanarayanan

NUMBER OF PAGES: xi, 160

ABSTRACT

The *Xenopus laevis* oocyte was examined as a model system for investigating insulin receptor function.

The role of extracellular calcium on insulin-stimulated deoxyglucose uptake (ISDU) in the *Xenopus laevis* oocyte was investigated. It was determined that removal of calcium from the medium did not alter the rate of $^{45}Ca^{2+}$ release from oocytes preloaded with $^{45}CaCl_2$. In contrast to earlier reports using tissue explants and cultured cells, the insulin response in oocytes is not sensitive to a range of extracellular calcium concentrations from 1 μM to 10 mM. However, treatment of oocytes with 1 mM EGTA, in the absence of Ca^{2+}, prior to, during or within 5 minutes of insulin addition resulted in a 2-4 fold inhibition of ISDU.

To further investigate the event(s) in insulin signalling inhibited by EGTA, the number of receptors for insulin on the oocyte must be increased. To this end we have investigated the effects of the 5′ and 3′ untranslated regions as well as the coding region of mRNA on translational efficiency in reticulocyte lysate and oocytes. The results obtained in Xenopus oocytes are consistent with earlier cell-free data (Falcone and Andrews, 1991). We have demonstrated that replacing the cognate 5′ UTR with the Xenopus beta globin 5′ UTR appropriately linked to a consensus sequence for efficient translation initiation (ACCATGG) results in increased in translation in Xenopus oocytes. *In vitro* synthesized preprolactin transcript injected into oocytes was found to be functionally stable for several days (D. Andrews - unpublished data). Stabilization of the preprolactin transcript was localized to the 3′ UTR. Furthermore, inserting the preprolactin 3′ UTR downstream of another coding region resulted in stabilization of the modified transcript. These results provided a basis for improving expression of cloned human insulin receptor in Xenopus oocytes. By optimizing the 5′ and 3′ UTR's of the insulin receptor clone we were successful in expressing high levels of insulin receptors in Xenopus oocytes. Effects of the coding region on translation were also investigated and we provide evidence that sequences in the coding region modulate translational efficiency.

ACKNOWLEDGEMENTS

I wish to thank all the people who have supported my efforts:

Dr. David Andrews for his guidance, patience and unique perspective on this project.

Dr. V. S. Ananthanarayanan for many useful discussions.

Mina Falcone for her friendship, support and interest. I could always count on her technical advice, but more importantly I could count on her honest opinion.

Jason Young, Gil Abisdris, Josie Ursini and all the people who passed through the Andrews lab for many useful discussions, both scientific and otherwise, and especially for their friendship.

Paul MacPherson for his patience, love and support. Without him I would be computer illiterate and often stranded at 3 a.m. without transportation home.

My sister Helen, my niece Erin, my mother Panagiota and Jim and Mary MacPherson for their love and support over the years.

Dr. A. Dingle for helping with the care of the Frogs.

I would like to gratefully acknowledge the financial support of Dr. D.W. Andrews, Dr. V.S. Ananthanarayanan and the Ontario Graduate Scholarship Program (OGS).

TABLE OF CONTENTS

ABBREVIATIONS

ISDU - Insulin stimulated deoxyglucose uptake

SRPRα - Signal Recognition Particle Receptor - alpha subunit

EGTA - ethylene glycol bis(ß-amino-ethyl ether)-N,N,N',N'-tetraacetic acid

BAPTA - 1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid

^{3}H deoxyglucose - 2-[1,2-^{3}H(N)]-deoxy-D-glucose

nt - nucleotide

RRL - rabbit reticulocyte lysate

WG - wheat germ extract

UTR - untranslated region (of mRNA molecule)

Da - dalton

kDa - kilodalton

DNS - data not shown

SDS-PAGE - sodium dodecyl sulfate - polyacrylamide gel electrophoresis

REFERENCES FOR DATA

Data for two figures (Fig. 1 Chapter III-2 and Fig. 1 Appendix B) in this thesis were produced by D. Falcone (unpublished data). These data are included to provide a rationale for the experiments which followed.

FIGURE	RESEARCHER
Chapter III-2 FIG 1	D. FALCONE
Appendix B FIG 1	D. FALCONE

RECOMBINANT DNA

The recombinant plasmids used in chapter III were made by several members of Dr. D. W. Andrews lab. The researcher responsible for constructing the version of a clone used in this thesis is referenced in TABLE 1 in each section of Chapter III and in TABLE 1 Appendix B.

LIST OF ILLUSTRATIONS

CHAPTER I
INTRODUCTION

I-i
INTRODUCTION

Insulin, like most peptide hormones, does not cross the plasma membrane of its target cells. To elicit a cellular response, insulin interacts with the extracellular domain of a plasma membrane bound receptor which, upon insulin binding, is responsible for signalling intracellular events associated with insulin action (reviewed in Olefsky, 1990). The insulin receptor belongs to a family of hormone receptors and tyrosine kinase oncogenes which share homology in their intracellular kinase domains (Ullrich et al., 1985). Many of the short-term (receptor autophosphorylation, aggregation of receptors, internalization, tyrosine kinase activation, cellular protein phosphorylations) and long term metabolic effects of ligand binding to tyrosine kinase receptors have been characterized (insulin receptor reviewed in Olefsky, 1990; general receptor phosphorylation reviewed in Sibley et al., 1987). However, the mechanism of transmembrane signalling, presumably the first event in the signalling process, is not clearly understood for this family of receptors. Understanding the mechanism of transmembrane signalling of the insulin receptor may provide information on the mechanisms involved in signalling by other members of this receptor family (Appendix A contains a summary of the current literature on insulin receptor signalling).

Calcium has been implicated in the transmembrane and

intracellular signalling mechanisms of various hormones and growth factors (reviewed in Williamson et al., 1981; Berridge and Irvine, 1989; Ullrich and Schlessinger, 1990; Berridge, 1993). While a role for calcium has been established for a variety of cellular signalling events, the role of calcium (specifically that of extracellular calcium) in insulin receptor function has remained controversial. Brimble and Ananthanarayanan (1991) have shown that insulin and insulin B chain, the bioactive subunit of insulin, bind Ca^{++} and transport it into lipid vesicles. They suggested that extracellular Ca^{++} interacts with insulin and is involved in the interaction between insulin and insulin receptor. This project focused on developing an *in vivo* experimental system which could be used to test the above possibility.

The cell system in which we chose to study the effects of extracellular calcium on insulin receptor function was the *Xenopus laevis* oocyte. The oocyte is well characterized and has been used extensively in a variety of studies in areas such as developmental biology, gene expression, protein trafficking, ion transport and signal transduction (for a review of expression in oocytes see Soreg, 1985; some other examples: Gurdon and Wickens, 1983; Mohunet et al., 1983; Koren et al., 1983; Levi et al., 1987; Simon et al., 1987; Bement and Capco, 1989; Labbe et al., 1988; Shuttleworth and Coleman, 1988; Snyder et al., 1988; Rothman et al., 1988; Tate et al., 1989; Murray et al., 1992; Lechleiter and Clapham,

1992). The Xenopus oocyte, as a model system for extracellular calcium studies, potentially has several advantages over tissue explants and cultured cells.

The cells used in previous studies, described below, are well characterized insulin sensitive cells, but are not suitable for studying extracellular calcium effects. Studies on the effects of intracellular and extracellular calcium on insulin function have yielded few consistent results. Two studies, both of which studied the effects of intracellular calcium on deoxyglucose uptake in isolated rat adipocytes illustrate the controversy in this area of research. Draznin et al. (1987) reported that, in isolated adipocytes, insulin stimulated uptake required an optimal range of cytosolic free calcium. Calcium levels outside this range resulted in decreased uptake. A report from Kelly et al. in 1989 refuted the results of Draznin et al. (1987). A related study in 3T3-L1 adipocytes found that depletion of intracellular calcium by quin2/AM had no effect on insulin-stimulated deoxyglucose uptake(ISDU) (Klip and Ramial, 1987). The results from groups studying the effects of extracellular calcium on insulin receptor function are no more consistent (McDonald et al., 1976; Akhtar and Perry, 1979 I and II; Desai et al., 1979; Eckel and Reinauer, 1984; Emmami and Perry, 1986; Williams and Turtle, 1981 and 1984; Williams et al., 1990). The results of these studies are discussed in more detail in Chapter II. While insulin binding to the receptor, amino acid

uptake and deoxyglucose uptake may be maximal at 5 mM or 10 mM calcium in cell culture (Williams et al., 1990), this may not be relevant to physiological events because changes in extracellular Ca^{++} can be linked to changes in intracellular Ca^{++} (Taylor et al., 1979). For example, Taylor et al. (1979) reported that both basal and insulin stimulated deoxyglucose uptake (ISDU) was stimulated by incubation of adipocytes in media containing 5 mM calcium. This was not observed when inhibitors of calcium flux across the plasma membrane were included in the incubation, suggesting that the effect was due to changes in intracellular Ca^{++} resulting from increased extracellular Ca^{++}. Consistent with this suggestion was the observed decrease in intracellular cAMP levels after incubation with 5 mM calcium (Taylor et al 1979). Therefore, while insulin binding, amino acid uptake and deoxyglucose uptake may be maximal at 5 mM or 10 mM calcium in cell culture, this may not be relevant to the physiological events. As suggested above high extracellular calcium may increase influx of Ca^{++} to the cytosol. In the unstimulated state the cell has in place mechanisms to maintain cytosolic calcium concentrations but when stimulated these mechanisms may be impaired. For example, examination of the effects of insulin on the plasma membrane Ca/ATPase, (the major site of calcium extrusion from the cell) showed that binding of insulin to the insulin receptor inhibited the Ca ATPase (Reviewed in Pershadsingh and McDonald, 1984). Calmodulin, which is

tyrosine phosphorylated in respose to insulin stimulation, has been implicated in the mechanism of inhibition (Graves et al., 1986; Delfert et al., 1988; Sacks et al., 1989)). Inhibition of calmodulin phosphorylation by the insulin receptor is Ca^{++} dependent and at 30 μM Ca^{++} phosphorylation by the insulin receptor is totally inhibited (Sacks et al., 1989). It has been suggested that *in vivo* calmodulin is a direct substrate for insulin receptor kinase and may be an important link between insulin receptor activation and intracellular signalling pathways (Wong et al., 1988). Given the above reports, changes in micro-environmental calcium concentrations at the plasma membrane, may have important implications for insulin action. Therefore high extracellular calcium, as was used in previous studies, at the time of insulin stimulation may lead to an exaggerated influx of calcium at the plasma membrane. If micro-environmental calcium influxes at the plasma membrane affect calmodulin phosphorylation, presumably involved in insulin signalling, nonphysiological levels of extracellular calcium may be affecting intracellular signalling events, not extracellular events.

Removal of calcium from the extracellular media may also affect internal calcium stores, especially in cells that have limited mechanisms for controlling internal calcium levels over a broad range outside physiological conditions (Ahktar and Perry, 1979I; Terepka et al., 1976). Mammalian

cells, such as adipocytes and hepatocytes, are maintained in an environment that is tightly controlled within a narrow calcium concentration range in the organism (Terepka et al., 1976). Maintaining calcium concentrations within such a narrow range makes it difficult to design experiments using isolated cells that can establish a role for extracelluar calcium. Furthermore, Reed et al. (1990) reported that hepatocytes isolated in calcium-free media undergo oxidative stress due to increased mitochondrial Ca^{++} cycling and loss of membrane potential (Reed et al., 1990). Therefore the effects of extracellular calcium depletion as reported by Eckel and Reinaur (1984), Draznin et al. (1987) and Ahktar and Perry (1979), may also be complicated by changes in intracellular calcium as well as by cell viability. This may contribute to the problem of establishing a clear requirement for extracellular calcium in insulin function. This has resulted in few, if any, reports on either the nature of the molecular interactions between calcium and the insulin - insulin receptor complex or the functional significance of these interactions. These can be investigated only after an experimental system is set up in which extracellular calcium can be studied independent of intracellular calcium.

Xenopus laevis reproduce by external fertilization, where oocytes are released and fertilized in an aqueous environment. Xenopus laevis oocytes are therefore exposed to an environment which has a fluctuating calcium concentration.

Therefore, it is possible that the oocyte has in place homeostatic mechanisms which allow it to maintain intracellular calcium stores independent of a range of external conditions. Experiments designed to test whether removal of external calcium would lead to a loss of intracellular calcium stores in the oocyte are described in chapter II. The results provided a basis for experiments to test the effects of extracellular calcium on insulin action in the oocyte.

An insulin response has been previously demonstrated in Xenopus oocytes (Morgan et al., 1986; Stefanovic et al., 1986; Janicot and Lane, 1989; Vera and Rosen, 1990). Furthermore, two nonallelic preproinsulin genes were cloned from Xenopus laevis and the products of these genes were found in pancreatic extracts from adult frogs suggesting insulin and the insulin receptor are functional in Xenopus (Schuldiner et al., 1989). While the number of receptors for insulin is low in oocytes there is a dose-dependent response in both phosphorylation of oocyte proteins and glucose uptake (chapter II). The observation that oocytes respond to insulin permits us to test the effects of extracellular calcium on the endogenous insulin response. Experiments were designed to determine if there is a role for extracellular calcium in ISDU in the oocyte. The results of these experiments are presented in chapter II.

In trying to establish a general role for calcium in

insulin signalling it will be necessary to extend the results obtained using the endogenous oocyte receptors to other insulin receptors. If calcium is involved in the interactions between insulin and insulin receptor then expression of mutant receptors in oocytes may be useful to investigate these interactions. Alternatively, calcium may be involved in an early post-insulin-binding event such as receptor aggregation or internalization (reviewed in Olefsky 1990 and discussed in Appendix A). Given the low numbers of receptors for insulin on the oocyte surface, expression of high numbers of receptors in the oocyte will be necessary for crosslinking, immunofluorescence and EM studies designed to determine which of these early events are being affected. The oocyte is a well characterized expression system making it suitable for the above studies (reviewed in Soreq, 1985). Cloned insulin receptors have been functionally expressed in oocytes and were found to increase glucose uptake and phosphorylation, but the receptor numbers were not high enough to visualize the immunoprecipitated ^{35}S labelled proteins (Vera and Rosen, 1990). Our initial attempts to express a construct of the human receptor both in cell-free translation systems and in oocytes were unsuccessful. This problem prompted us to re-examine expression of foreign proteins in Xenopus oocytes. The results presented in chapter III provide a practical approach to improving expression in the oocyte by optimizing the 3′ and 5′ untranslated regions of the molecules being translated.

The effects of these regions (Chapter III), as well as the coding region itself (Apendix B), on the functional stability and translational efficiency, of microinjected RNA, and the implications for protein translation in general are discussed. Based on the results of the above study we made an insulin receptor construct containing optimized 5' and 3' untranslated regions. This construct was expressed very efficiently in both cell free translations and in Xenopus oocytes (Chapter III-3).

The oocyte has been shown to have an effector-coupling mechanism similar to mammalian cells (Vera and Rosen, 1990). Exogenous receptors can couple to the existing intracellular signalling pathways to produce an insulin stimulated deoxyglucose response. The observation that the oocyte has similar intracellular insulin signalling mechanisms to other well characterized insulin responsive cells has important short and long-term implications for this research. While there is controversy as to whether the Xenopus receptors are more structurally similar to mammalian insulin receptors or insulin-like growth factor I receptors, the observation that early and late events can be coupled suggests that the mechanism of signalling is comparable (Baxter and Williams, 1983; Froesh et al., 1985; Janicot and Lane, 1989; Vera and Rosen, 1990). Furthermore, the ability of exogenous receptors to couple efficiently to the endogenous oocyte insulin signalling pathways will make it possible to assay mutant

receptors for both early post insulin binding functions such as receptor internalization and for downstream events such as glucose uptake.

I-ii

EXPERIMENTAL SUMMARY

In this thesis, two different aspects of the oocyte as a model system are examined. The experimental approach and immediate goals are different for the two sets of experiments and for this reason the thesis is presented as two independent units (Chapters II and III). The final chapter will integrate the results of the two chapters and place the results into context of the oocyte as a model system for examining the effects of extracellular calcium on insulin receptor function.

CHAPTER II
THE EFFECTS OF EXTRACELLULAR CALCIUM ON INSULIN STIMULATED DEOXYGLUCOSE UPTAKE IN XENOPUS OOCYTES

II-i
ABSTRACT

We have investigated the role of extracellular calcium on insulin-stimulated deoxyglucose uptake (ISDU) in the Xenopus laevis oocyte. It was determined that removal of calcium from the medium did not alter the rate of ^{45} Ca release from oocytes preloaded with $^{45}CaCl_2$. In contrast to earlier reports using tissue explants and cultured cells, the insulin response in oocytes is not sensitive to extracellular calcium concentrations ranging from 1 μM to 10 mM. However, treatment of oocytes with 1 mM EGTA prior to, during or within 5 minutes of insulin addition resulted in a 2-4 fold inhibition of insulin-stimulated deoxyglucose uptake.

II-ii
INTRODUCTION

Although the effects of extracellular calcium on insulin action have been studied using both tissue explants and cultured mammalian cells the optimal calcium concentrations for insulin binding to the receptor, glucose uptake, amino acid uptake, inhibition of adrenaline induced lipolysis and receptor phosphorylation are not consistent (McDonald et al., 1976; Akhtar and Perry, 1979 I and II; Desai et al., 1979; Eckel and Reinauer, 1984; Emmami and Perry, 1986; Williams and Turtle, 1981 and 1984; Williams et al., 1990). Removal of calcium from isolated cardiocytes by incubation with EDTA led to decreased insulin binding due to inactivation of high-affinity sites on the receptor. As a consequence, the rate of glucose uptake was reduced, however, neither maximal glucose uptake nor internalization of the receptor-ligand complex were altered (Eckel and Reinauer, 1984). In the above study maximal insulin binding and half-maximal rate of glucose uptake rate were reported with 5 mM extracellular calcium. In contrast, experiments using isolated adipocytes indicated that 1-2 mM Ca^{++} was optimal for glucose uptake while 3 mM was found to be inhibitory (Draznin et al., 1987). Washing adipocytes with calcium-free media prior to hormone stimulation was also reported to alter a variety of insulin mediated processes (Akhtar and Perry, 1979II). Both amino acid and glucose uptake were almost completely inhibited. Adrenaline-induced lipolysis and insulin binding

were not abolished, even when the cells were treated with EDTA. Furthermore, 20-50 μM calcium restored amino acid and glucose uptake but not high affinity insulin binding suggesting that the high affinity site is not necessary for these insulin mediated processes (Akhtar and Perry, 1979II). Finally, in cultured hepatoma cells 10 mM calcium was reported necessary for maximal glucose and amino acid uptake (Williams et al., 1990). In this system, the effect of calcium depletion was more pronounced for glucose than for amino acid uptake.

One possible explanation for the wide variety of effects attributed to extracellular calcium on insulin action may be the cells used in the studies. While hepatocytes and adipocytes are insulin-sensitive cells, they may not be resistant to large changes in extracellular calcium concentrations (Terepka et al., 1976). Therefore, when isolated cells are placed in media containing calcium concentrations outside the physiological range it results in a re-distribution of intracellular calcium to maintain cytosolic calcium concentrations (Terepka et al., 1976). Furthermore, extracellular calcium concentrations outside the physiological range may alter Ca^{++} flux across the plasma membrane and thus affect intracellular processes (Taylor et al., 1979).

We have chosen the Xenopus laevis oocyte as a model system for studying the effects of extracellular calcium on

insulin stimulated deoxyglucose uptake. The oocyte is responsive to insulin and has been shown to have expected autophosphorylation, kinase activation and deoxyglucose uptake stimulation (Morgan et al., 1986; Vera and Rosen, 1989; Janicot and Lane, 1989). Furthermore, *Xenopus laevis* reproduce by external fertilization in an aqueous environment where calcium concentrations are not controlled. Therefore, the oocyte may have homeostatic mechanisms that allow it to maintain intracellular calcium concentrations independent of the external medium. We present here the results of experiments to determine the suitability of the oocyte for studying the effects of extracellular calcium on insulin action. Specifically, we determined that removal of extracellular calcium does not result in release of calcium to the media from intracellular stores. Further, we present data which define a role for extracellular calcium in an early post-insulin-binding event leading to ISDU. The insulin response required only μM concentrations of calcium in the media, as treatment of oocytes with EGTA or BAPTA was required in order to demonstrate inhibition of ISDU.

II-iii
MATERIALS AND METHODS

MATERIALS

Adult female breeding Xenopus laevis were purchased from Boreal (St. Catherines Ontario Canada). The radiochemicals, $^{45}CaCl_2$ and ^{3}H-deoxyglucose were from DuPont-New England Nuclear and ^{32}P was from ICN. Protease inhibitors were from Boerhinger Mannheim and Sigma. The calcium chelators, EGTA and BAPTA were from Sigma. Hepes buffer was from Boerhinger Mannheim. All other reagents were from BDH or Baker Chemicals.

OOCYTE ISOLATION AND MICROINJECTION

Adult female X.laevis were obtained from Boreal, St. Catherines. Ovarian fragments were surgically removed from anaesthetized females. The oocytes were either manually dissected from the ovary or released by incubation at room temperature in ND96 (96mM NaCl, 2mM KCl, 1mM $MgCl_2$, 5 mM hepes, pH 7.6) containing 2mg/ml collagenase (ICN or Sigma) for 2 hours (Vera and Rosen, 1989). The oocytes were extensively washed and then stored, at 19°C, until use in ND96 media supplemented with 1mM $CaCl_2$, pen/strep (100 u/ml each penecillin and streptomycin) and gentamycin (0.01 mg/ml). Sutter borosilicate micropipettes (I.D. 0.5, O.D. 1.0) were pulled on a Sutter-instruments K.T. Brown Type Puller, and bevelled using a Sutter K.T. Brown Type beveller, Model BV-10 from Sutter instruments. Oocytes were microinjected using a Sutter, Model NA-1, injection system.

CALCIUM EFFLUX

Oocytes were loaded with $^{45}Ca^{++}$ by incubation in MBSH(88 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 0.82 mM $MgSO_4$, 0.33 mM $Ca(NO_3)_2(4\ H_2O)$, 0.41 mM $CaCl_2$, 10 mM hepes, pH 7.4) containing 50 μCi/ml $^{45}Ca^{++}$ for 4 hours. After the incubation the oocytes were divided into two groups and washed 3 times with MBSH supplemented with either calcium (1mM final) or EGTA (1mM). The oocytes were divided into groups of 30 in flat bottomed 2 ml tubes containing 0.5 ml medium. The media were removed and replaced with fresh media every 5 min for the first 75 min then every 10 min. Radioactivity of the medium from each time point was determined by scintillation counting using a Beckman scintillation counter. The activity inside the oocytes was determined by lysing the oocytes in 2% SDS. The results were plotted as percentage of the total cpm (outside and inside the oocyte) versus time.

PHOSPHORYLATION ASSAY

Oocytes were microinjected with 50nl each ^{32}P orthophosphate (1mCi/10 μl) and allowed to equilibrate for one hour. The oocytes were stimulated with increasing concentrations of insulin, as indicated, for 20 minutes in the presence of 1 mM calcium. Then the medium was removed and a crude membrane fraction produced by homogenization in isotonic buffer and centrifugation at 12,000 rpm (16,000 x g) in a micro centrifuge for 15 minutes. This membrane fraction was extracted at high pH (11.5) with sodium carbonate to isolate integral membrane proteins. Carbonate extractions of oocyte membranes were as described in Simon et al. (1987). The membrane pellets were boiled for 5 minutes in SDS-PAGE loading buffer and separated on 10 % polyacrylamide gels using the Tris-Tricine buffer system (Schagger and Von Jagow, 1987). The gels were dried and exposed to film (KODAK X-OMAT) at -70°C for 1-4 days.

DEOXYGLUCOSE UPTAKE ASSAY

Sets of 5-15 oocytes were used for the deoxyglucose uptake experiments as indicated. Preliminary experiments combined insulin stimulation and deoxyglucose uptake in a single incubation. To study the effects of calcium on insulin mediated stimulation separately from the effects of Ca^{++} on glucose transport, experiments were designed with two incubations, a stimulation (5-30 minutes as indicated) followed by uptake. All uptake incubations were in 0.5 ml ND96(in the presence or absence 1mM calcium) supplemented with 2mM deoxyglucose and 3 or 6 μCi/ml 3 H deoxyglucose for 2 hours. In initial experiments 0.3 mM phloretin, an inhibitor of glucose transport, was used as a negative control.

Oocytes were stimulated with insulin (0.2-20 μM) for the indicated time in 0.5 ml ND96 (with or without 1 mM calcium as indicated). For conditions where calcium was not included oocytes were washed several times with ND96. Oocytes were treated with ND96 supplemented with 1 mM EGTA or BAPTA (as indicated) for 5 minutes. The oocytes were washed several times in ND96 before and after addition of EGTA to prevent a sudden pH change due to H^+ released upon calcium binding to EGTA. The oocytes were stimulated in ND96+/-Ca either before or after the EGTA treatment as indicated. Buffer pH was adjusted to avoid alterations of pH when comparing the different conditions.

To examine the effects of Ca^{++} concentration on ISDU, oocytes were stimulated in media with extracellular calcium concentrations ranging from 1 μM to 10 mM. For the lower calcium concentrations oocytes were washed twice with ND96 buffered with Ca^{++},EDTA and EGTA to calcium concentrations ranging from 1 μM to 1 mM and then incubated for 30 minutes prior to addition of insulin (Computer program for calcium

buffer preparation used by permission of Dr. R. Haslam). For the higher range,1-10 mM, ND96 media were made with the appropriate $CaCl_2$ concentration.

Basal uptake was determined for each condition by omitting insulin.

After uptake, the oocytes were quickly washed several times in ice-cold ND96 and lysed in 2% SDS. Radioactivity, in the media and cell homogenates, was determined by scintillation counting. Samples of the final wash were also counted to ensure that contributions from residual media transferred to the scintillation vials along with the oocytes were negligible (less than 20 cpm/oocyte).

CALCULATIONS AND DATA PRESENTATION

Data are presented as ISDU (insulin stimulated uptake = uptake with insulin - basal uptake) or as DU (total uptake). With the exception of $^{45}Ca^{++}$ experiments data were plotted as cpm/oocyte versus the stimulation condition. When data from several experiments were included in calculations for a figure, the data were normalized to the indicated condition in each experiment. This was done only if different concentrations of ^{3}H deoxyglucose were used in the experiments or if a different condition was introduced in one of the experiments.

Student t test analysis of the data was used to determine the significance of observed differences between conditions.

II-iv
RESULTS

REMOVAL OF EXTRACELLULAR CALCIUM BY EGTA DOES NOT RESULT IN LOSS OF INTRACELLULAR CALCIUM TO THE MEDIUM

Xenopus oocytes loaded with $^{45}Ca^{++}$ for 4 hours were washed with MBSH medium to remove the label that remained in the medium. The medium was then replaced and counted at 5 or 10 minute intervals to determine if there was release of label into the medium. (Figure 1). The high activity in the early time points varies considerably from experiment to experiment, approximately, adding up to approximately 10-20% of total, and probably reflects residual label left in the media from the loading incubation. After the initial washes a low level, approximately 1%, of the total $^{45}Ca++$ was released to the medium in each 10 minute incubation. The amount of $^{45}Ca++$ released did not vary significantly between oocytes washed with medium containing 1 mM calcium, or medium containing 1 mM EGTA (Figure 1). These data demonstrate that removal of calcium from the media, using chelating agents, does not lead to a loss of intracellular calcium above that observed in media containing Ca^{++}.

FIGURE 1
EFFECT OF REMOVING Ca^{++} FROM THE MEDIA ON Ca^{++} RELEASE FROM OOCYTE

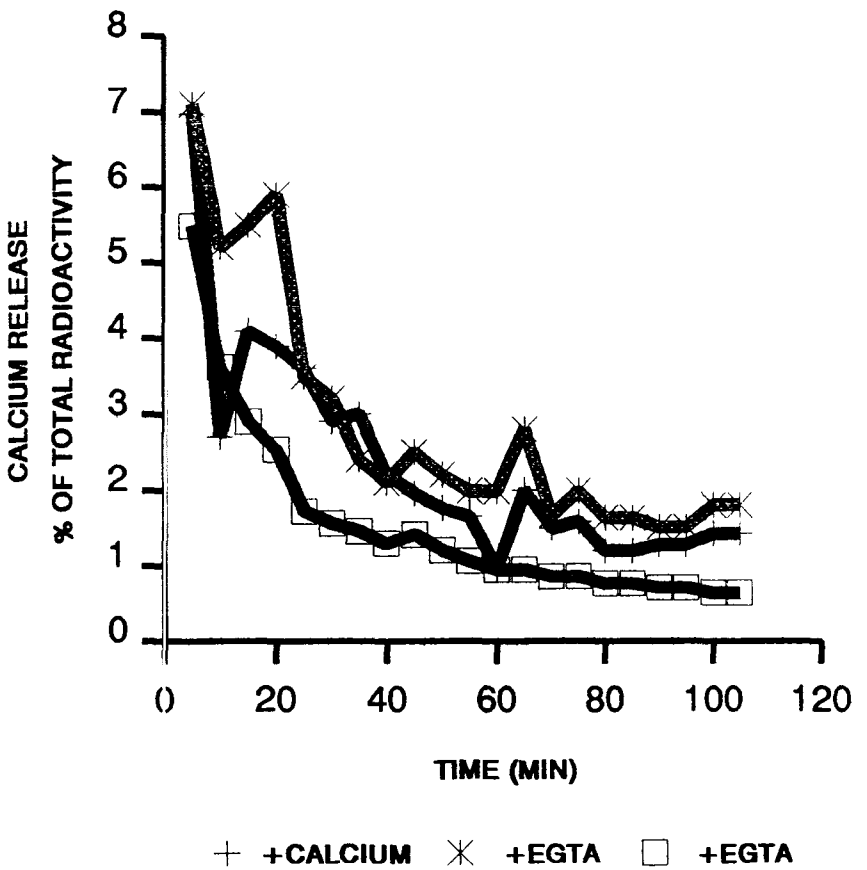


FIGURE 1
EFFECT OF REMOVING Ca^{++} FROM THE MEDIA ON Ca^{++} RELEASE FROM OOCYTE
Groups of 20 oocytes were loaded with $CaCl_2$ for 4 hours and then were washed 6 times with 5 mL of either MBSH(1mM Ca^{++}) or MBSH(1mM EGTA-two separate experiments are shown) as indicated. The oocytes were then placed in 0.5 mL MBSH +/- calcium, as indicated. At 5 minute intervals, for 105 minutes, the medium was removed and replaced with fresh medium. The radioactivity of the media at each time point was determined by scintillation counting.

XENOPUS OOCYTES RESPOND TO INSULIN IN A DOSE-DEPENDENT MANNER

Biological response to insulin was assayed in the oocytes using deoxyglucose uptake and phosphorylation. As expected, in the presence of physiologic Ca^{++}, the oocytes respond to insulin in a dose dependent manner (Figures 2a and b). Increasing concentrations of insulin result in an increase in deoxyglucose uptake that is maximal at 5-10 μM insulin (Figure 2a). Consistent with previously reported data in oocytes (Janicot and Lane, 1989), 10 μM insulin stimulated deoxyglucose uptake 2-3 fold over basal (basal 487+/- 52 CPM/oocyte cf 1109 +/- 88 CPM/oocyte). 10 μM insulin is higher than reported for adipocytes (Taylor et al., 1979). However, this may reflect differences in sensitivity of oocytes and adipocytes to porcine insulin. Furthermore, at this insulin concentration it is expected that both the insulin receptor and the IGF-1 receptor will be activated by insulin, although the affinity of insulin for IGF-I receptor is 1000X lower than for insulin receptor (reviewed in Froesch et al., 1985). Nevertheless, there is evidence that the insulin-like effects of IGF I are mediated through the insulin receptor and not the IGF I receptor and conversely the growth factor effects of insulin are mediated through the IGF I receptor (for review see Froesch et al., 1985). As discussed in Appendix A the kinase activity of insulin and IGF-1 receptors share a common substrate,pp185/IRS-1, an important intracellular signalling molecule which couples activation of

the receptors to SH2 containing molecules such as PI3 kinase (see Appendix A for discussion and references). Given the overlap in biological effects mediated by insulin and IGF-I receptors, the effects of calcium on either receptor are difficult to separate.

FIGURE 2a
INSULIN STIMULATION OF DEOXYGLUCOSE UPTAKE

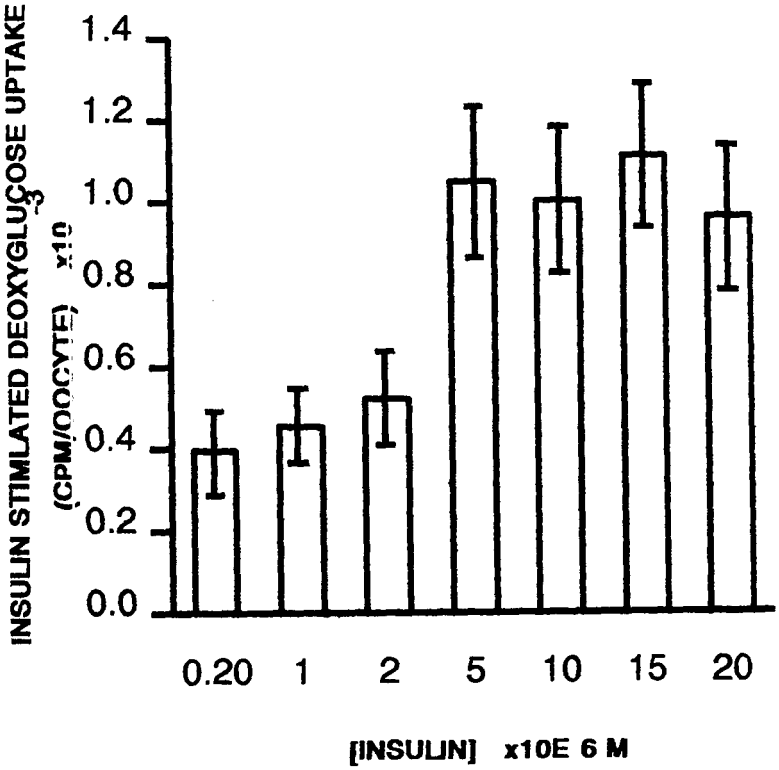


FIGURE 2a
INSULIN STIMULATION OF DEOXYGLUCOSE UPTAKE
Groups of 10 oocytes were stimulated for 30 minutes with insulin ([insulin] as indicated). Insulin not bound after 30 minutes was removed by washing the oocytes 3 X 3 ml ND96. Deoxyglucose uptake was determined by incubating the oocytes for 2 hours in ND96 supplemented with 2 mM unlabelled deoxyglucose and 6 μCi ^{3}H deoxyglucose/ml. Uptake was terminated and external label removed by 3 X 3 ml, ice cold ND96 washes. Oocytes were lysed in 2% SDS and radioactivity quantified by scintillation counting. ISDU=(uptake with insulin - basal uptake). The data presented are from two separate experiments each done in triplicate.

Examination of total membrane proteins labelled with ^{32}P demonstrated a similar concentration-dependent response to insulin (Figure 2b).

FIGURE 2b
INSULIN-STIMULATED PHOSPHORYLATION OF OOCYTE MEMBRANE PROTEINS

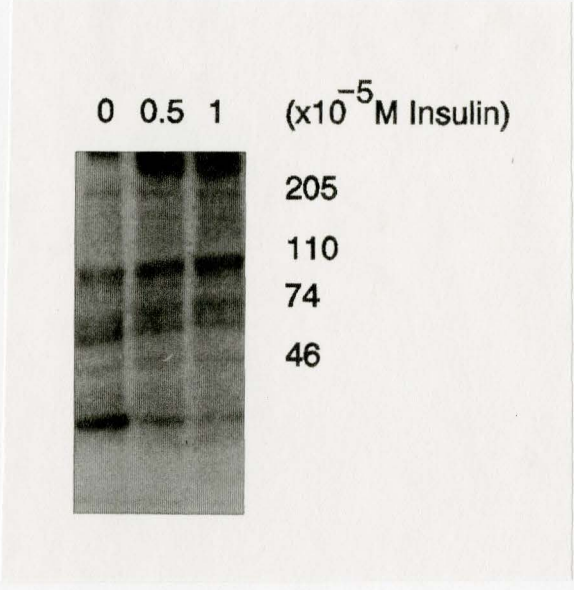


FIGURE 2b
INSULIN-STIMULATED PHOSPHORYLATION OF OOCYTE MEMBRANE PROTEINS
Four sets of 10 oocytes were microinjected with 50 nl each ^{32}P orthophosphate (1 mCi/10 μl). After 1 hour the oocytes were challenged for 20 minutes with insulin (concentration of insulin indicated). Oocytes were homogenized and the membrane fractions were enriched by extraction with sodium carbonate (pH 11.5). The membrane proteins were separated on SDS-PAGE (4-15%), dried and exposed to film for 4 days.

Most notable was an increase in phosphorylation of a very high molecular weight species (approximately 250 kDa). However phosphorylation of a 100 kDa polypeptide was also increased. In addition, a decrease in phosphorylation of a 30 kDa polypeptide was observed.

The above results suggest that in oocytes 10 μM insulin is sufficient to elicit maximal deoxyglucose uptake.

EFFECT OF CALCIUM ON INSULIN-STIMULATED DEOXYGLUCOSE UPTAKE

To examine the effects of extracellular calcium on insulin stimulated deoxyglucose uptake oocytes were incubated with insulin and ^{3}H deoxyglucose in the presence or absence of calcium in the media. When insulin and calcium are added to oocytes deoxyglucose uptake is approximately 2-fold higher than without insulin (Figure 3 compare lanes 1 and 3). If calcium is not included in the medium (lane 2) deoxyglucose uptake drops to the basal level observed without insulin (lane 4) or when uptake is inhibited by the addition of phloretin (lane 5). Paired t test analysis of lane 1 (+Ca) and lane 2 (-Ca) confirmed that the difference observed when calcium and insulin were present was significant ($F>0.95$). Moreover removal of Ca^{++} did not affect basal uptake, Figure 3 compare lanes 3 and 4. Therefore, it is possible to determine the amount of ISDU by subtracting basal uptake from the total.

FIGURE 3
CALCIUM DEPENDENCE OF DEOXYGLUCOSE UPTAKE

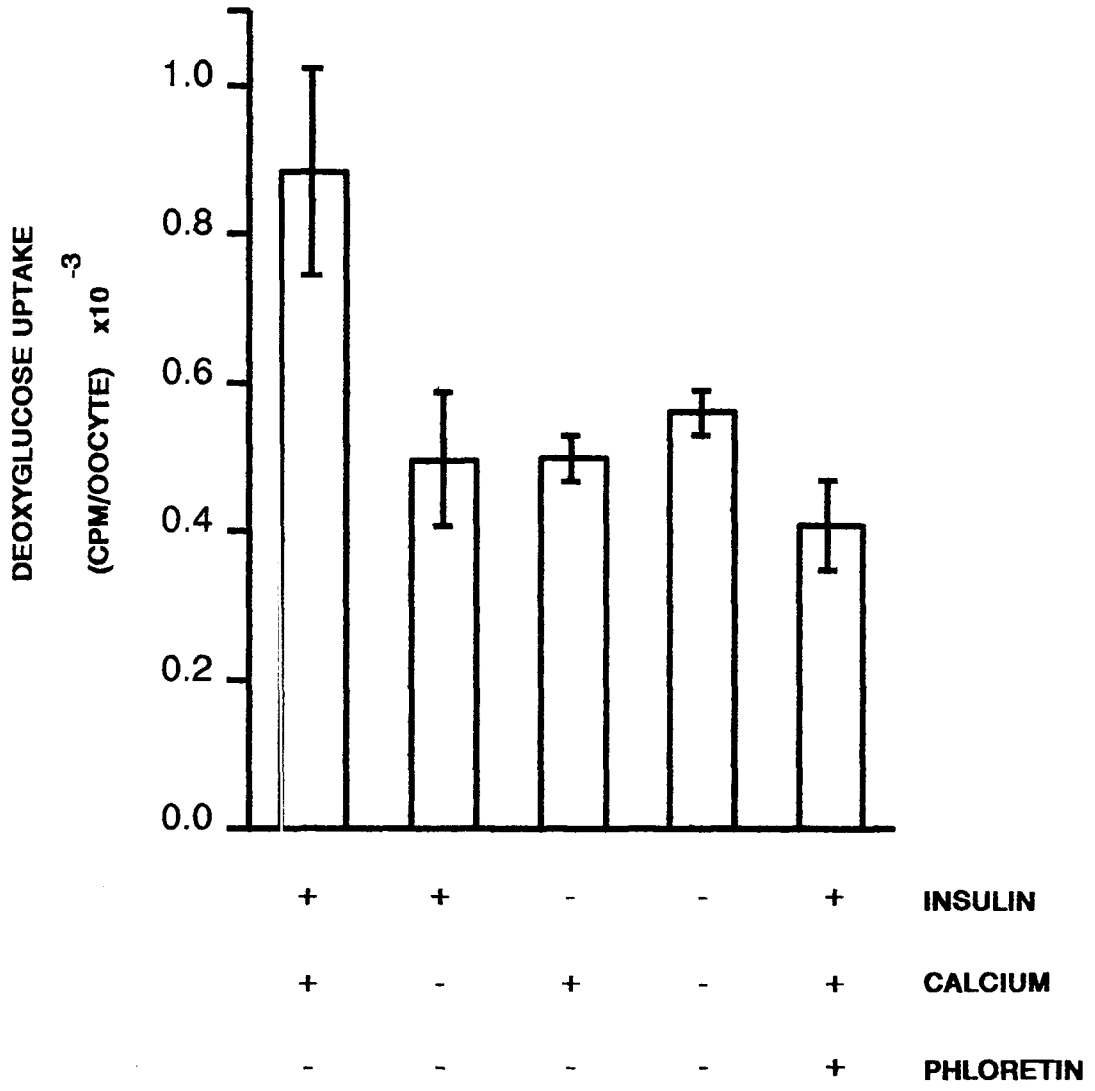


FIGURE 3
CALCIUM DEPENDENCE OF DEOXYGLUCOSE UPTAKE
Groups of 10 oocytes were incubated for 145 minutes, at room temperature, in ND96 media supplemented with 2 mM 2-deoxyglucose, 6μCi/mL ^{3}H deoxyglucose and insulin (10 μM), calcium (1 mM) and phloretin (0.03 mM) as indicated. Oocytes for the -Ca conditions were washed several times in ND96(without Ca^{++}) prior to insulin and deoxyglucose addition. Uptake was stopped by 3 X 1 ml washes of ice cold ND96(0.3mM phloretin), followed by 3 X 1 ml washes of ice cold ND96. Oocytes were lysed with 2% SDS and activity quantified by scintillation counting. The results presented are from 2 experiments, each performed in duplicate.

While these data support a role for physiological concentrations of extracellular calcium in insulin action they do not identify the stage at which the calcium is required. Calcium could be involved in an early stage of insulin signalling, for glucose transport, or for both processes.

After stimulation of oocytes with insulin, the earliest significant increase in deoxyglucose uptake was observed after 30 minutes (Figure 4).

FIGURE 4
INSULIN STIMULATED DEOXYGLUCOSE UPTAKE IN XENOPUS OOCYTES
-RATE OF DEOXYGLUCOSE UPTAKE OVER TIME

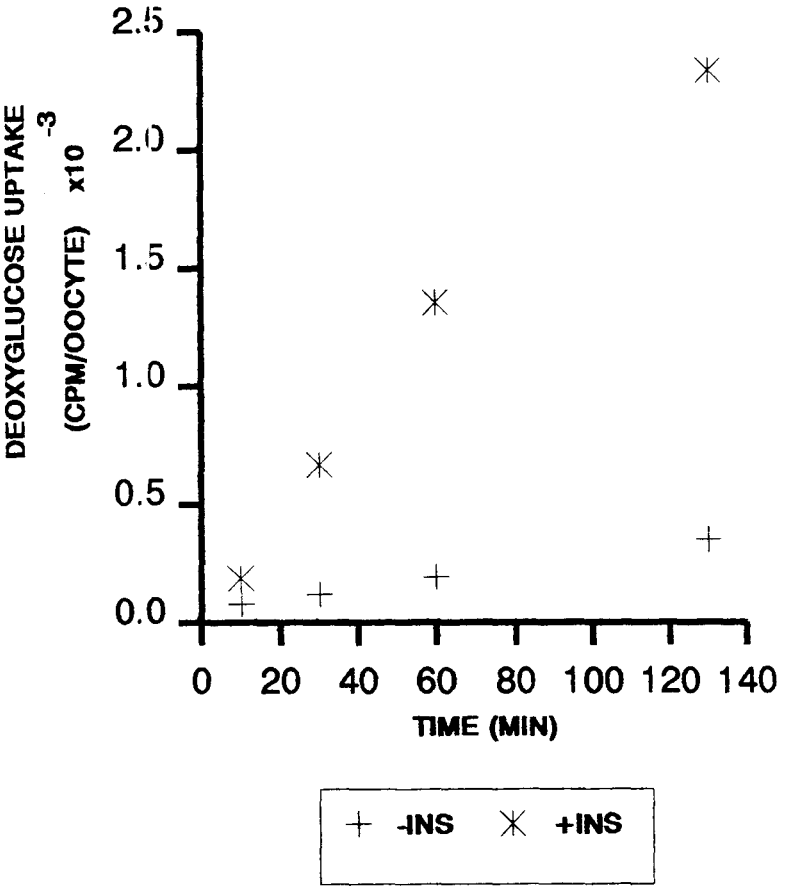


FIGURE 4
INSULIN STIMULATED DEOXYGLUCOSE UPTAKE IN XENOPUS OOCYTES
Oocytes were incubated in ND96[1mM Ca^{++}, 2mM deoxyglucose, 6μCi ^{3}H deoxyglucose/ml] in the presence (*) or absence (+) of 10 μM insulin. At 10, 30, 60 and 120 minutes sets of 10 oocyts were washed 3 X 3 ml ND96, lysed in 2% SDS and radioactivity determined by scintillation counting. The results are fron one experiment performed in triplicate. The results from this and other similar experiments indicate that observable stimulated deoxyglucose occurs at 20-30 minutes after addition of insulin.

Therefore, to separate signalling from glucose transport, oocytes were stimulated with insulin for not longer than 30 minutes and washed several times to remove unbound insulin. The ability to separate stimulation and uptake phases of ISDU is another advantage of the oocyte system compared to isolated adipocytes or cultured cells (oocytes are large and unlike adipocytes, do not require centrifugation when removing insulin by repeated washing with fresh media - as an approximation if aspiration after each wash removed all but 0.1 ml (overestimate) of media with 3 washes, 3 ml each, the final insulin concentration, if the starting concentration was 10 μM, would be less than 0.3 nM - this is not sufficient to ellicit a significant response in the oocyte) (Janicot and Lane 1989). After insulin was removed, ^{3}H deoxyglucose was added to the oocytes and uptake was allowed to proceed for 2 hours in the presence or absence of 1 mM calcium as indicated. In control experiments, in which Ca^{++} was present for both stimulation and uptake phases, the removal of insulin after 30 minutes was shown to have no effect on maximal deoxyglucose uptake. Therefore, the effects of calcium on the <u>early events</u>, (i.e. before a significant increase in deoxyglucose uptake), in insulin signalling could be examined by altering the Ca^{++} concentration during stimulation without interfering with glucose transport. When the experiment was performed in this manner, washing the oocytes (5 times for 5 minutes each wash) in media not containing added Ca^{++}(ND96 - $CaCl_2$) was not

sufficient to abolish insulin stimulated deoxyglucose uptake provided the uptake phase of ISDU was in media containing 1 mM Ca^{++} (Figure 5a lanes 1,2). However chelation of residual Ca^{++} with either 1 mM EGTA or BAPTA at pH 7.2 (for 5 minutes) prior to insulin stimulation (in media containing neither Ca^{++} nor chelator) reduced ISDU 2 fold (Figure 4a lanes 3 and 4, paired t test lane 1 and 4 - $F>0.99$ highly significant, lane 1 and 3 - $F>0.975$ significant). We conclude from this experiment that insulin stimulation i.e. the early phase of ISDU, does require extracellular calcium, but at non-physiological concentrations achieved only by addition of chelators to the media.

FIGURE 5
EFFECTS OF CALCIUM CHELATORS ON INSULIN STIMULATED DEOXYGLUCOSE UPTAKE

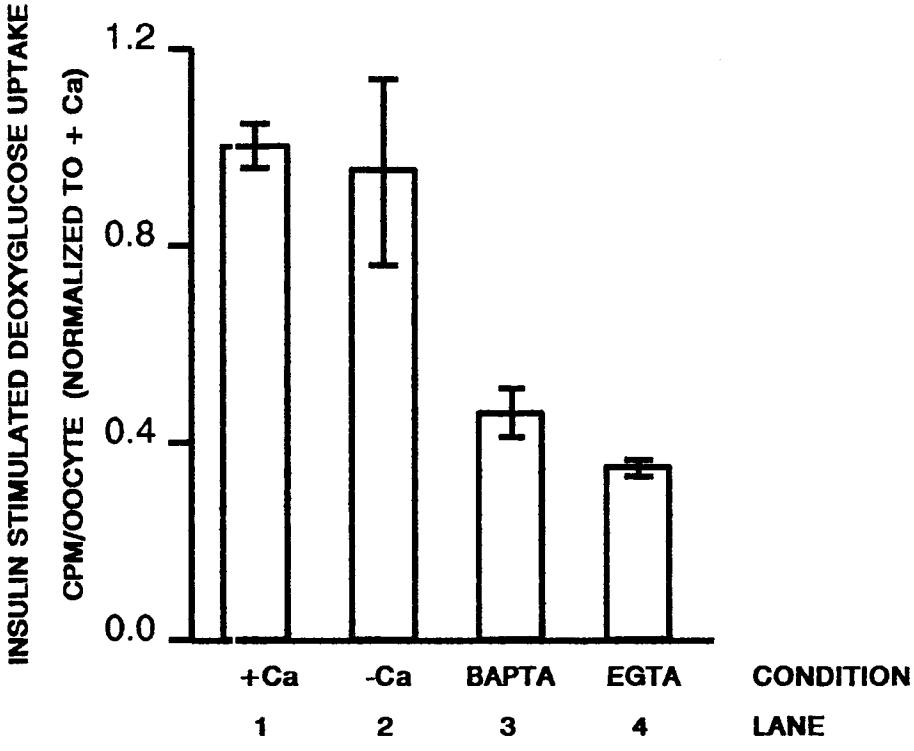


FIGURE 5
EFFECTS OF CALCIUM CHELATORS ON INSULIN STIMULATED DEOXYGLUCOSE UPTAKE
Groups of 10 oocytes were washed 3 X 3 ml ND96(no calcium added) supplemented with 1 mM Ca^{++} (lane 1), no additions (lane 2), 1 mM BAPTA (lane 3) or 1 mM EGTA (lane 4). Oocytes were incubated in the medium for 5 minutes. EGTA and BAPTA were removed by 3 X 3 ml ND96 washes prior to the 30 minute insulin ([10 μM]) stimulation. Stimulation was in ND96/1 mM Ca^{++} (lane 1) or ND96 (lanes 2, 3 and 4). Unbound insulin was removed by 3 x 3 ml ND96 washes. Deoxyglucose uptake was determined by incubation of stimulated oocytes in ND96[+1mM Ca^{++}, 2mM deoxyglucose and ^{3}H deoxyglucose (6μCi/ml)] for 2 hours. Washes, cell lysis and scintillation counting were as described in Figure 3. The results presented are from 2 experiments, performed in triplicate. Data were normalized to +Ca^{++} (lane 1) condition.

The oocytes used in the above experiments were dissected manually from the ovarian fragment. Incubation of oocytes overnight in plastic culture dishes removes the majority of the surrounding follicle cells (Bement and Capco, 1989). While the above authors incubated the oocytes in calcium- and magnesium-free medium we did not find it necessary for defolliculating the oocytes in plastic culture dishes. When these experiments were repeated using oocytes defolliculated using collagenase (as controls) a similar pattern of inhibition was observed (Figure 6). These results suggest that EGTA and BAPTA affect receptors on the oocyte and that the observed results are not influenced by residual follicle cells.

FIGURE 6
EFFECTS OF EGTA ON ISDU - COLLAGENASE TREATED OOCYTES

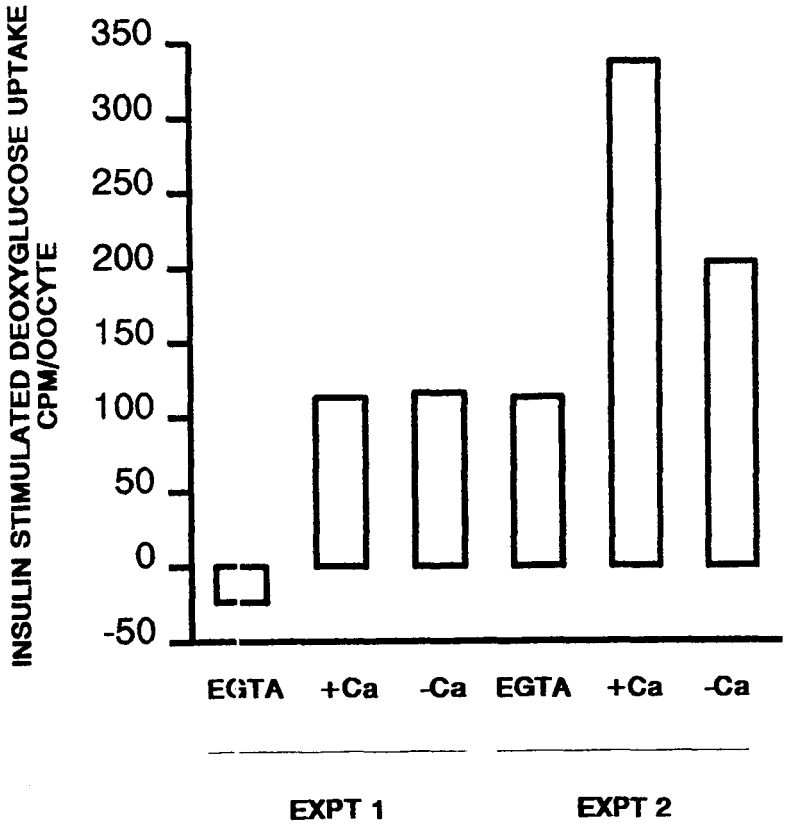


FIGURE 6
EFFECTS OF EGTA ON ISDU - COLLAGENASE TREATED OOCYTES
In lanes marked EGTA and -Ca, sets of 10 oocytes were washed with ND96/-Ca^{++} prior to stimulation. In the EGTA sets the oocyte were also treated with ND96/1 mM EGTA prior to stimulation. The oocytes were stimulated with 10 μM insulin for 30 minutes in ND96/1mM Ca^{++} (+Ca) or ND96/-Ca^{++} (-Ca and EGTA). Uptake and scintillation counting were as described above. The results are from two separate experiments.

To further identify the stage at which EGTA inhibits insulin stimulation, oocytes were treated with EGTA at different times before, during or after insulin stimulation. To determine the time required for maximal insulin stimulation oocytes were stimulated with insulin, for 5 and 15 minutes, prior to uptake. The insulin stimulated deoxyglucose uptake observed after exposure to insulin for 5 minutes was equal to that after 15 minutes (Figure 7a lanes 1 and 2) suggesting that 5 minutes is sufficient to produce a maximal response in the oocyte. EGTA blocked insulin stimulation when oocytes were treated both immediately before or during insulin addition (Figure 7a compare lanes 1 with 3 and 4). When oocytes were incubated in media containing EGTA after insulin stimulation there was a time dependent loss of inhibition of ISDU (Figure 7a lanes 5, 6 and 7). Inhibition was observed when oocytes were treated with EGTA after 5 minutes of insulin stimulation, but by 15 and 30 minutes the inhibition was no longer significant (paired t test analysis - lanes 5 and 1 $F=0.99$ highly significant, lanes 6 and 1 $F<0.95$ significant, lanes 7 and 1 $F<0.75$ not significant). From these data it appears that extracellular calcium is required early in the insulin response. Our observation that by 5 minutes maximal stimulation by insulin has occurred but EGTA can still inhibit ISDU up to 5 minutes after insulin addition suggests that extracellular calcium is required after insulin binding but

early in the response before stimulated deoxyglucose uptake is observed. Nevertheless the extent of inhibition 5 minutes after insulin addition is not as large as seen when the oocytes are treated with EGTA before or during stimulation with insulin suggesting extracellular calcium may also be involved in insulin binding to the receptor or the early Ca^{++} sensitive event has already started. Finally, to exclude any effects of EGTA treatment on basal uptake we measured the deoxyglucose uptake without added insulin. In the above experiments basal uptake was not significantly affected by the EGTA treatment suggesting the inhibition observed early in the stimulation was insulin-dependent.

FIGURE 7a
EFFECT OF EGTA ON INSULIN STIMULATED DEOXYGLUCOSE UPTAKE

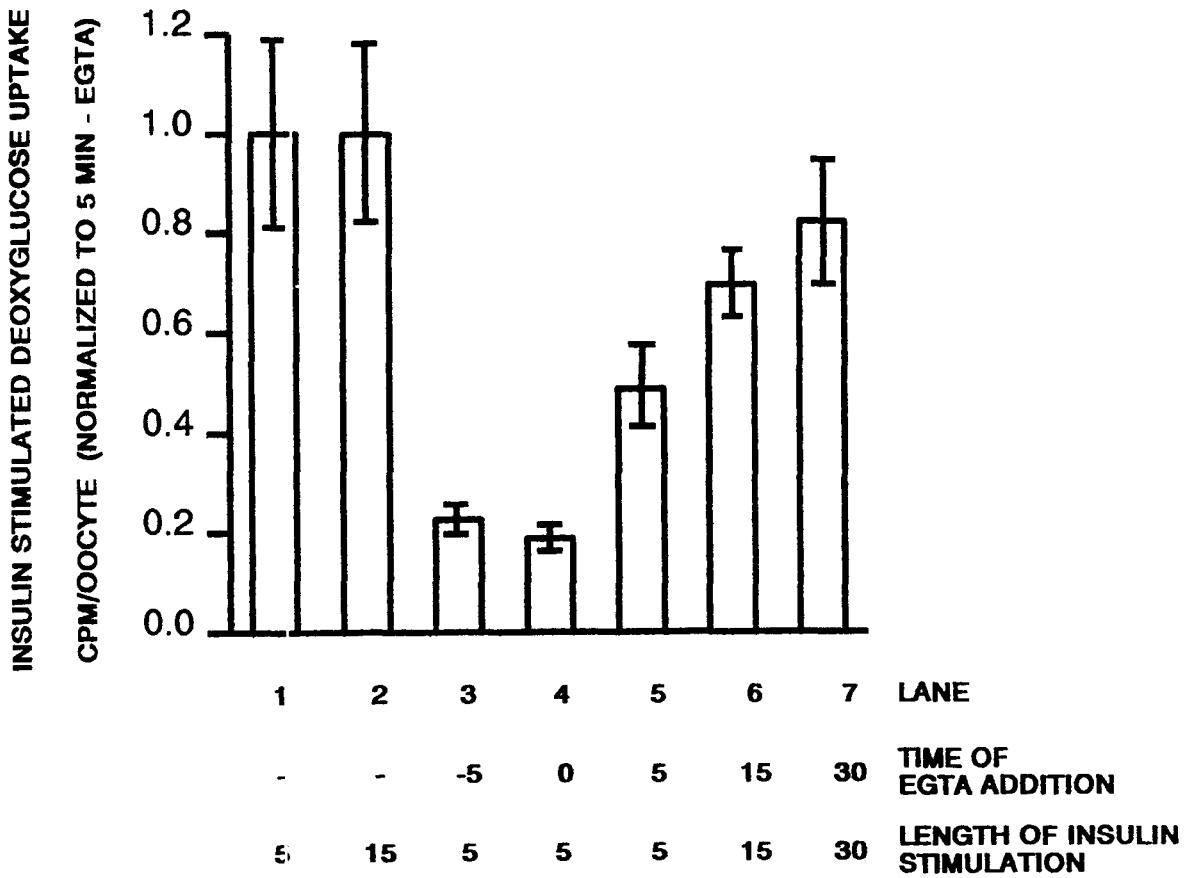


FIGURE 7a
EFFECT OF EGTA ON INSULIN STIMULATED DEOXYGLUCOSE UPTAKE
In lanes 1 and 2 oocytes were stimulated with 10 μM insulin in ND96/1mM Ca^{++} for 5 or 15 minutes as indicated. Unbound insulin was removed by 3 X 3ml ND96-calcium washes prior to uptake in ND96+1mM calcium. The effect of EGTA on ISDU, before, during and after stimulation, was determined by washing oocytes for approximately 5 minutes with ND96/1 mM EGTA and comparing uptake to untreated oocytes (lane 1 and 2). Lane 3 shows the effect of treating oocytes with 1mM EGTA in ND96(no calcium) prior to a 5 minute insulin stimulation in ND96+calcium. In lane 4 oocytes were treated with 1 mM EGTA during the 5 minute insulin stimulation in ND96(no calcium). In lanes 5, 6, and 7 oocytes were stimulated for the indicated time prior to treatment with 1 mM EGTA. Uptake and scintillation counting were as described above. The data presented were from 5 experiments,each performed in triplicate. The results from each experiment were normalized to the 5 minute/+Ca^{++} condition. Each point is from at least 4 experiments done in triplicate except lane 7 which was from one experiment done in triplicate.

The calcium free media used in the above experiments contained Mg^{++}. To exclude the possibility that Mg^{++} is functionally substituting for Ca^{++} ISDU was examined in Ca^{++} and Mg^{++} free media (figure 7b). Removal of both Mg^{++} and Ca^{++} from the media, during stimulation, did not have an effect on ISDU (Figure 7b lanes 1 and 2). This result suggests that Mg^{++} is not functionally substituting for Ca^{++} in the experiments where only Ca^{++} was ommitted from the media (Figure 4). This result is consistent with the earlier conclusion that the stimulation phase of ISDU in the oocyte does not require physilogical amounts of Ca^{++} in the extracellular media.

We also examined the toxicity of EGTA on the oocytes to exclude the possibility that EGTA and BAPTA, being anionic, inhibit ISDU by binding directly to oocyte proteins and not by chelating Ca^{++} (Figure 7b). EGTA, in the stimulation phase, was not effective in inhibiting ISDU when in the presence of excess Ca^{++} suggesting that the presence of EGTA is not sufficient to inhibit ISDU (Figure 7b, lanes 3, 4 and 5).

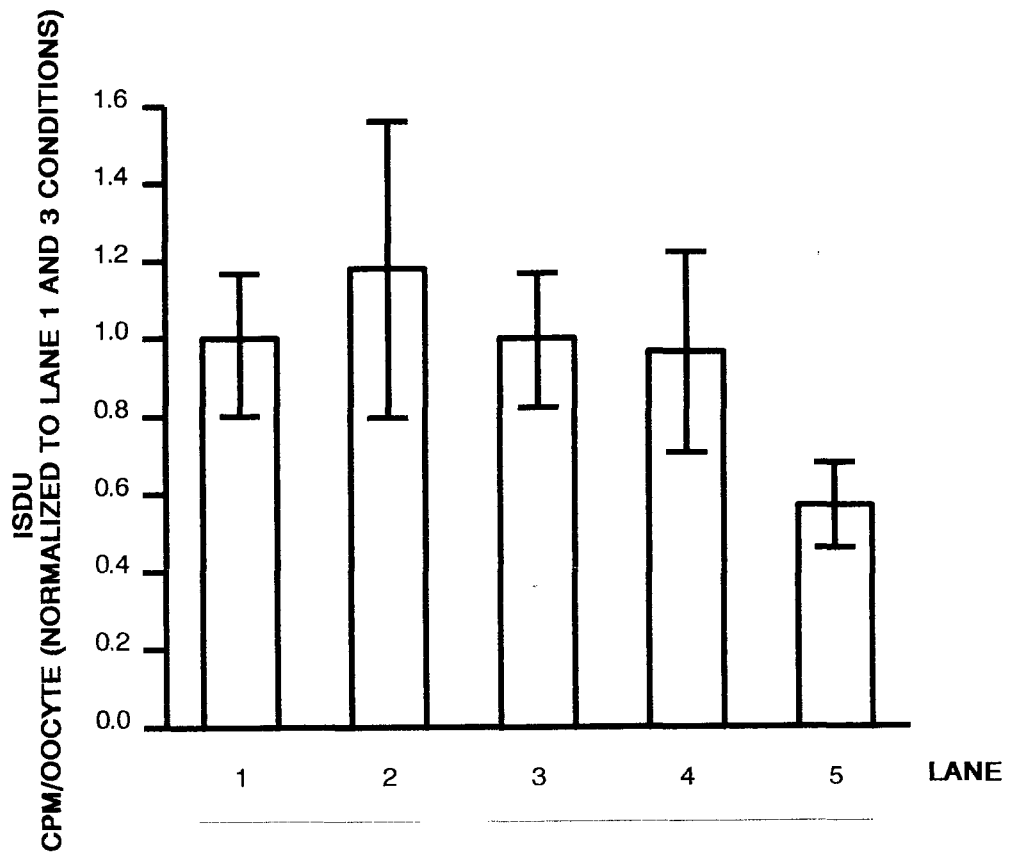
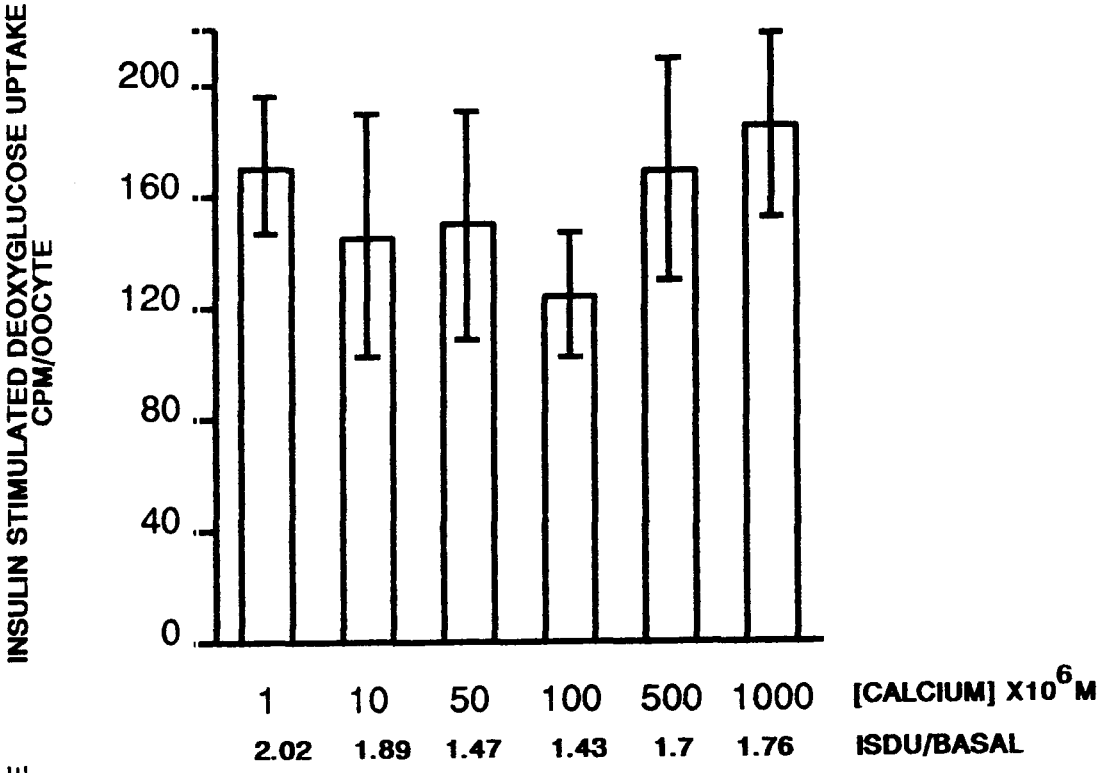


FIGURE 7b
EFFECTS OF CALCIUM, MAGNESIUM AND EGTA ON INSULIN STIMULATED DEOXYGLUCOSE UPTAKE
Groups of 10 oocytes were washed 3 X 3 ml ND96(no calcium or magnesium added) supplemented with 1 mM Ca^{++} and Mg^{++} (lane 1 and 3), no additions (lane 2), 1 mM EGTA (lane 5) or 1 mM EGTA, 1mM Mg^{++} and 2 mM Ca^{++} (lane 4) [the media were adjusted to pH 7.2 after the addition of EGTA, Mg^{++} and Ca^{++}] . Oocytes were stimulated with 10 μM insulin, in the same media, for 5 minutes. EGTA and unbound insulin were removed by 3 X 3 ml ND96 washes prior to uptake. Deoxyglucose uptake was determined by incubation of stimulated oocytes in ND96[+1mM Ca^{++}, 2mM deoxyglucose and ^{3}H deoxyglucose (6μCi/ml)] for 2 hours. Washes, cell lysis and scintillation counting were as described in Figure 3. The results presented are from 2 experiments, each performed in triplicate. Data were normalized to the $+Ca^{++}/+Mg^{++}$ condition.

To determine the concentration of calcium required for insulin stimulation, the calcium concentration in the media was adjusted from 1 μM to 10 mM. The concentration ranges from 1 μM to 1 mM were buffered using EGTA and EDTA while 1 mM to 10 mM range media were prepared using $CaCl_2$ at appropriate concentrations. ISDU was not significantly affected by calcium concentrations greater than or equal to 1 μM (Figure 8a). The same results were obtained for calcium concentrations ranging from 1 mM to 10 mM (Figure 8b). The extent of stimulation above basal was not different between the buffered (Figure 8a) and unbuffered (Figure 8b) media suggesting that EGTA and EDTA were not inhibitory in a situation where Ca^{++} and Mg^{++} are in excess.

FIGURE 8a and 8b
EFFECT OF EXTRACELLULAR CALCIUM CONCENTRATION ON INSULIN STIMULATED DECXYGLUCOSE UPTAKE

8a



8b

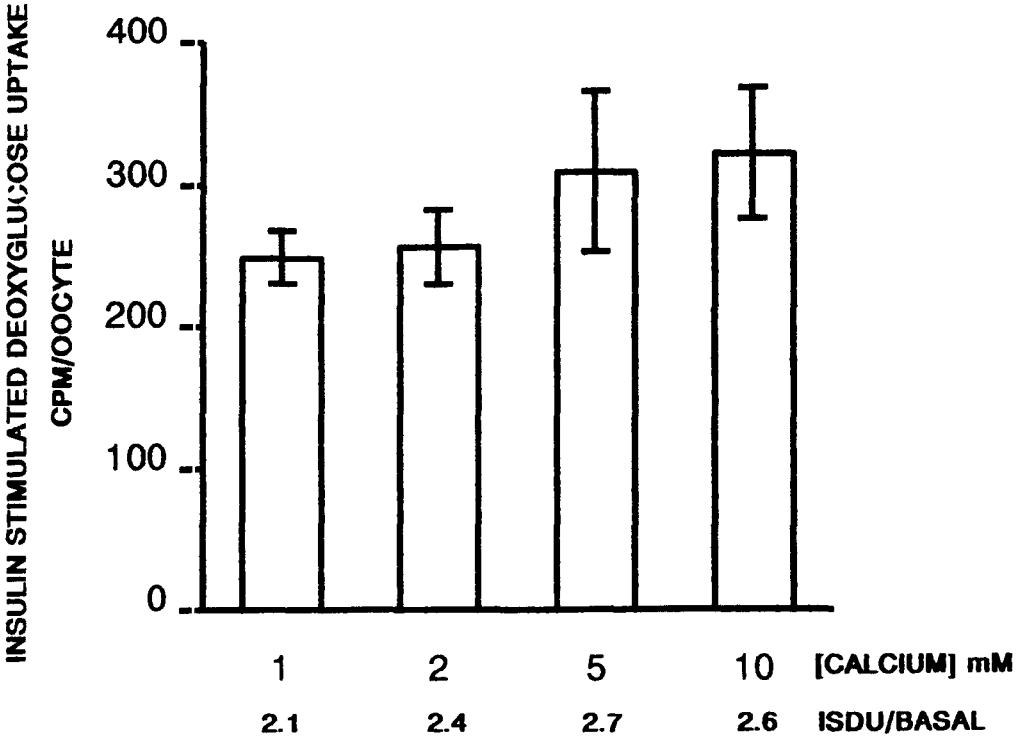


FIGURE 8a and 8b
EFFECT OF EXTRACELLULAR CALCIUM CONCENTRATION ON INSULIN STIMULATED DEOXYGLUCOSE UPTAKE
Groups of 10 oocytes were washed several times in one of a series of calcium buffers ranging from 1 to 1000 μM calcium (8a - buffered with EGTA/EDTA) or 1-10 mM Ca^{++} (8b - unbuffered). The oocytes were incubated for 30 minutes and then stimulated for 30 minutes with 10 μM insulin. Insulin not bound after 30 minutes was removed by washing the oocytes 3 X 3 ml ND96. Stimulation of deoxyglucose uptake was determined by incubating the oocytes for 2 hours in ND96(1 mM Ca^{++}) supplemented with 2 mM deoxyglucose and 3 μCi ^{3}H deoxyglucose/ml. Uptake was terminated and external label removed by 3 X 3 ml, ice cold ND96 washes. Oocytes were lysed in 2% SDS and activity determined by scintillation counting. The results for 5a are from 3 experiments , each performed in triplicate and 5b are from 2 experiments performed in triplicate. The number below each calcium concentration represents the extent of insulin stimulation at each concentration (ISDU/BASAL).

S. Brimble in Dr. V. S. Ananthanarayanan's laboratory has found that insulin B chain transports calcium into lipid vesicles in a 1:1 ratio. Insulin also transports calcium into vesicles (Brimble and Ananthanarayanan, 1992). These results along with results from similar studies on other peptides led to the suggestion that calcium plays a cofactor like role in the binding of insulin to the insulin receptor. According to this model calcium interactions are necessary for signal transduction. The delivery of a calcium to the receptor by insulin could act as the "message" for signal transduction (Ananthanarayanan, 1991).

Preliminary results on the effects of the A and B chain on deoxyglucose uptake are shown in Figure 9. Insulin A and B chains both stimulated deoxyglucose uptake to a small extent (1/10 that of insulin). In contrast to insulin stimulation, stimulation by A and B chains was abolished by removal of calcium, suggesting a different mechanism of action. Further experiments are necessary to determine if the response is specific for insulin derived peptides or whether the result is a non-specific peptide effect. The observation that A chain was as effective as the bioactive B chain in stimulating deoxyglucose uptake suggests the effect is non specific. Furthermore the ability of B chain, and not A chain, to bind to and transport calcium in an artificial system does not correlate with stimulation of deoxyglucose uptake in Xenopus oocytes.

FIGURE 9
STIMULATION OF DEOXYGLUCOSE UPTAKE BY INSULIN, A OR B CHAIN

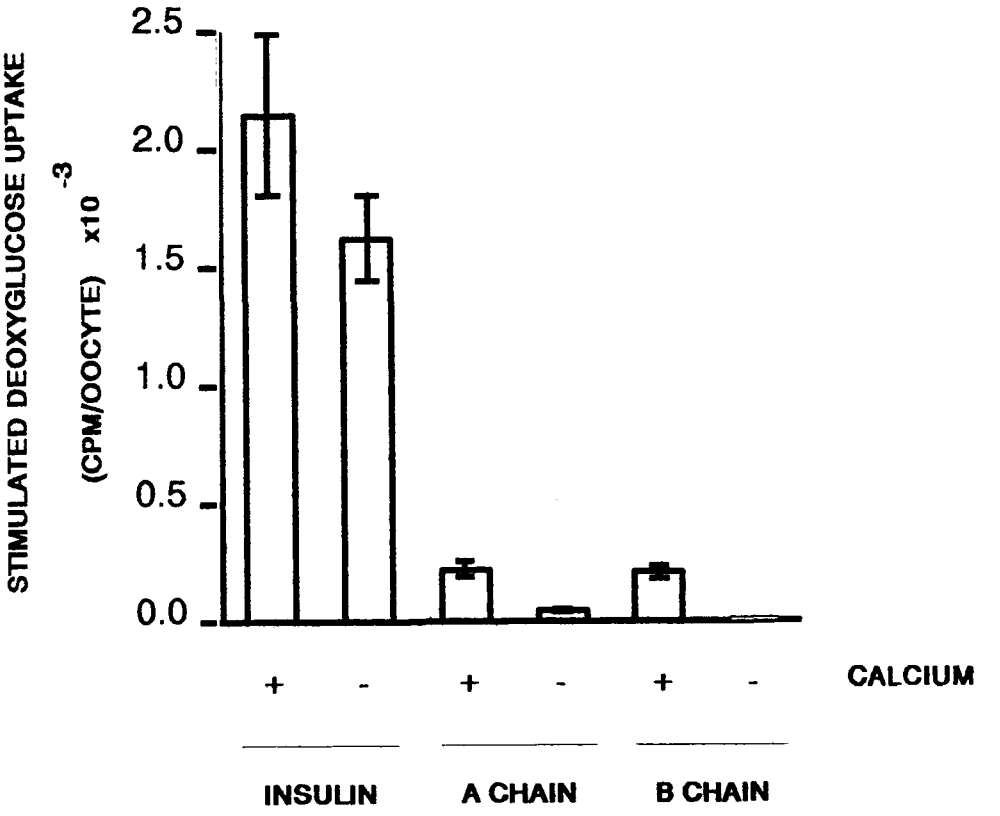


FIGURE 9
STIMULATION OF DEOXYGLUCOSE UPTAKE BY INSULIN, A OR B CHAIN
Groups of 10 oocytes were washed several times with ND96(1mM Ca^{++}) or ND96(without calcium) as indicated and subsequently stimulated in the same media, for 30 minutes, with insulin (10 μM), insulin A chain (25 μM) or insulin B chain (25 μM). Peptide not bound after 30 minutes was removed by 3 X 3 ml washes. Stimulation of deoxyglucose uptake was determined by incubating the oocytes for 2 hours in ND96(1mM Ca^{++}) supplemented with 2 mM and 3 μCi ^{3}H deoxyglucose/ml. Uptake was terminated and external label removed by 3 X 3 ml, ice cold ND96 washes. Oocytes were lysed in 2% SDS and specific activity determined by scintillation counting. The data presented were from two experiments, each performed in triplicate.

II-v
DISCUSSION

To examine the role of extracellular calcium in insulin receptor transmembrane signalling we measured deoxyglucose uptake in response to insulin using the Xenopus oocyte. In a variety of other cell systems chelation of extracellular Ca^{++} with EGTA depletes intracellular Ca^{++} with a resulting loss of Ca^{++} to the medium (Terepka, 1976; Ahktar and Perry, 1979; Taylor et al., 1979). However, after labelling intracellular stores in Xenopus oocytes with $^{45}Ca^{++}$ we were unable to detect differences in the rate of loss of the labelled Ca^{++} when oocytes were treated with EGTA (Figure 1). The small amount of calcium released to the media over time does not necessarily represent a loss of calcium from the intracellular stores but may instead represent an exchange for calcium associated with the plasma membrane (Inoue et al. 1992 and references within). This is likely since the plasma membrane the Ca^{++}/ATPase pump, which is regulated by calmodulin, is activated only when there is a large increase in the cytosolic Ca^{++} concentrations. To distinguish clearly between loss from intracellular stores and exchange for Ca^{++} on the membrane inhibitors of Ca^{++} action, such as cobalt and nickel, as well as inhibitors of Ca^{++} flux, such as verapamil, would be useful (Taylor et al. 1979). If the observed loss over time is due to exchange with membrane bound Ca^{++} then these agents would have no effect on the observed pattern. In

addition, further experiments will be necessary to determine if removal of calcium from the media results in changes in intracellular calcium distribution.

As expected, exposing the oocytes to insulin resulted in a concentration dependent stimulation of both deoxyglucose uptake and changes in polypeptide phosphorylation typical of an insulin sensitive cell. However, in contrast to previous reports with tissue explants, our results suggest that for Xenopus oocytes physiological concentrations of extracellular calcium are not required for insulin stimulated deoxyglucose uptake. During the stimulation phase reducing the calcium concentration in the media to as low as 1μM or as high as 10 mM did not affect insulin stimulated deoxyglucose uptake (Figures 5, 8a and 8b). In other studies, the optimal extracellular calcium concentration reported for maximal stimulation of deoxyglucose uptake ranged from 1-2 mM, with concentrations greater than 3 mM inhibitory (adipocytes, Draznin et al., 1987) to 10 mM (HTC hepatoma cell line, Williams et al., 1990). However, it is possible that the cells used in previous studies were not resistant to changes in extracellular calcium concentrations. For example, removal of extracellular calcium from the media of isolated hepatocytes results in oxidative stress and decreased cell viability (Reed et al., 1990). Alternatively, if sustained chelation of extracellular Ca^{++} depletes intracellular calcium stores then the inhibition of insulin stimulated deoxyglucose

uptake may be due to changes in cell physiology rather than due to alterations in receptor activation. As was discussed in the introduction high extracellular Ca^{++}, as was used in some other studies, may also affect intracellular Ca^{++} and the response to insulin. It is also possible that the oocyte receptor for insulin may be different structurally from that of mammalian cells. Further studies using human insulin receptors expressed in Xenopus oocytes may permit us to evaluate this possibility.

While physiological levels of free calcium are not required for insulin signalling in oocytes, chelating agents reduced the response to insulin. Furthermore, the observed inhibition is time dependent, such that 15 minutes after stimulation with insulin EGTA was no longer effective in blocking insulin mediated deoxyglucose uptake. EGTA was most effective in inhibiting stimulated deoxyglucose uptake if added before or during stimulation with insulin, but was still effective 5 minutes after insulin addition. These results suggest that calcium is involved in an early event or events in insulin receptor function. Our observation that EGTA treatment was only partially effective in inhibiting insulin action 5 minutes after insulin addition suggests the calcium-sensitive event is relatively early in the insulin response (i.e. before the onset of stimulated deoxyglucose uptake).

Purified insulin receptors have been shown to bind calcium in a specific manner (Williams and Turtle, 1984).

Therefore, calcium chelation with EGTA or BAPTA may be altering the structure of the insulin receptor and therefore inhibiting either the binding of insulin to the receptor or the ability of the receptors to transduce a signal. We think these possibilities are unlikely given that the extent of insulin stimulation of deoxyglucose uptake is the same whether insulin is added for 5 minutes or 15 minutes (lanes 1 and 2 Figure 4b). This result suggests that either all of the receptor sites are saturated within 5 minutes of insulin addition or alternatively the maximal deoxyglucose response has been stimulated by 5 minutes at this insulin concentration. Furthermore, it is expected that both high- and low-affinity sites will be occupied and functional at this concentration of insulin (Akhtar and Perry, 1979I; Eckel and Reinaur, 1987). Therefore inhibition of ISDU by EGTA, 5 minutes after insulin addition, is not likely due to impaired insulin-insulin receptor interactions. Alternatively, the chelators may be affecting membrane calcium pools necessary for normal membrane dynamics (Calcium binding to phosholipids: Inoue et al., 1992; Effects of EGTA and EDTA on erythrocyte membrane: Bramley and Coleman, 1972; muscle cell surface Ca^{++} binding to phospholipids: Moggio et al., 1992 and references contained within). This in turn may alter an early post-ligand-binding signalling event such as ability to transduce ligand dependent conformational changes through the transmembrane region, insulin receptor aggregation, internalization or kinase

activation (reviewed in Olefsky, 1990 and disscussed in Appendix A). The lack of good antibodies to Xenopus receptors coupled with the small number of endogenous receptors makes it difficult to examine these possibilities.

Calcium concentrations in the media as low as 1 μM are sufficient for normal insulin stimulation. The oocyte, even after externalization, would not encounter calcium concentrations as low as 1 μM. These results are consistent with the above suggestion that EGTA or BAPTA are causing bulk membrane perturbations, not necessarily specific to ISDU, which affect an early signalling event such as aggregation or internalization (reviewed in Olefsky 1990 and discussed in Appendix A).

The observation that EGTA is no longer effective in blocking insulin stimulated deoxyglucose uptake 15 minutes after insulin addition suggests that changes in membrane structure are not involved in inhibiting the uptake step of ISDU (Figure 4b). Uptake is more likely inhibited by removal of free Ca^{++} in the media. This is supported by the observation that incubation of oocytes in media containing chelating agents is not necessary to inhibit the uptake phase of ISDU (Figure 3). Treatment with EGTA 30 minutes after insulin addition did not inhibit uptake provided the uptake phase was in media containing 1 mM Ca^{++} (Figure 7a).

While it is clear that physiological concentrations of extracellular calcium are not necessary for insulin

stimulation of deoxyglucose uptake, inhibition by the presence of chelating agents suggests a mechanistic requirement for calcium (at the plasma membrane), in insulin signalling in Xenopus oocytes. While we have defined, temporally, a Ca^{++} sensistive step in insulin action in Xenopus oocytes, further studies are necessary to establish the signalling event being inhibited and the mechanism of inhibition.

CHAPTER III
EFFECTS OF THE 5′ AND 3′ UTRS AND CODING REGION ON TRANSLATIONAL EFFICIENCY IN XENOPUS OOCYTES AND RETICULOCYTE LYSATE TRANSLATION SYSTEMS

III-i
INTRODUCTION

In chapter II a requirement for extracellular calcium was established for the insulin response mediated by Xenopus receptors. To extend these results we would like to express cloned insulin receptors in the oocyte. Expression of human insulin receptors in the oocyte and subsequent testing of the effects of extracellular calcium on signalling through the exogenous receptors will establish whether the results in chapter II are specific to Xenopus receptors or whether there is a common mechanistic requirement for extracellular calcium in insulin action. Furthermore, expression of cloned insulin receptors in the oocyte would enable further studies on the mechanisms involved in receptor signalling and the effects of calcium on signalling. For this reason we were interested in optimizing expression of cloned genes, in particular the insulin receptor in oocytes.

The 5′ untranslated region (UTR) has been implicated in the translational efficiency of mRNA in a variety of systems (Johansen et al., 1984; Tyc et al., 1984; Berkner and Sharp, 1984; Jobling and Gehrke, 1987; Gallie et al., 1987 a and b; Jobling et al., 1988; Lazarus, 1992). Studies done previously in our laboratory suggested that the 5′ UTR is an important element in improving translation in cell free systems. We have shown that the 5′ UTR of the Xenopus ß-globin gene increases the rate of translation initiation and

therefore expression, of a variety of coding regions both in reticulocyte lysate (RRL) and wheat germ extract (WG) translation systems (Falcone and Andrews, 1991). Translational efficiency was further improved by positioning the starting AUG in a favourable context for translation initiation (Kozak 1987). A vector (pspUTK) was constructed, previously, which contained a 5′ UTR (termed UTK), with both of the above elements for efficient translation initiation along with a multiple cloning site 3′ of the UTK leader. While the UTK leader was found to be effective in cell-free translations it had not been rigorously examined in an *in vivo* system. Therefore the effect of the UTK 5′ leader was tested in the oocyte translation system and the results were consistent with the *in vitro* data (chapter III-1).

In Xenopus oocytes, as opposed to cell free translation systems, the stability of the mRNA must also be considered (Nudel et al. 1976; Kreig and Melton, 1984). Functional expression of foreign proteins in oocytes occurs over several hours to days. Therefore increased stability of the messenger RNA would increase the levels of protein expressed. The expression of bovine prolactin in Xenopus oocytes was found previously to be stable over several days. The half-life of mRNA molecules in oocytes is on the order of several hours and considerable destabilization is observed after 24 hours (Nudel et al. 1976; Kreig and Melton 1984; Drummond et al. 1985), suggesting the prolactin RNA has a

stabilization sequence. Based on previous studies on mRNA stability we investigated the effects of the prolactin 3′ UTR on the functional stability of *in vitro* synthesized transcripts in Xenopus oocytes. The 3′ UTR of prolactin was transferred to an unrelated coding region to test whether the 3′ UTR could stabilize RNA independent of the coding region. The prolactin 3′ UTR was found to be responsible for stabilizing prolactin transcripts and the stabilization effect was transferable to SRPRα, an unrelated coding region. These experiments suggest that this sequence may be useful as a general mRNA stabilization sequence. These results are presented in chapter III-2.

A construct containing a cDNA encoding the insulin receptor in the UTK vector along with the 3′ UTR of prolactin was constructed and tested in both RRL and in Xenopus oocytes (chapter III-3). The constructs containing the UTK 5′ leader and the 3′ UTR of prolactin were translated better than other constructs in both RRL and Xenopus oocytes (chapter III-3).

We have also observed dramatic effects, on translational efficiency, due to differences within the coding region. We examined more closely the effects of the coding region of SRPRα on translational efficiency in RRL and Xenopus oocytes (Appendix B). These effects could not be attributed to codon usage or the length of the coding region. The mechanisms by which the coding region affects translation are not obvious, therefore optimization of the insulin receptor

coding region, for translation, is not feasible at this time. In the future, once the effects of the coding region are better understood, it may be possible to optimize the insulin receptor coding region as well as the 5′ and 3′ UTR's.

This chapter is divided into 3 sections. Chapter III-1 and III-2 deal with the 5′ UTR and 3′ UTR and the effects of each on translational efficiency in cell-free systems as well as in Xenopus oocytes. Chapter III-3 describes the results of experiments designed to improve functional expression of the insulin receptor in RRL and Xenopus oocytes. The effects of the coding region were examined at the same time as the 5′ and 3′ UTR's (Appendix B).

III-ii
MATERIALS AND METHODS
(CHAPTER III AND APPB)
MATERIALS

Adult female breeding Xenopus laevis were purchased from Boreal (St. Catherines Ontario Canada). Molecular biology reagents, restriction enzymes, *in vitro* transcription and translation reagents and sequencing kits were from New England Biolabs, Pharmacia and Cedarlane laboratories and used according to manufacturers instructions. ^{35}S methionine was from Dupont-New England Nuclear. Protease inhibitors were from Boerhinger Mannheim and Sigma. All other reagents were from BDH or Baker Chemicals.

The insulin receptor clone was a kind gift from A. Ullrich. All other plasmids have been described previously (Falcone and Andrews, 1991). Sutter borosilicate micropipettes (I.D. 0.5, O.D. 1.0) were pulled on a Sutter instruments K.T. Brown Type Puller, and bevelled using a Sutter K.T. Brown Type beveller, Model BV-10. Oocytes were microinjected using a Sutter, Model NA-1, injection system.

OOCYTE ISOLATION

Adult female X.laevis were obtained from Boreal, St. Catherines. Ovarian fragments were surgically removed from anaesthetized females. The oocytes were manually dissected from the ovarian fragment and stored until use in ND96 media supplemented with pen/strep (100 u/ml each) and gentamycin (0.01 mg/ml) at 19 C.
ND96 contains 96mM NaCl, 2mM KCl, 1mM $MgCl_2$, 1mM $CaCl_2$, 5 mM Hepes, pH 7.6.

SEQUENCING

The constructs were sequenced using NEB vent polymerase sequencing system with instructions according to the manufacturer.

TRANSCRIPTS - SP6 Polymerase Reaction and Normalization of RNA by Fluorometry

All plasmids were linearized using a restriction site in the polylinker at the 3′ end of the 3′ UTR. All the ends produced were 5′ overhangs or blunt ends produced by a Klenow reaction. This is necessary when producing transcripts for injection into oocytes as circular or 3′ overhangs result in anomalous transcription products which produce anomolous translation products (M.T. Andrews, 1989). The problem of 3′ overhangs results in only a small percentange of incorrect transcription and translation products (5% or less). As a

result, even if the Klenow reaction is incomplete there would be an insignificant population of artifacts. The first available unique site was used to reduce effects of vector sequences on normalization and translation. All linearized DNA was run on 1 % agarose gels and stained with ethidium bromide to check for complete digestion. Restriction sites within the prolactin 3′ UTR were also used to produce transcripts containing different portions of the 3′ UTR.

SP6 polymerase reactions were as described previously in Gurevich et al. (1991) except for the replacement of DTT in the reaction with glutathione buffer, 50 mM reduced glutathione, 10 mM oxidized glutathione and 20 mM Hepes pH 7.5. Capped transcript was produced for all the oocyte injections.

Transcripts were normalized using fluorometry as described by Falcone and Andrews (1991). In most cases only transcripts containing the same coding region were normalized against each other to avoid fluorescence differences resulting from coding region secondary structure and not RNA concentration.

TRANSLATION - Reticulocyte lysate (RRL), wheat germ extract (WG) and Xenopus oocytes.

All RRL and WG translations, including transcript functional stability in RRL, were as described previously (Andrews et al., 1989; Falcone and Andrews, 1991). In all experiments RRL and WG translations were incubated for 1 hour and performed in duplicate for each transcript. All RRL experiments were repeated at least 3 times. 1-2 μl of translation product was run on SDS-PAGE using the tris-tricine buffer system (Schagger and Von Jagow, 1987). The gels were fluorographed using PPO, dried and exposed to film. Rate of translation initiation in RRL was determined as described in Falcone and Andrews (1991).

Translational efficiency in Xenopus oocytes was determined by microinjection of *in vitro* synthesized transcription products. 50 nl of normalized SP6 transcription product:^{35}S methionine (50:50) was microinjected per oocyte. 30-40 oocytes were injected per transcript. Oocytes were incubated, in ND96, 4 hours for prolactin and for 6 hours for SRPRα unless otherwise indicated. After the incubation sets of 5-10 (as indicated) healthy oocytes were homogenized and labelled protein was recovered by immunoprecipitation. Immunoprecipitation of oocyte homogenates was as described by Simon et al. (1987). Immunoprecipitation of SRPRα translation products was modified slightly, using SuperSWB (1.5% triton X-100, 500 mM NaCl, 100 mM tris-Cl pH 8, 10 mM EDTA), including protein G agarose, and pelleting the protein A/G agarose coupled to the antibody/antigen over a 0.5 M sucrose cushion before the washes. Antibodies were USB anti-ovine prolactin antisera, Oncogene Science AB-1 anti-insulin receptor

monoclonal antibody and Sparky anti-SRPRα monoclonal antibody described previously (Tajima et al., 1986). Protein A coupled to agarose was purchased from BIORAD and protein G coupled to agarose from USB. Immunoprecipitation products were separated on SDS-PAGE using the tris-tricine buffer system, fluorographed with PPO and exposed to film (Schagger and Von Jagow, 1987) At least 2 complete sets, i.e. each transcript done in duplicate, were produced per experiment to ensure consistent results.

To test translational competence, or RNA functional stability, over time, transcript was injected without added ^{35}S methionine. 40-50 oocytes were injected per transcript, 50 nl per oocyte and then incubated in ND96. At the times indicated in the figure legends 5-10 healthy oocytes were placed in 0.5 ml ND96:^{35}S methionine (0.05mCi/ml) and incubated 4-5 hours for prolactin and 6 hours for SRPRα clones. After the incubation the labelled oocytes were frozen in liquid N_2 and stored at -70°C until all the time points were complete. Prolactin and SRPRα proteins were immunoprecipitated from oocyte hommogenates as described above.

TRANSCRIPT STABILITY IN RRL

Transcripts were radiolabelled during transcription with ^{35}S-α-UTP. Labelled transcripts were translated in RRL reactions as above but omitting ^{35}S methionine. 4μl of the translation reaction was sampled at 0, 15, 30, 45, and 60 minutes, filtered through glass microfibre filter (Whatman glassmicrofibre GF/C filters, 2.4 cm) and washed extensively to remove free NTPs. The radioactivity bound on the filters was quantified by scintillation counting and the results plotted as intact mRNA (CPM) vs time (min). These experiments were performed by D. FALCONE

QUANTIFICATION OF PROTEIN SYNTHESIS

The relative amount of protein synthesis was quantified by densitometric scanning of the fluorograms using a Hoefer Scientific GS300 scanning densitometer. In all cases only full length products were considered as translated. When comparing the translation of the same coding regions synthesis is given as O.D. When comparing different coding regions the O.D. was corrected for the number of methionines.

III-1-i
ABSTRACT

The following is the first of three sections in which the problem of insulin receptor expression is addressed. In this section, the effect of the 5′ untranslated region on expression of cloned genes in both reticulocyte lysate and Xenopus oocytes is examined.

III-1-ii
INTRODUCTION

It has been shown previously in our laboratory that the 5′ untranslated region of the Xenopus ß-globin gene when placed at the 5′ end of a variety of cloned genes increased the rate of translation initiation several fold *in vitro* (Falcone and Andrews, 1991). The Xenopus ß-globin 5′ UTR contains regions of homology to both eukaryotic viral [picornovirus polypyrimidine stretch, TTTTCCTTT(E. Lovinsky, 4B6 project)] and prokaryotic [T7 bacteriophage gene 10 leader, AGAAAATA and TTAACTTTA (E. Lovinsky, 4B6 project)] 5′ regions (Elroy-Stein et al., 1989; Olins et al., 1988 and 1989). In the original contexts these sequences have been shown to improve translational efficiency (Elroy-Stein et al., 1989; Olins et al., 1988 and 1989).

In addition to the 5′ UTR the initiation sequence also contributes to translational efficiency. For most coding regions tested, maximum translational efficiency was obtained using both the Xenopus ß-globin 5′ UTR and a good consensus sequence for translation initiation (Falcone and Andrews, 1991). The consensus sequence for efficient initiation at the correct start site (ACCAUGG) was determined by analysis of the 5′ regions of several hundred eukaryotic genes (Kozak, 1987). Analysis of this region showed that it is a consensus sequence for efficient translation initiation (Kozak, 1986). The scanning model for translation initiation has been proposed to explain the role of this consensus sequence in translation

initiation (reviewed in Kozak, 1989). The scanning model proposes that the translation machinery binds to the CAP site at the extreme 5′ end of the mRNA and then moves along the RNA until an AUG in proper context is encountered. Studies examining the effects of secondary structure in the 5′ UTR on translation initiation as well as CAP dependence support this model (reviewed in Kozak, 1989). While there is some data to support the scanning model for translation initiation there is growing evidence that the mechanism is more complicated. For example, the observed CAP- independent initiation of mRNA containing picornovirus 5′ leader sequences does not support the scanning model (Elroy-Stein et al., 1989). Furthermore, studies using picornovirus leaders, sendai viral leaders and some cellular mRNA's have demonstrated that ribosomes can initiate by entering the RNA at an internal site in the 5′ UTR (Pelletier and Sonnenberg, 1988; Curran and Kolakofsky, 1989; Macejak and Sarnow, 1991). The sequences responsible for this observation are found within the 5′ leader and may represent either direct sites for ribosome binding or a binding site for a protein involved in recruiting the ribosome (reviewed in Jackson et al., 1990; Jackson, 1990; Merrick, 1990).

While the mechanism(s) of the enhanced rate of translation initiation is(are) unknown we can use the above information to improve expression of proteins of interest *in vitro* and *in vivo*. Here we report the results of experiments examining the effects of the 5′ UTR in oocytes using some of

the constructs from a previous study *in vitro* (Falcone and Andrews, 1991). These results demonsrate for the first time that the UTK leader sequence is effective in increasing expression in an *in vivo* translation system.

III-1-iii
RESULTS AND DISCUSSION

ROLE OF THE 5′ UTR ON TRANSLATION IN XENOPUS OOCYTES

To extend the results obtained *in vitro* to *in vivo*, the effects of the Xenopus ß-globin 5′ UTR and Kozak consensus sequence on translation were tested in the oocyte. As shown in Figures 1a and 1b the results obtained are consistent with the cell-free data. A summary of each clone and 5′ leader sequence is presented in Table 1. Results of densitometric scanning of the fluorograms from 2 independent experiments, one of which was done in quadruplicate, are presented in Figure 2. A construct of P_T, a deletion mutant of prolactin, containing the Xenopus ß-globin 5′ UTR (UTR) was translated 3-4 fold better than the SD construct (Figure 2 compare UTR and SD). The construct containing an optimal Kozak consensus sequence, ACCATGG, was also found to increase translation efficiency 3-4 fold (Figure 2 compare SD and KD). When both of these elements are included in the 5′ region (UTK) the effect was found to be additive, with a 7-8 fold increase in translation (Figure 2, compare UTK, UTR, KD, and SD). These results suggest that the two elements are acting independently to increase translational efficiency.

TABLE 1

CONSTRUCTS

MADE BY OR REF.	5'UTR	CODING REGION	3'UTR	MAC PLASMID
D.FALCONE D.W. ANDREWS (1991)	UTK	PT BOVINE PREPROLACTIN 3' DELETION MUTANT (amino acids 2-57 after signal sequence)	PL 3'	106
"	UTR	"	"	120
"	KD	"	"	156
"	SD	"	"	157
D.FALCONE	EMC	"	"	266
D.W.ANDREWS	UTE/K	SRPRalpha (FULL LENGTH)	SRPR 3'	1
	UTE/UTK	"	"	13
D.FALCONE	UTK	"	"	191
GIFT FROM A.ULLRICH	UTR + CCCATGG	INSULIN RECEPTOR	IR 3'	65
A.VASSIL.	UTK	"	IR 3'	222
A.VASSIL.	UTK	"	PL 3'	475

Leader sequence
SD GAATACAAGCTCATGG
KD GAATACAAGCTCATCTACCATGG
UTR GAATACAAGCTTGCTTGTTCTTTTTGCAGAAGCTCAGAATAAACGCTCAACTTTGGCAGATCCATGG
UTK GAATACAAGCTTGCTTGTTCTTTTTGCAGAAGCTCAGAATAAACGCTCAACTTTGGCAGATCTACCATGG

TABLE 1
The 5′ UTR, coding region and 3′ UTR for each construct. Each construct is referenced to the researcher responsible for constructing this version of the prolactin, SRPRα or insulin receptor clones. The SD, UTR, KD, and UTK leader sequences are given below the table. UTE/K SRPRα contains the endogenous SRPRα 5′ UTR and a Kozak consensus initiation site. UTE/UTK has the Xenopus ß-globin 5′ UTR uptream of 35 nucleotides of the endogenous SRPRα 5′ UTR as well as the Kozak consensus sequence. EMC is the 5′ leader sequence derived from the EMC piconovirus (see reference in text).

FIGURE 1a and 1b
EFFECT OF 5′ UTR ON TRANSLATIONAL EFFICIENCY IN XENOPUS OOCYTES - P_T SYNTHESIS

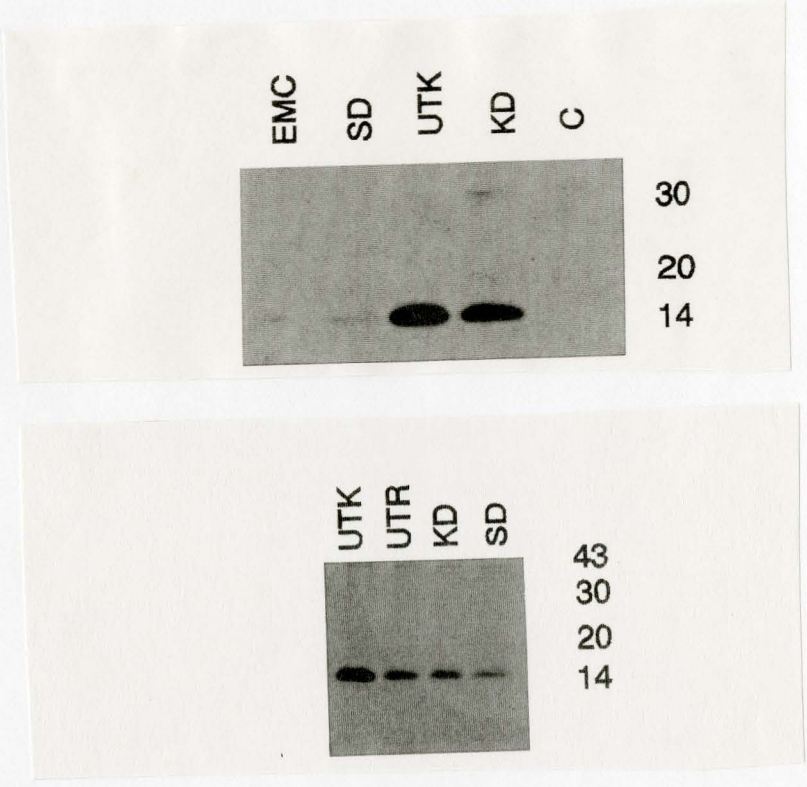


FIGURE 1a and 1b
EFFECT OF 5′ UTR ON TRANSLATIONAL EFFICIENCY IN XENOPUS OOCYTES - P_T SYNTHESIS

In vitro synthesized transcripts were normalized by fluorometry and subsequently injected into Xenopus oocytes in a 1:1 ratio of transcription product to ^{35}S methionine (1 mCi/1 ml). Oocytes were incubated for 4 hours after which the P_T translation products were immunoprecipitated and separated on SDS-PAGE gels. The 5′ leader region is indicated above each lane. UTK, UTR, KD and SD leader sequences are given in Table 1. Oocytes in lane C were injected with ^{35}S methionine alone as a control.

FIGURE 2
QUANTIFICATION OF THE EFFECT OF 5′ UTR ON TRANSLATIONAL EFFICIENCY - P_T SYNTHESIS

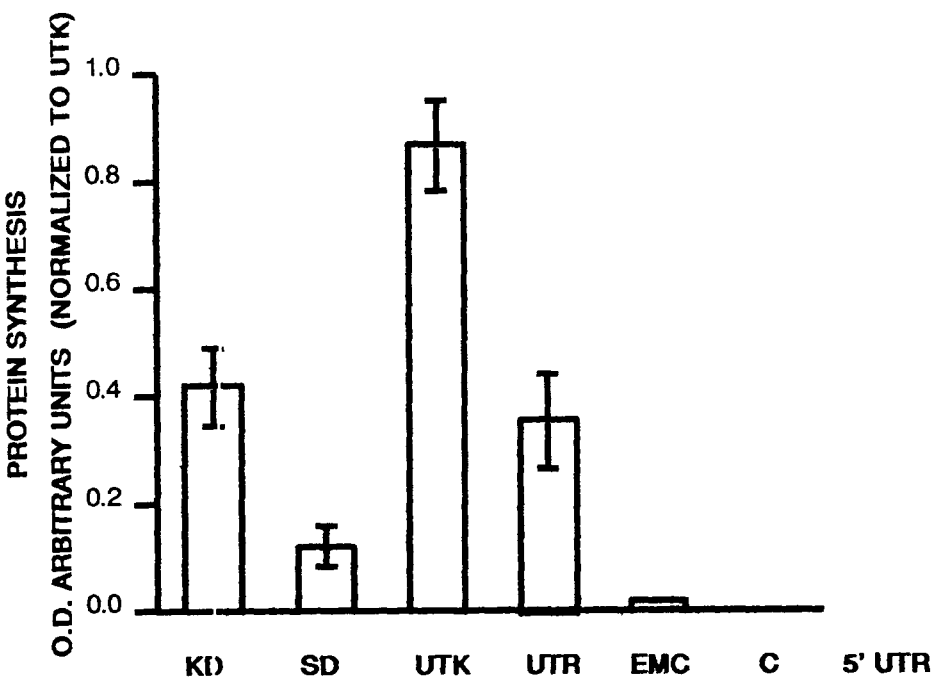


FIGURE 2
QUANTIFICATION OF THE EFFECT OF 5′ UTR ON TRANSLATIONAL EFFICIENCY - P_T SYNTHESIS
The effects of the 5′ UTR on translational efficiency in the Xenopus oocyte were quantified by densitometric scanning of the fluorograms from experiments identical to Figure 1. Relative protein synthesis was measured as O.D.(arbitrary units) and resulting data normalized to UTK. The densitometry data are from two experiments, one done in quadruplicate.

The observation that regions in the 5′ UTR increase translation initiation, independently of the consensus sequence for efficient initiation, is not predicted by the scanning model for initiation (reviewed in Kozak, 1989). While the model does suggest that secondary structure in the 5′ region may inhibit translation initiation by preventing movement of the preinitiation complex along the RNA it does not allow for regions of the 5′ UTR being involved in enhancing initiation. The ability of the Xenopus 5′ UTR to increase translational efficiency in such diverse systems as rabbit reticulocyte lysate, wheat germ extract and Xenopus oocytes suggests this region acts as a general enhancer of eukaryotic translation initiation. For this reason the Xenopus 5′ UTR provides a useful tool for increasing the translation of almost any cloned gene, both *in vitro* and *in vivo*.

The EMC (encephalomyocarditis) viral 5′ leader has also been found to improve translation both *in vivo* and in cell-free systems (Pelham, 1978; Svitkin et al., 1978; Elroy-Stein et al., 1989). The possibility that the mechanism by which the Xenopus ß-globin 5′ UTR enhances translation initiation is similar to eukaryotic viral leaders is presently being examined in our laboratory. While the EMC and Xenopus leaders share homology, in a polypyrimidine stretch, they are not equally effective in increasing expression in oocytes (Figure 1b and Figure 2 compare EMC, SD and UTK). This has

been observed for other viral leaders as well and may be due to requirements for species specific host translation factors (Gallie et al., 1987b).

To establish that the UTK leader is effective in improving expression of at least one more coding region in the oocyte three constructs of the SRP-receptor alpha subunit (SRPRα), containing different 5′ UTRs, were expressed in the oocyte. These constructs were convenient choices since antisera against the translation products were available in the laboratory. The expression of SRPRα was also improved 4-5 fold by cloning the coding region into the UTK vector [Figure 3 lanes E/K(cognate 5′ leader with Kozak consensus sequence), UTK/E(Xenopus β-globin 5′ UTR inserted 5′ of 35 nt of SRPRα cognate 5′ UTR) and UTK]. While the optimal 5′ leader sequence was UTK the hybrid UTK/E improved translation 2-3 X over E/K. This suggests that the position of the Xenopus beta globin 5′ UTR does not have to be an exact match to the initiating AUG to be effective in enhancing translation initiation.

FIGURE 3
EFFECT OF 5′ UTR ON SRPRα SYNTHESIS

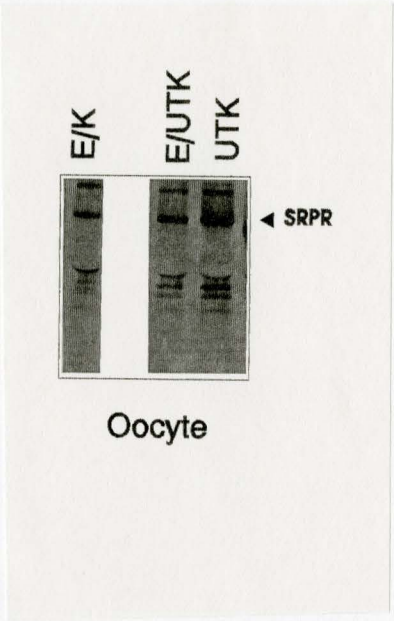


FIGURE 3
EFFECT OF 5′ UTR ON SRPRα SYNTHESIS
The effect of replacing the 5′ UTR of SRPRα with the 5′ UTR of Xenopus beta globin, on translational efficiency in Xenopus oocytes. Oocytes were injected with normalized *in vitro* synthesized transcript in a 1:1 mix with ^{35}S methionine and incubated for 6 hours. SRPRα translation products were immunoprecipitated and separated on SDS-PAGE. The leader region is indicated above each lane. E/UTK is a hybrid leader containing the Xenopus beta globin 5′ UTR and 35 nucleotides of the SRPRα 5′ UTR.

Based on these results the coding region of the insulin receptor was inserted into the UTK vector and receptor expression tested in both RRL and in Xenopus oocytes (see section III-3).

III-1-iv
CONCLUSION

The UTK leader sequence, shown previously to increase expression in cell-free translation systems, was tested in the Xenopus oocyte. The results in the oocyte were consistent with those in RRL and WG extract suggesting that the UTK leader can be used to increase expression of the insulin receptor in cell-free translations as well as in Xenopus oocytes.

III-2-i
ABSTRACT

In the previous section the effect of the 5′ UTR on translation in RRL and Xenopus oocytes was examined in an attempt to improve expression of the insulin receptor in cell free and oocyte translations. What follows, in this section, is an examination of the role of the 3′ UTR. The results suggest that addition of the Sac I-Sph I fragment of the prolactin 3′ UTR followed by 30-50 A's at the 3′ end of the insulin receptor construct will result in insulin receptor transcripts which, when injected into Xenopus oocytes, will be functionally stable for several days.

III-2-ii

INTRODUCTION

One of the factors that determines the level of expression of a gene is the abundance of the corresponding mRNA molecule. The level of mRNA is determined by the rate at which the gene is transcribed, the rate of processing of the transcript to produce a mature mRNA and finally the half-life of the mRNA once it is transported to the cytosol (reviewed in Brawerman, 1989; Jackson and Standart, 1990; Saini et al., 1990). Because RNA is injected directly into Xenopus oocytes the only parameter relevant to expression of *in vitro* synthesized insulin receptor in Xenopus oocytes is the rate of degradation of the RNA. The sequences responsible for determining the half-life (i.e. stability) of a given mRNA (or *in vitro* synthesized transcript) have been localized predominantly to the 3′ untranslated region of the molecule (reviewed in Jackson and Standart, 1990). There is considerable evidence that the poly A tail, at the 3′ end of the 3′ UTR of most mRNAs, is involved in preventing mRNA degradation and may also influence translation initiation (reviewed in Bernstein and Ross, 1989). The tail is added post transcriptionally to the 3′ end of an mRNA containing an AAUAAA signal found within the 3′ UTR (reviewed in Wickens 1990, also Sheets et al. 1990, Bienroth et al. 1993). A poly A tail, 30 nucleotides in length, was found to be as effective as longer tails in preventing degradation of RNA microinjected

into oocytes (Nudel et al. 1976).

Both the stability of the mRNA molecule and the functional stability (protein synthesis) were improved by the presence of the poly A tail (Nudel et al. 1976). mRNAs containing poly A tails 16 nucleotides in length were found to be as functionally unstable as completely deadenylated RNA, suggesting a minimum length requirement for poly A function (Nudel et al. 1976). Since many eukaryotic mRNAs are polyadenylated to some extent it seems likely that other sequences in the RNA molecule are responsible for the vast differences in stability between different mRNA species (Ahron and Schneider, 1993 and references within). This is supported by the discovery of sequences which increase or decrease the rates of deadenylation and degradation of adenylated and deadenylated RNA (reviewed in Klausner and Harford., 1989; Ratnasabapathy et al., 1990; Laird-Offringa et al., 1990; Saini et al., 1990; Saini and Summerhayes, 1991; Aharon and Schneider, 1993). In general these sequences are found in the non-poly A untranslated sequences 3′ of the stop codon but there have been reports of 5′ UTR sequences which are involved in RNA stability (reviewed in Saini et al., 1990).

The Xenopus oocyte has been a useful model system for determining the stability of *in vitro* synthesized mRNA (Kreig and Melton, 1984). Transcript can be radioactively labelled during *in vitro* transcription of a cloned gene. This can then be injected into oocytes and the molecular stability of the

RNA can be determined by extracting the RNA at different times post injection (Drummond et al., 1985). However, the stability of the RNA molecule does not always correlate with the ability of the RNA to be translated over time (Drummond et al., 1985; Galili et al., 1988). Therefore it is more useful to monitor the functional stability of an RNA in the oocyte by following new protein synthesis by incorporation of ^{35}S methionine. Functional stability is the most important variable when attempting to express insulin receptors, both wild type and mutant, in Xenopus oocytes.

The preprolactin transcript is functionally stable for up to several days in Xenopus oocytes (D.W. Andrews, submitted). Here we report the results of experiments designed to characterize the sequences responsible for the observed stability and to test the transferability of the 3′ UTR (untranslated region) to other genes.

III-2-iii

RESULTS AND DISCUSSION

PLASMIDS

The constructs used in the following experiments are summarized in Table 1. The predicted sequence of the junction at the 3′ end of the coding region and the 3′ UTR was verified for each clone by sequencing. The complete preprolactin 3′ UTR was sequenced and found to contain a stretch of 17 A and 11 T residues at the end of the prolatin 3′ UTR sequence. Unique sites within the 3′ UTR of prolactin are indicted in Table 1. These sites were used to produce transcripts with truncated prolactin 3′ UTRs.

TABLE 1

CONSTRUCTS

MADE BY OR REF.	5'UTR	CODING REGION	3'UTR	MAC PLASMID
D.FALCONE/ A.VASSIL.	UTK	BOVINE PREPROLACTIN	PROL 3'/A17T11	274
"	UTK	"	XENOPUS BETA GLOBIN 3' UTR (Kreig and Melton 1984)	253
"	UTK	"	Poly A30 (Promega/ psp64polyA)	250
D.W.ANDREWS	UTR SRPRalpha	MUTANT (termination codon at n: 1300)	Coding region past stop + endogenous 3'	74
D.FALCONE	UTR	"	PROLACTIN 3' inserted after the stop at 1300	446

PROLACTIN 3' UTR SEQUENCE

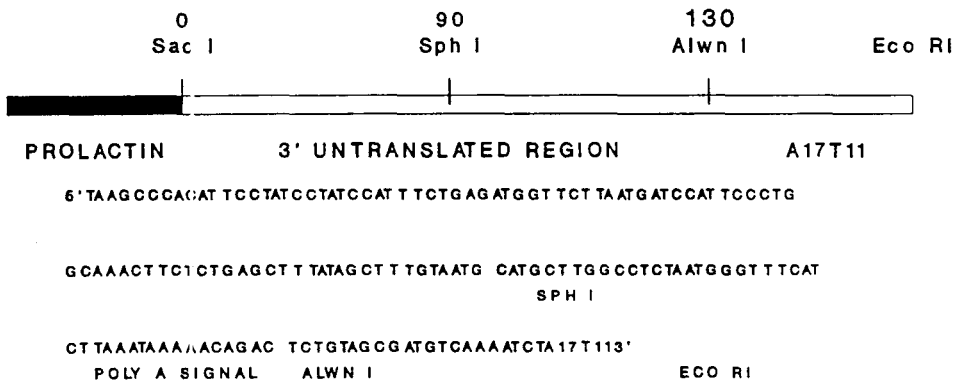


TABLE 1
CONSTRUCTS
Table one summarizes the 5′ UTR, coding region and 3′ UTR for each construct. Unique restriction sites in the 3′ UTR of prolactin are indicated. Each construct is referenced to the researcher responsible for constructing this version of the prolactin or SRPRα clones.

EFFECT OF THE 3′ UTR IN RRL TRANSLATION

Previously, Domina Falcone in our laboratory was examining the effects of the 5′ and 3′ UTR's on translational efficiency in RRL an WG extracts. Figure 1 was plotted from her unpublished data (with permission).

The sequence 3′ of the coding region does not appear to have an effect on the expression of RNA *in vitro* translation reactions (D. Falcone unpublished data, Kreig and Melton 1984). The results of experiments to test the stability of *in vitro* synthesized RNA in reticulocyte lysate translation reactions are presented in Figure 1. In these experiments, the stability of src and prolactin transcripts containing different 3′ regions was tested in reticulocyte lysate translation reactions. The 3′ UTR had little if any effect on both the functional and molecular stability of the src transcript (Figure 1a and 1b respectively). Functional stability was determined by incubation of transcript in a RRL reaction for a given time prior to addition of ^{35}S methionine and comparing the amounts of labelled full length product made after a 1 hour incubation. Molecular stability was determined by incubation of ^{35}S UTP labelled transcript in RRL reactions not containing methionine label and subsequently examining the amount of RNA bound to nylon filters (D. Falcone, unpublished data). It is obvious from these data that cell-free translation systems are not suitable for RNA stability studies.

FIGURE 1a and 1b
FUNCTIONAL STABILITY OF PROLACTIN AND SRC TRANSCRIPTS IN RRL

FIGURE 1a

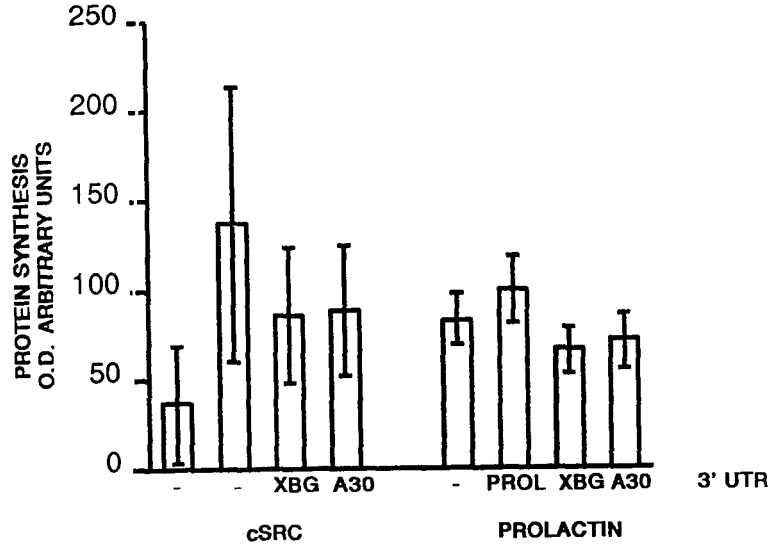


FIGURE 1b

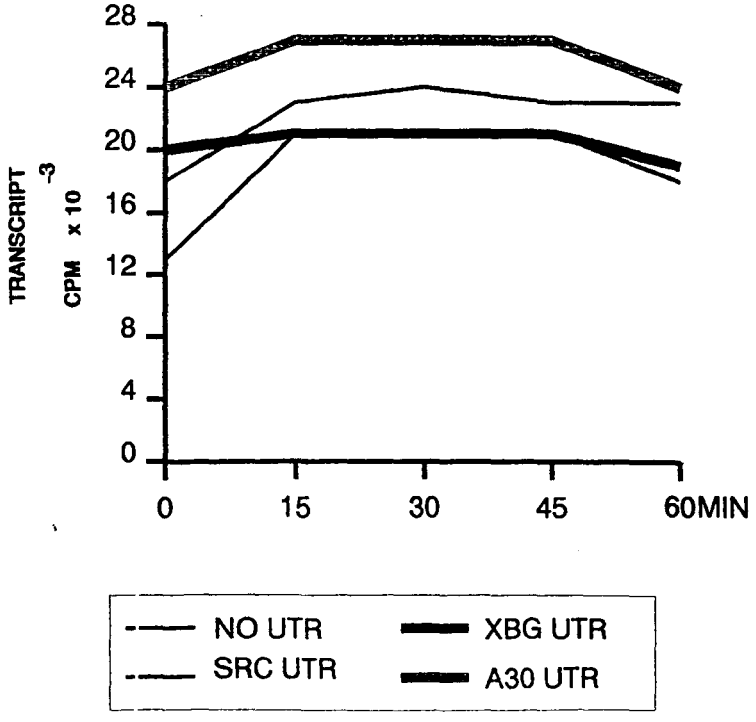


FIGURE 1a
FUNCTIONAL STABILITY OF PROLACTIN AND SRC TRANSCRIPTS IN RRL
The effect of the 3′ UTR on functional stability in RRL is shown. cSRC and preprolactin constructs containing no UTR (-), Xenopus ß-globin 3′ UTR (XBG), poly A30 (A30) or prolactin 3′ UTR (PROL) were transcribed *in vitro*, normalized by fluorometry and incubated in RRL reactions for 30 minutes without ^{35}S methionine label. After 30 minutes ^{35}S methionine was added and reactions incubated for an additional hour. Translation products were separated by SDS-PAGE and the gels fluorographed, dried and exposed to film. Synthesis of full length protein was quantified by densitometric scanning of the fluorograms and results plotted as synthesis vs 3′ UTR. Data provided by MINA FALCONE

FIGURE 1b
MOLECULAR STABILITY OF SRC TRANSCRIPTS IN RRL
The effect of the 3′ UTR on transcript stability in RRL is shown. cSRC constructs containing no 3′ UTR (NO UTR), Xenopus ß-globin 3′ UTR (XBG UTR), cSRC partial 3′ UTR containing only approximately 20 nt (cSRC UTR) or poly A30 (A30 UTR) were transcribed *in vitro* in the presence of ^{35}S-alpha-UTP. Labelled transcript was translated in RRL not containing ^{35}S methionine. At the times indicated samples were removed and RNA content determined by binding of intact RNA to glass filters, washing to remove free nucleotides and quantifying by scintillation counting. Data provided by MINA FALCONE

EFFECT OF THE 3′ UTR ON TRANSLATIONAL EFFICIENCY IN XENOPUS OOCYTES

Before attempting to dissect the regions of the prolactin 3′ UTR involved in stabilization of RNA, the effect of the 3′ region on translational efficiency was determined. If the 3′ UTR is involved in translation efficiency, results from experiments to test RNA stability may be difficult to interpret. The translational efficiencies of prolactin transcripts with different 3′ UTR sequences (polyA$_{30}$, Xenopus ß-globin 3′ UTR and prolactin 3′ UTR) were tested both in reticulocyte lysate and in Xenopus oocytes. The concentration of each transcript was normalized by fluorometry and translated in a one hour reticulocyte translation reaction and/or injected into oocytes and incubated for 4 hours. The amount of transcript injected into the oocyte is not likely to be saturating since the level of expression observed in this experiment was not maximal (unpublished observation - in other experiments, using considerably higher concentrations of transcript, translation is not saturated). In both cases ^{35}S methionine was included in the reactions to label the translation products. Total prolactin synthesis was determined by separating total reticulocyte products and immunoprecipitation products from oocytes on SDS-PAGE, followed by fluorography and densitometric scanning of the fluorograms (Figure 2 - oocyte data only). In both the one hour reticulocyte translation and 4 hour oocyte translation,

prolactin synthesis was not affected by the 3′ untranslated region.

FIGURE 2
TRANSLATIONAL EFFICIENCY OF PREPROLACTIN CONSTRUCTS CONTAINING DIFFERENT 3′ UTR SEQUENCES

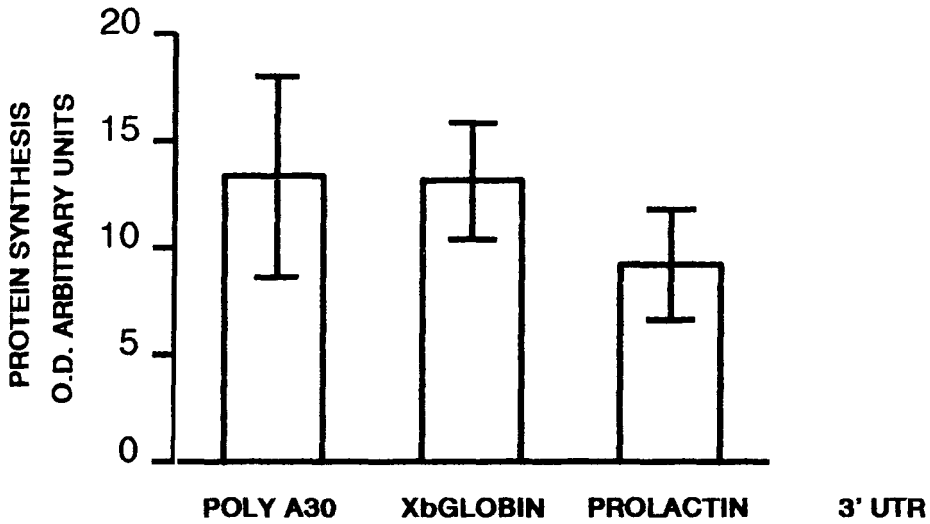


FIGURE 2
Translational efficiency of preprolactin constructs containing different 3′ UTRs in Xenopus oocytes. Oocytes were injected with equal amounts of *in vitro* synthesized transcript in a 1:1 mix with ^{35}S methionine and incubated for 4 hours. Prolactin translation products were isolated by immunoprecipitation, separated on SDS- PAGE, and the gels fluorographed and exposed to film. Synthesis of full length protein was quantified by densitometric scanning of the fluorogram and results plotted as synthesis vs 3′ UTR. The results are from one experiment performed in triplicate except poly A_{30} which was in duplicate.

The above results suggest that the 3′ UTR does not infuence the rate of translation initiation or elongation.

Furthermore, while cell-free translation systems are useful for a variety of translational studies they are not suitable for observing differences in RNA stability. In a relatively short translation incubation, such as the reticulocyte lysate system, the effects of known RNA stabilization signals, such as the Xenopus ß-globin 3′ UTR, are not observed (Kreig and Melton 1984b; and Figure 1a and 1b).

EFFECT OF THE PROLACTIN 3′ UTR ON RNA FUNCTIONAL STABILITY IN XENOPUS OOCYTES

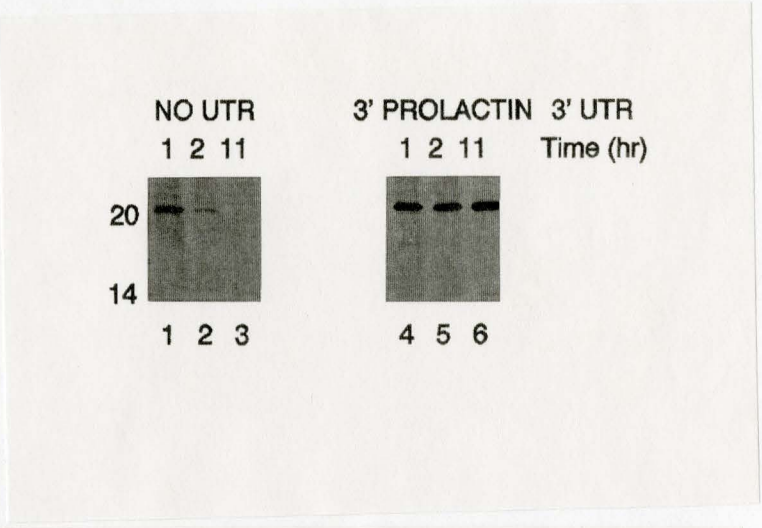
To characterize the region of the prolactin transcript responsible for the previously observed functional stability (D. Andrews unpublished data), several plasmids were constructed, all with different sequences 3′ of the stop codon (table 1). A SacI restriction site was introduced immediately after the stop codon to facilitate the construction of the various clones. Linearizing the clone at this site also provided a prolactin transcri
pt with no 3′ UTR region.

The functional stability of prolactin transcripts with or without the 3′ UTR was tested in the oocyte (Figure 3a). Oocytes were pulse labelled for 4-5 hours, as indicated, with ^{35}S methionine at different times post injection of the

transcript. Therefore, labelled proteins will be isolated only if the RNA is still competent for translation at the time of the pulse. Removal of the 3′ UTR dramatically decreased the functional stability of the transcript in oocytes. By 2 hours post injection there was little prolactin synthesis and by 11 hours there was no synthesis (Figure 3a lanes 2 and 3). In contrast, transcript containing the 3′ UTR was stable for at least 11 hours after injection (Figure 3a lane 6).

FIGURE 3a/3b
FUNCTIONAL STABILITY OF PROLACTIN AND SRPRα TRANSCRIPTS IN XENOPUS OOCYTES

3a



3b

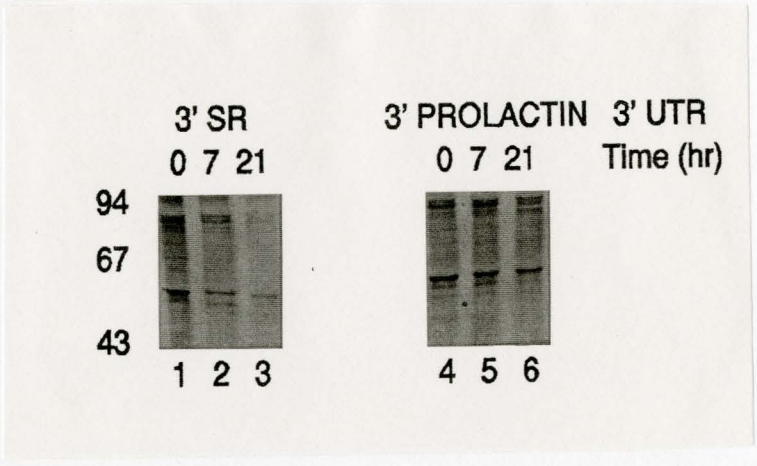


FIGURE 3a
FUNCTIONAL STABILITY OF PROLACTIN TRANSCRIPTS IN XENOPUS OOCYTES
The functional stability of preprolactin transcripts containing either no 3′ UTR (NO UTR) or the prolactin 3′ UTR (3′ PROLACTIN) is shown. *In vitro* synthesized transcript was injected into oocytes. 1, 2 and 11 hours after injection sets of 5 oocytes were removed and incubated for 5 hours in media containing ^{35}S methionine (0.5mCi/10ml). Prolactin translation products were immunoprecipitated, separated on SDS-PAGE and the gels fluorographed, dried and exposed to film.

FIGURE 3b
FUNCTIONAL STABILITY OF SRPRα TRANSCRIPTS IN XENOPUS OOCYTES
The functional stability of SRPRα transcripts containing either endogenous SRPRα 3′ UTR sequence (3′SR) or the prolactin 3′ UTR (3′ PROLACTIN) is shown. *In vitro* synthesized transcript was injected into oocytes. At the time of injection (0) and at 7 and 15 hours after injection sets of 10 oocytes were removed and incubated for 6 hours in media containing ^{35}S methionine (0.5mCi/10ml). SRPRα translation products were immunoprecipitated, separated by SDS-PAGE and the gels fluorographed, dried and exposed to film.

The above experiment suggests that the prolactin 3′ UTR is necessary for the prolactin transcript to be stable in oocytes. However, it does not show that the sequence is sufficient to stabilize RNA in general. In some cases the 3′ UTR has been found to stabilize a transcript by interacting with sequences in the 5′ UTR and/or the coding region (reviewed in Saini et al., 1990). In these cases the 3′ UTR is necessary but not sufficient for stabilization of foreign RNA. To test this the 3′ UTR of prolactin was added to a plasmid at the 3′ end of the coding region of an SRPRα(signal recognition particle receptor, α subunit) deletion mutant. Addition of the prolactin 3′ UTR stabilized the SRPRα transcript up to 20 hours (Figure 3b panel 1). The 3′ UTR of the control transcript contained both SRPRα coding sequences and endogenous 3′ UTR sequences. This transcript was relatively unstable (Figure 3b panel 2).

The polyA tract in the prolactin 3′ UTR is only 17 bases in length. Studies in oocytes have shown that a 16 base polyA tail is not sufficient to stabilize transcripts (Nudel et al., 1976). Furthermore, transcripts with 16 A residues were found to lose functional stability at the same rate as transcripts with no poly A tail (Nudel et al., 1978). The prolactin poly A tail is only 1 A longer than what was previously found to be ineffective for stabilization of transcripts in oocytes. This suggested the possibility that another sequence in the 3′ UTR was involved in stabilization

of the RNA either in conjunction with the poly A sequence or independently. Two unique restriction sites, SphI and AlwNI, in the cDNA of prolactin are not present in the region of the plasmid between the SP6 promoter and the stop codon. Digestion of the prolactin clone with either of these enzymes produced DNA which contained only part of the 3′ UTR of prolactin (table 1). The Sph I site is found 5′ of the AAUAAA, therefore digestion with Sph I removes the poly adenylation signal. The functional stability of prolactin transcripts made from this DNA was tested in the oocytes along with transcripts containing the full length prolactin 3′ UTR and the Xenopus ß-globin 3′ UTR. The full length prolactin 3′ UTR stabilized prolactin transcript as well as the Xenopus ß-globin sequence previously reported to stabilize RNA (Figure 4 panels 1, 2). The transcripts containing the truncated prolactin 3′ UTR were stable longer than the transcript containing no UTR sequences (Figure 3a panel 1 compared to Figure 4 panels 3 and 4). By 20 hours the amount of prolactin made had decreased by half for both truncated UTRs, suggesting a loss of functional transcript and a requirement for a poly A stretch for full stabilization (Figure 4).

FIGURE 4
FUNCTIONAL STABILITY OF PREPROLACTIN TRANSCRIPTS CONTAINING DELETIONS IN THE 3′ UTR

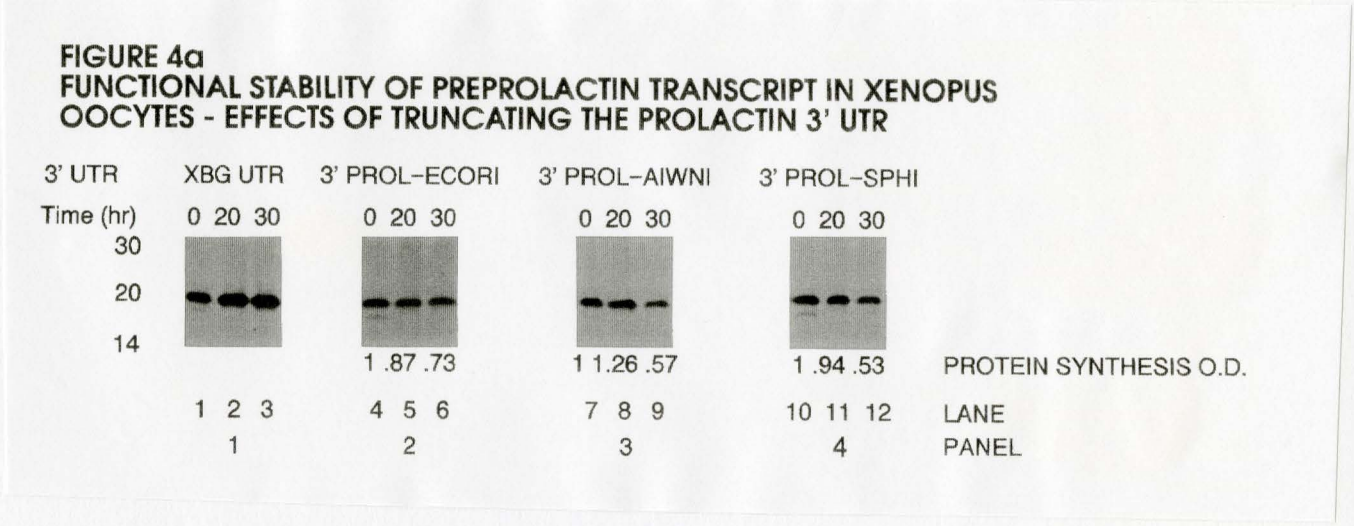


FIGURE 4
Functional stability of preprolactin transcripts containing different regions of the prolactin 3′ UTR. The different transcripts were produced by restriction digestion of the DNA within the 3′ UTR (3′PROL-ECORI/full length 3′ UTR, 3′PROL-ALWNI/130 nt of 3′ UTR, 3′PROL-SPHI/90 nt of 3′ UTR). Transcript was injected into oocytes and 0 (at time of injection), 20 and 30 hours after injection sets of 5 oocytes were removed and incubated for 5 hours in media containing ^{35}S methionine (0.5mCi/10ml). Prolactin translation products were analyzed after immunoprecipitation by SDS- PAGE and fluorography. The differences in functional stability, although small, were consistent in other experiments.

Removal of segments of the prolactin 3′ UTR did not result in a dramatic loss of stability as was observed when the entire region was deleted. This is not surprising given that 16 A residues, only one less than the deleted A stretch, was as unstable as fully deadenylated RNA. The first 90 bases (Sac I - Sph I) and regions downstream of this region may be acting either together or independently. The first 90 bases of the 3′ UTR are sufficient to stabilize the transcript, relative to transcripts without a 3" UTR, but may require interactions with downstream regions of the 3′ UTR for further stabilization. This possibility remains to be tested.

III-2-iv
CONCLUSION

The results presented here confirm that the 3′ UTR of prolactin contains sequences necessary and sufficient to stabilize mRNA in Xenopus oocytes. Given the above results we should be able to use the 3′ UTR of prolactin coupled to a poly A tail, 30-50 nt in length, to stabilize insulin receptor transcripts in Xenopus oocytes. In the next section (III-3), the effects of the prolactin 3′ UTR on the expression of insulin receptor in RRL and Xenopus oocytes is examined.

III-3-i
ABSTRACT

Sections III-1 and III-2 provide an experimental basis for optimizing the expression of cloned insulin receptor in Xenopus oocytes. Based on these results an insulin receptor construct containing the UTK 5′ leader sequence and the 3′ UTR of prolactin was made. The results of experiments to test expression of this construct are presented in this section.

III-3-ii
INTRODUCTION

A common feature in the upstream region of many hormone receptors, such as the insulin receptor, is a large number of G an C residues. These stretches have been shown to inhibit translation and are thought to be involved in the regulation of expression of the receptors *in vivo* (reviewed in Kozak, 1991). To avoid the effects of the cognate 5′ UTR the insulin receptor had been previously cloned into psp64T, a vector containing an SP6 transcription promoter and the Xenopus ß-globin 5 ′ untranslated region (UTR). Initial attempts to express this insulin receptor construct in cell free reticulocyte lysate (RRL) translation system were unsuccessful. The insulin receptor clone was sequenced to determine the exact sequence of the 5′ UTR that resulted from construction of the plasmid. Upon sequencing, it was discovered that the context of the start site had been altered to CCCATGG, thus the initiating AUG was not in a favourable context for initiation of translation.

Having established in section III-1 that the Xenopus ß-globin 5′ UTR improved translation in the oocytes as well as in cell lysates, an insulin receptor clone was constructed that had an optimized 5′ UTR (UTK leader). The construction of this clone was simplified by use of the pspUTK vector made by M. Falcone (Falcone and Andrews, 1991). Given the results in section III-1 addition of the UTK leader alone should increase the expression of the insulin receptor in the

oocytes. In section III-2 it was demonstrated that the 3′ UTR of prolactin contains a transferable mRNA stabilization sequence. These results along with the results of section III-1 suggest that a construct in which both the UTK leader and the prolactin 3′ UTR are added to the coding region for insulin receptor will increase expression in Xenopus oocytes. The results of expressing these constructs of the insulin receptor in RRL and in Xenopus oocytes are presented below.

III-3-iii

RESULTS AND DISCUSSION

SEQUENCING OF THE INSULIN RECEPTOR CONSTRUCTS

The 5′ UTR and start site of the original insulin receptor construct was sequenced by exo- vent polymerase reaction (used as specified by the manufacturer). The sequence surrounding the start site was CCCATGG (UTR-IR). Therefore the insulin receptor coding region was subcloned out of this vector into pspUTK (UTK-IR). A construct of the insulin receptor was also made containing both the UTK 5′ leader as well as the 3′ UTR of prolactin (UTK-IR-UTRPROL)

EXPRESSION OF THE INSULIN PRORECEPTOR IN RRL

Expression of three constructs encoding the insulin receptor were compared in RRL translation reactions (Figure 1). The 180 kDa proreceptor with the xenopus beta globin 5′ UTR and the Kozak consensus start site was expressed to higher levels than a similar construct without the Kozak consensus start site [Figure 4 compare UTR-IR (not quantifiable by densitometry) and UTK-IR]. As expected for a cell free expression system, the construct with both 5′UTK and 3′ UTR of prolactin was expressed as well as the 5′UTK construct (compare UTK-IR and UTK-IR-UTRPROL).

FIGURE 1
EXPRESSION OF INSULIN RECEPTOR CONSTRUCTS IN RRL

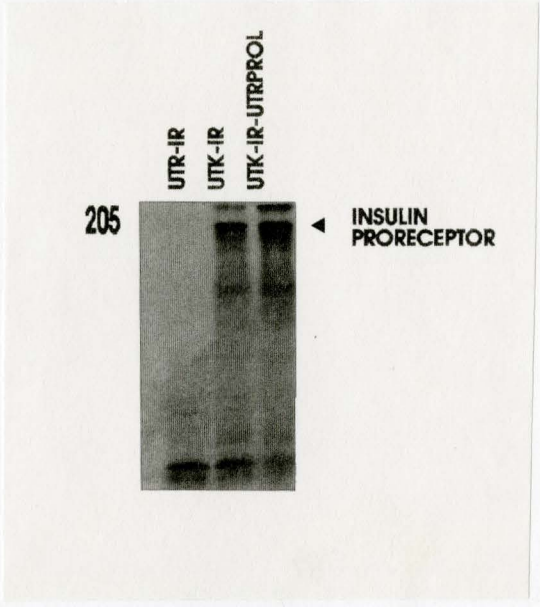


FIGURE 1
EFFECT OF 5′ UTR AND 3′ UTR ON INSULIN PRORECEPTOR SYNTHESIS
Translational efficiency of *in vitro* synthesized insulin receptor transcripts in one hour RRL translations. Effect of the 5′ UTR on expression. The leader region is indicated above each lane. UTR-IR has the Xenopus ß-globin 5′ UTR, UTK-IR has both the Xenopus ß-globin 5′ UTR and a Kozak consensus sequence for efficient translation initiation, UTK-IR-PROLUTR has the prolactin 3′ UTR after the insulin receptor coding region.

EXPRESSION OF THE INSULIN RECEPTOR IN XENOPUS OOCYTES

Although we had sucess in optimizing expression of human insulin proreceptor in RRL, initial attempts to express the insulin receptor construct with the optimized 5′ and 3′ UTR's in oocytes were unsucessful. After examining possible reasons for this, it was concluded that the polyclonal antibody used for isolating the receptor was unstable and not suitable. The experiment was repeated using a monoclonal antibody specific for human receptors and which has no cross reactivity with Xenopus receptors and we found to efficiently immunoprecipitate proreceptor from RRL translations (Oncogene Science AB-1).

The 5′ UTK leader had a dramatic effect on expression of the insulin receptor in the oocytes (figure 2, compare lane5 with 6 and 7). Expression of UTR-IR was not detected (figure 2, compare lanes 4 and 5). The construct containing the prolactin 3′ UTR expressed the same level of processed receptors (α-135 kDa and ß-95 kDa subunits) but higher levels of proreceptor (uncleaved precursor - approximately 200 kDa) than UTK-IR (figure 2, compare lanes 3 and 4). This result suggests that since this clone is, after 40 hours, making higher levels of proreceptor, the transcript may be more functionally stable than UTK-IR. More experiments are required to verify this suggestion. From the results it appears that the oocyte is able to efficiently process the receptor into α and ß subunits. Size comparison to the

molecular weights reported previously corresponds to glycosylated and further modified forms characteristic of the mature receptor. (Appendix A for references on insulin receptor molecular weights). The larger molecular weight of the oocyte proreceptor compared to RRL translation also suggests the efficient addition of post-translational modifications. The expression we observe with the optimized construct is considerably higher than previous reports, in which it was necessary to use high specific activity ^{32}P labelling of the ß-subunit to demonstrate expression (Vera and Rosen, 1990). In our experiments, using the optimized construct, we are able to demonstrate insulin receptor expression by ^{35}S metionine labelling, immunoprecipitation, SDS-PAGE and short exposure times (overnight exposures).

FIGURE 2
EXPRESSION OF INSULIN RECEPTOR CONSTRUCTS IN XENOPUS OOCYTES

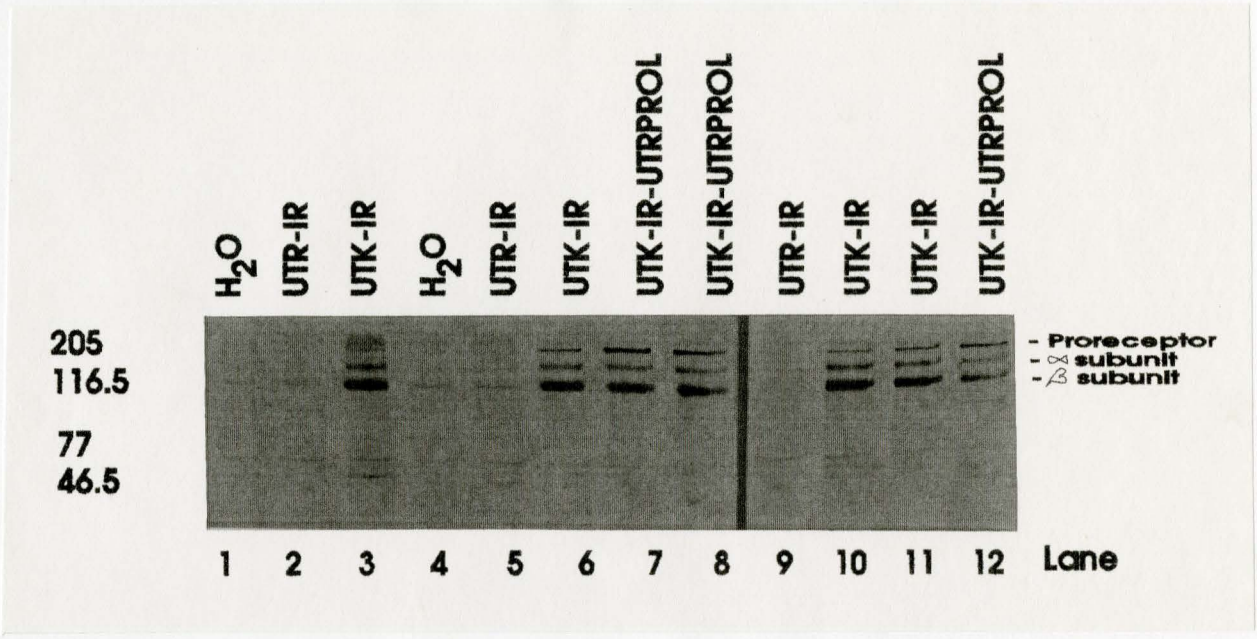


FIGURE 2
EFFECT OF 5′ UTR AND 3′ UTR ON INSULIN INSULIN RECEPTOR SYNTHESIS
Oocytes were injected with *in vitro* synthesized transcript, 50 nl/oocyte and incubated in 1 mCi ^{35}S methionine/10 ml ND96(1mM Ca^{++}) for 40 hours with a media change at 24 hours. The oocytes were then homogenized, in sets of 6 oocytes, and insulin receptor immunopprecipitated using AB-1 anti-human insulin receptor antibody. The leader region is indicated above each lane. Control lane- H_2O injected oocytes, UTR-IR has the Xenopus ß-globin 5′ UTR, UTK-IR has both the Xenopus ß-globin 5′ UTR and a Kozak consensus sequence for efficient translation initiation, UTK-IR-PROLUTR has the prolactin 3′ UTR after the insulin receptor coding region.
The proreceptor, α-subunit and ß-subunit are indicated. A background band at approximately 60 kDa serves as an internal control to ensure consistent injection and labelling. Coomassie Blue staining of the SDS-PAGE gel indicated equivalent recovery of antibody in each lane (not shown). Included in this experiment were injections of two different transcription reaction products for each construct in duplicate for each construct at least for one of the two transcripts (as indicated). In all cases the results were consistent.

III-3-iv
CONCLUSIONS

We have demonstrated, in this section, the highly efficient expression of cloned human insulin receptors in Xenopus oocytes by optimization of the 3′ and 5′ UTR's. As discussed below, the expression of the insulin receptor provides an important tool for further investigations of insulin receptor function.

CHAPTER IV
CONCLUSION

IV-i
CONCLUSIONS

In examining the Xenopus oocyte as a potential model system for studying the effects of extracellular calcium on insulin action several short and long term experimental factors were considered. The results, presented in chapters II and III, establish a basis for the oocyte as a model system for examining the role of extracellular calcium in insulin receptor function.

Prior to the experiments described here there were several reports linking extracellular Ca^{++} and various insulin stimulated processes. However the extent to which Ca^{++} is involved, the stage at which Ca^{++} is required, and the concentration required for maximal stimulation all vary depending on which insulin stimulated process is investigated. In contrast to previous studies, the results in chapter II do not suggest a role for physiological concentrations of extracellular calcium in insulin action. However, the time dependent inhbition of ISDU by EGTA does suggest a mechanistic requirement for calcium at the plasma membrane in an early event in insulin action. While we have defined temporally an event which is sensitive to EGTA we have not determined the mechanism by which EGTA is inhibiting ISDU. Furthermore, the results obtained made use of the endogenous Xenopus receptors. Therefore contrasting reports on the effects of extracellular Ca^{++} on stimulation of mammalian receptors may be a result of structural differences between the receptors (i.e. mammalian

receptor function may be more sensitive to removal of physiological concentrations of Ca^{++} from the media than Xenopus receptors). Alternatively, the conflicting results may be due to differences in the the sensitivity of mammalian cells and Xenopus oocytes to fluctuations in extracellular calcium.

The above possibilities can be tested by expressing human receptors in the oocyte and looking for changes in calcium dependence. If the function of human receptors is affected by free calcium it will be interesting to study the structural differences between the human and Xenopus receptors. If, however, the response mediated by the human receptor is also resistant to free calcium it will support the second possibility. If this is the case then the oocyte will provide an ideal model system for further studies to determine the signalling step(s) affected by calcium chelators. Expression of normal and mutant insulin receptors in the oocyte will be essential in determining which event is inhibited by EGTA. Antibodies specific for human insulin receptors can be used as probes for signalling events. Immunofluoresence, chemical crosslinking and electron microscopy (using gold cluster labelled antibodies) may be used to follow aggregation and internalization of insulin receptors at various times after insulin exposure (see Appendix A for discussion of post-insulin-binding events). Furthermore, autophosphorylation and tyrosine kinase

activition of the expressed receptors can be determined by ^{32}P labelling the oocytes and immunoprecipitating with anti-human insulin receptor antibodies and anti-phosphotyrosisne antibodies after exposure, of oocytes expressing human insulin receptors, to insulin (see Appendix A for discussion and references for tyrosine kinase activity of the insulin receptor). The effects of extracellular calcium and EGTA on these events may provide clues to the mechanisms involved in inhibition by EGTA. These studies as well as mutational analysis may also determine whether the Ca^{++} required is associated with the membrane or directly with the receptor.

Initial attempts to express the cloned human insulin receptor in oocytes were unsucessful. For this reason we addressed the problem of optimizing expression of cloned genes in the Xenopus oocyte. Expression of the insulin receptor was dramatically improved by optimizing the 5′ and 3′ UTR's. Furthermore the results of this study provide a good strategy for increasing expression of many cloned genes by replacing the cognate 5′ and 3′ UTRs with sequences found to increase translation and RNA stability. In addition, results from examining the effects of the coding region on translational efficiency suggest that, once the mechanisms involved are better understood, expression may be further improved by optimizing the nucleotide sequence of a gene without changing the amino acid sequence. As outlined above we should now be able to use receptors expressed in the oocyte to investigate

further the role of extracellular calcium in the mechanism of transmembrane signalling through the insulin receptor.

It has been suggested that changing the extracellular concentration of Ca^{++} outside physiological limits leads to perturbations in intracellular calcium levels and this may in turn affect insulin signalling. While the results in chapter II show that removal of Ca^{++} from the media does not result in a loss of bulk intracellular Ca^{++} it does not exclude the possibility that intracellular distribution of Ca^{++} is altered. Further studies, possibly using injection of Ca^{++} sensitive dyes such as Fura-2, on the effects of changing extracellular Ca^{++} concentration on the intracellular distribution of Ca^{++} will be necessary to determine whether the oocyte does control intracellular calcium independent of extracellular conditions. If this is so then the Xenopus oocyte system will be useful for examining the effects of extracellular calcium on other processes as well.

The results presented here demonstrate that the Xenopus oocyte is suitable for both preliminary and long term studies of the effects of extracellular calcium on insulin receptor function. Using the Xenopus oocyte, we have demonstrated a requirement for extracellular calcium, likley associated at the surface either on the receptor or the membrane, in an early post-insulin-binding event in the stimulation of deoxyglucose uptake by insulin. Furthermore, having expressed the human insulin receptor a more extensive

analysis of the role of extracellular calcium in insulin receptor function will be possible. In addition, having achieved high expression in a cell which has efficient effector-coupling for insulin receptors opens the possibility for studying other aspects of insulin receptor function in addition to extracellular Ca^{++} dependence.

REFERENCES

Aharon, T. and R.J. Schneider. 1993. Selective destabilization of short-lived mRNAs with the granulocyte-macrophage colony-stimulating factor AU-rich noncodingregion is mediated by a cotranslational mechanism. Molecular and Cellular Biology 13,No.3:1971-1980.

Akhtar, R.A. and M.C. Perry. 1979. Insulin action in isolated fat cells I. Effects of divalent cations on the stimulation by insulin of glucose uptake. Biochimica et Biophysica Acta 585:107-116.

Akhtar, R.A. and M.C. Perry. 1979. Insulin action in isolated fat cells II. Effects of divalent cations on stimulation by insulin of protein synthesis, on inhibition of lipolysis by insulin, and on the binding of ^{125}I-labelled insulin to isolated fat cells. Biochimica et Biophysica Acta 585:117-127.

Ananthanarayanan, V.S. 1991. Peptide Hormones, neurotransmitters, and drugs as Ca^{2+} ionophores: implications for signal transduction. Biochem. Cell Biol. 69:93-95.

Andrews, D. 1989. Examining protein translocation in cell-free systems and microinjected xenopus oocytes. BioTechniques 7:960-967.

Andrews, M.T. 1989. Microinjection of *in vitro* derived mRNA into amphibian eggs. From Promega Notes, No.17:1.

Baxter, R.C. and P.F. Williams. 1983. Reciprocal modulation of insulin and insulin-like growth factor-I receptor affinity by calcium. Biochemical and Biophysical Research Communications 116,No.1:62-67.

Bement, W.M. and D.G. Capco. 1989. Activators of protein kinase C trigger cortical granule exocytosis, cortical contraction, and cleavage furrow formation in xenopus laevis oocytes and eggs. Journal of Cell Biology 108:885-892.

Berkner, K.L. and P.A. Sharp. 1985. Effect of the tripartite leader on synthesis of a non-viral protein in an adenovirus 5 recombinant. Nucleic Acids Research 13,no3:841-856.

Bernstein, P. and J. Ross. 1989. Poly(A), poly(A) binding protein and the regulation of mRNA stability. TIBS 14:373-377.

Berridge, M.J. and R.F. Irvine. 1989. Inositol phosphates and cell signalling. Nature 341:197-205.

Berridge, M.J. 1993. Inositol trisphosphate and calcium signalling. Nature 361:315-325.

Bienroth, S., W. Keller and E. Wahle. 1993. Assembly of a processive messenger RNA polyadenylation complex. EMBO J. 12,No.2:585-594.

Bramley, T.A. R. Coleman. 1972. Effects of inclusion of Ca^{2+}, Mg^{2+}, EDTA or EGTA during the preparation of erythrocyte ghosts by hypotonic haemolysis. Biochimica et Biophysica Acta 290:219-228.

Brawerman, G. 1989. mRNA decay: finding the right targets. Cell 57:9-10.

Brimble, K.S. and V.S. Ananthanarayanan. 1992. Induction of Ca^{2+} Transport in liposomes by insulin. Biochimica et Biophysica Acta 1105:319-327.

Curran, J. and D. Kolakofsky. 1989. Scanning independent ribosomal initiation of the sendai virus Y proteins *in vitro* and *in vivo*. EMBO J. 8,No.2:521-526.

Delfert, D.M., C.B. Graves and J. McDonald. 1988. Interaction of the insulin receptor with calcium and calmodulin. Chapter 12 in Insulin Receptors, Part A: Methods for the Study of Structure and Function. Alan R. Liss, Inc. 189-201.

Desai, K.S., E. Zinman, G. Steiner and C. H. Hollenberg. 1978. Effect of calcium on [^{125}I]insulin binding to rat adipocytes. Canadian Journal of Biochemistry 56,No.9:843-848.

Draznin, B., K. Sussman, M. Kao, D. Lewis and N. Sherman. 1987. The existence of an optimal range of cytosolic free calcium for insulin-stimulated glucose transport in rat adipocytes. Journal of Biological Chemistry. 262, No.30:14385-14388.

Drummond, D.R., J. Armstrong and A. Colman. 1985. The effect of capping and polyadenylation on the stability, movement and translation of synthetic messenger RNAs in xenopus oocytes. Nucleic Acids Research 13,No.20:7375-7394.

Eckel, J. and H. Reinauer. 1984. Effect of EDTA on insulin binding and insulin action in isolated cardiocytes from adult rat. Evidence for a functional role of low-affinity insulin receptors. Diabetes 33:214-218.

Elroy-Stein, O., T.R. Fuerst and B. Moss. 1989. Cap-independent translation of mRNA conferred by encephalomyocarditis virus 5′ sequence improves the performance of the vaccinia virus/bacteriophage T7 hybrid expression system. PNAS USA Biochemistry 86:6126-6130.

Emami, S. and M.C. Perry. 1986. Requirement for bivalent cations in the actions of insulin and sodium nitroprusside on metabolism in rat adipocytes. FEBS 200:51-57.

Falcone, M. and D.W. Andrews. 1991. Both the 5′ untranslated region and the sequences surrounding the start site contribute to efficient initiation of translation *in vitro*. Molecular and Cellular Biology 11,No.5:2656-2664.

Froesch, E.R., C. Schmid, J. Schwander and J. Zapf. 1985. Actions of insulin-like growth factors. Annual Reviews in Physiology 47:443-467.

Gallie, D.R., D.E. Sleat, J.W. Watts, P.C. Turner and T.M.A. Wilson. 1987. The 5′-leader sequence of tobacco mosaic virus RNA enhances the expression of foreign gene transcripts *in vitro* and *in vivo*. Nucleic Acids Research 15,No.8:3257-3273.

Gallie, D.R., D.E. Sleat, J.W. Watts, P.C. Turner and T.M.A. Wilson. 1987. A comparison of eukaryotic viral 5′-leader sequences as enhancers of mRNA expression *in vivo*. Nucleic Acids Research 15,No.21:8693-8711.

Galili, G., E.E. Kawata, L.D. Smith and B.A. Larkins. 1988. Role of the 3′-poly(A) sequence in the translational regulation of mRNAs in xenopus laevis oocytes. Journal of Biological Chemistry 263,No.12:5764-5770.

Graves, C.B., R.D. Gale, J.P. Laurino and J.M. MsDonald. 1986. The insulin receptor and calmodulin. Calmodulin enhances insulin-mediated receptor kinase activity and insulin stimulates phosphorylation of calmodulin. JBC 261,No.22:10429-10438.

Gurevich, V.V., I.D. Pokrovskaya, T.A. Obukhova and S.A. Zozulya. 1991. Preparative *in vitro* mRNA synthesis using SP6 and T7 polymerases. Analalytical Biochemistry 195:207-213.

Gurdon, J.B. and M.P. Wickens. 1983. The use of xenopus oocytes for the expression of cloned genes. Chapter 25 in Methods in Enzymology. Academic Press, Inc. 101:370-386.

Inoue, M., Y. In and T. Ishida. 1992. Calcium binding to phospholipid: structural study of calcium glycerophosphate. Journal of Lipid Research 33:985-994.

Jackson, R.J. and N. Standart. 1990. Do the poly(A) tail and 3′ untranslated region control mRNA translation? Cell 62:15-24.

Jackson, R.J., M.T. Howell and A. Kaminski. 1990. The novel mechanism of initiation of picornavirus RNA translation. TIBS 15:477-483.

Jackson, R.J.. 1991. Initiation without an end. Nature 353:14-15.

Janicot, M. and M.D. Lane. 1989. Activation of glucose uptake by insulin and insulin-like growth factor I in xenopus oocytes. PNAS USA Biochemistry 86:2642-2646.

Jobling, S.A. and L. Gehrke. 1987. Enhanced translation of chimaeric messenger RNAs containing a plant viral untranslated leader sequence. Nature 325:622-625.

Jobling, S.A., C.M. Cuthbert, S.G. Rogers, R.T. Fraley and L. Gehre. 1988. *in vitro* transcription and translational efficiency of chimeric SP6 messenger RNAs devoid of 5′ vector nucleotides. Nucleic Acids Research 16,No.10:4483-4498.

Johansen, H., D. Schumperli and M. Rosenberg. 1984. Affecting gene expression by altering the length and sequence of the 5′ leader. PNAS USA Biochemistry 81:7698-7702.

Kelly, K.L., J.T. Deeney and B.E. Corkey. 1989. Cytosolic free calcium in adipocytes. Distinct mechanisms of regulation and effects of insulin action. Journal of Biological Chemistry 264:12754-12757.

Klausner, R.D. and J.B. Harford. 1989. Cis-Trans models for post-transcriptional gene regulation. Science 246:870-872.

Klip, A. and T. Ramlal. 1987. Cytoplasmic Ca^{2+}during differentiation of 3T3-L1 adipocytes. Effect of insulin and relation to glucose transport. Journal of Biological Chemistry 262:9141-9146.

Koren, R., Y. Burstein and H. Soreq. 1983. Synthetic leader peptide modulates secretion of proteins from microinjected xenopus oocytes. PNAS USA Cell Biology 80:7205-7209.

Kozak, M.. 1986. Point mutations define a sequence flanking the AUG initiator codon that modulates translation by eukaryotic ribosomes. Cell 44:283-292.

Kozak., M.. 1987. An analysis of 5'-noncoding sequences from 699 vertebrate messenger RNAs. Nucleic Acids Research 15,No.20:8125-8148.

Kozak, M.. 1989. The scanning model for translation: an update. Journal of Cell Biology 108:229-241.

Kozak, M.. 1991. An analysis of vertebrate mRNA sequences: intimations of translational control. Journal of Cell Biology 115,No.4:887-903.

Krieg, P.A. and D.A. Melton. 1984. Functional messenger RNAs are produced by SP6 *in vitro* transcription of cloned cDNAs. Nucleic Acids Research 12,No.18:7057-7070.

Labbe, J-C., A. Picard, E. Karsenti and M. Doree. 1988. An m-phase specific protein kinase of xenopus oocytes: partical purification and possible mechanism of its periodic activation. Developmental Biology 127:157-169.

Laird-Offringa, I.A., C.A. deWit, P. Elfferich and A.J. van der Eb. 1990. Poly(A) tail shortening is the translation-dependent step in c-mys mRNA degradation. Molecular and Cellular Biology 10,No.12:6132-6140.

Lazarus, P.. 1992. The regulation of translation by the 5' untranslated region of xenopus c-myc I mRNA during early development. Oncogene 7:1037-1041.

Lechleiter, J.D. and D.E. Clapham. 1992. Molecular mechanisms of intracellular calcium excitability in x. laevis oocytes. Cell 69:283-294.

Levi, G., K.L. Crossin and G.M. Edelman. 1987. Expression sequences and distribution of two primary cell adhesion molecules during embryonic development of xenopus laevis. Journal of Cell Biology 105:2359-2372.

Macejak, D.G. and P. Sarnow. 1991. Internal initiation of translation mediated by the 5' leader of a cellular mRNA. Nature 353:90-94.

McDonald, J.M., D.E. Bruns and L. Jarett. 1976. Ability of insulin to increase calcium binding by adipocyte plasma membranes. PNAS USA Biochemistry 73,No.5:1542-1546.

Melton, D.A., P.A. Krieg, M.R. Rebagliati, T. Maniatis, K. Zinn and M.R. Green. 1984. Efficient *in vitro* synthesis of biologically active RNA and RNA hybridization probes from plasmids containing a bacteriophage SP6 promoter. Nucleic Acids Research 12,No.18:7035-7056.

Merrick, W.C.. 1990. Overview: mechansim of translation initiation in eukaryotes. Enzyme 44:7-16.

Moggio, M., G. Fagiolari, A. Prelle, A. Gallanti, M. Sciacco and G. Scarlato. 1992. Lack of anionic phospholipid calcium binding sites in duchenne muscular dystrophy. Muscle and Nerve 325-331.

Mohun, T.J., C.D. Lane, A. Colman and C.C. Wylie. 1981. The secretion of proteins *in vitro* from xenopus oocytes and their accessory cells: a biochemical and morphological study. J. Embryol. Exp. Morph. 61:367-383.

Morgan. D.O., L. Ho, L.J. Korn and R.A. Roth. 1986. Insulin action is blocked by a monoclonal antibody that inhibits the insulin receptor kinase. PNAS USA Biochemistry 83:328-332.

Murray, M.T., D.L. Schiller and W.W. Franke. 1992. Sequence analysis of cytoplasmic mRNA-binding proteins of xenopus oocytes identifies a family of RNA-binding proteins. PNAS USA Biochemistry 89:11-15.

Nudel, U., H. Soreq and U.Z. Littauer. 1976. Globin mRNA species containing poly(A) segments of different lengths. Their functional stability in xenopus oocytes. European Journal of Biochemistry 64:115-121.

Olefsky, J.M. The insulin receptor - a multifunctional protein. Diabetes 39:1009-1016.

Olins, P.O., C.S. Devine, S.H. Rangwala and K.S. Kavka. 1988. The T7 phage gene 10 leader RNA, a ribosome-binding site that dramatically enhances the expression of foreign genes in e. coli. Gene 73:227-235.

Olins, P.O. and S.H. Rangwala. 1989. A novel sequence element derived from bacteriophage T7 mRNA acts as an enhancer of translation of the lacZ gene in Escherichia coli. Journal of Biological Chemistry 264,No.29:16973-16976.

Pelham, H.R.B.. 1978. Translation of encephalocyocarditis virus RNA *in vitro* yields an active proteolytic processing enzyme. European Journal of Biochemistry 85:457-462.

Pelletier, J. and N. Sonenberg. 1988. Internal initiation of translation of eukaryotic mRNA directed by a sequence derived from poliovirus RNA. Nature 334:320-325.

Pershadsingh, H.A. and J.M. McDonald. 1984. Hormone-receptor coupling and the molecular mechanism of insulin action in the adipocyte: a paradigm for Ca^{2+} homeostasis in the initiation of the insulin0induced metabolic cascade. Cell Calcium 5:111-130.

Plehwe, W.E., P.F. Williams, I.D. Caterson, L.C. Harrison and J.R. Turtle. 1983. Calcium-dependence of insulin receptor phosphoylation. Biochem J. 214:361-366.

Ratnasabapathy, R., S. L. Hwang and D.L. Williams. 1990. The 3'-untranslated region of apolipoprotein II mRNA contains two independent domains that bind distinct cytosolic factors. Journal of Biological Chemistry 265,No.23:14050-14055.

Reed, D.J., G.A. Pascoe and C.E. Thomas. 1990. Extracellular calcium effects on cell viability and thiol homeostasis. Environmental Health Perspectives 84:113-120.

Rothman, R.E., D.W. Andrews, M.C. Calayag and V.R. Lingappa. 1988. Construction of defined polytopic integral transmembrane proteins. Journal of Biological Chemistry 263,No.21:10470-10480.

Sacks, D.B., Y. Fujita-Yamaguchi, R.D. Gale and J.M. McDonald. 1989. Tyrosine-specific phosphorylation of calmodulin by the insulin receptor kinase purified from human placenta. Biochem. J. 263:803-812.

Saini, K.S., I.C. Summerhayes and P. Thomas. 1990. Molecular events regulating messenger RNA stability in eukaryotes. Molecular and Cellular Biochemistry 96:15-23.

Saini, K.S. and I.C. Summerhayes. 1991. Regulation of the turnover of mRNAs encoding cellular oncoproteins. Biochem. Cell Biol. 69:415-417.

Schagger, H. and G. von Jagow. 1987. Tricine-sodium dodecyl sulfate-polyacrylamide gel electorphoresis for the separation of proteins in the range from 1 to 100 kDa. Analalytical Biochemistry 166:368-379.

Shuldiner, A.R., S. Phillips, C.T. Roberts, Jr., D. LeRoith and J. Roth. 1989. Xenopus laevis contains two nonallelic preproinsulin genes. cDNA cloning and evolutionary perspective. Journal of Biological Chemistry 264,No.16:9428-9432.

Shuttleworth, L. and A. Colman. 1988. Antisense oligonucleotide-directed cleavage of mRNA in xenopus oocytes and eggs. EMBO 7,No.2:427-434.

Sibley, D.R., J.L. Benovic, M.C. Caron and R.J. Lefkowitz. 1987. Regulation of transmembrane signaling by receptor phosphorylation. Cell 48:913-922.

Simon, K., E. Perara and V. R. Lingappa. 1987. Translocation of globin fusion proteins across the endoplasmic reticulum membrane in xenopus laevis oocytes. Journal of Cell Biology 104:1165-1172.

Snyder, P.M., K.-H. Krause and M.J. Welsh. 1988. Inositol trisphosphate isomers, but not inositol 1,3,4,5-tetrakisphosphate, induce calcium influx in xenopus oocytes. Journal of Biological Chemistry 263,No.23:11048-11051.

Soreq, H. 1985. The biosynthesis of biologically active proteins in mRNA-microinjected xenopus oocytes. CRC Critical Reviews in Biochemistry 18,Issue3:199-238.

Stefanovic, D., E. Erikson, L.J. Pike and J.L. Maller. 1986. Activation of a ribosomal protein S6 protein kinase in xenopus oocytes by insulin and insulin-receptor kinase. EMBO 5,No.1:157-160.

Svitkin, Y.V. and V.I. Agol. 1978. Complete translation of encephalonyocarditis virus RNA and faithful cleavage of virus-specific proteins in a cell-free system from krebs-2 cells. FEBS Letters 87,No.1:7-11.

Tajima, S., L. Lauffer, V.L. Rath and P. Walter. 1986. The signal recognition particle receptor is a complex that contains two distinct polypeptide chains. Journal Cell Biology 103:1167-1178.

Tate, S.S., R. Urade, T.V. Getchell and S. Udenfriend. 1989. Expression of the mammalian Na^+-independent L system amino acid transporter in xenopus laevis oocytes. Archives of Biochem. and Biophys. 275,No.2:591-596.

Taylor, W.M., L. Hau and M. Halperin. 1979. Stimulation of glucose transport in rat adipocytes by calcium. Canadian Journal of Biochemistry 57:692-699.

Terepka, A.R., J.R. Coleman, H.J. Armbrecht and T.E. Gunter. 1976. Transcellular transport of calcium. From Calcium in Biological Systems. Symposia of the Society for Experimental Biology, No.XXX, Cambridge University Press. 117-140.

Tyc, K., M. Konarska, H.J. Gross and W. Filipowicz. 1984. Multiple ribosome binding to the 5'-terminal leader sequence of tobacco mosaic virus RNA. FEBS 140:503-511.

Ulrich, A., J.R. Bell, E.Y. Chen, R. Herrera, L.M. Petruzzelli, T.J. Dull, A. Gray, L. Coussens, Y.-C. Liao, M. Tsubokawa, A. Mason, C. Grunfeld, O.M. Rosen and J. Ramachandran. 1985. Human insulin receptor and its relationship to the tyrosine kinase family of oncogenes. Nature 313:756-761.

Ulrich, A. and J. Schlessinger. 1990. Signal transduction by receptors with tyrosine kinase activity. Cell 61:203-212.

Vera, J.C. and O.M. Rosen. 1989. Functional expression of mammalian glucose transporters in xenopus laevis oocytes: Evidence for cell-dependent insulin sensitivity. Molecular and Cellular Biology 9,No.10:4187-4195.

Vera, J.C. and O.M. Rosen. 1990. Reconstitution of an insulin signaling pathway in xenopus laevis oocytes: coexpression of a mammalian insulin receptor and three different mammalian hexose transporters. Molecular and Cellular Biology 10,No.2:743-751.

Wickens, M.. 1990. In the beginning is the end: regulation of poly(A) addition and removal during early development. TIBS 15:320-324.

Williams, P.F. and J.R. Turtle. 1981. Insulin inhibition of calcium binding by human placental membrane. Biochimica et Biophysica Acta 676:113-117.

Williams, P.F. and J.R. Turtle. 1984. Terbium, a fluorescent probe for insulin receptor binding. Evidence for a conformational change in the receptor protein due to insulin binding. Diabetes 33:1106-1111.

Williams, P.F., I.D. Caterson, G.J. Cooney, R.R. Zilkens and J.R. Turtle. 1990. High affinity insulin binding and insulin receptor-effector coupling: modultaion by Ca^{2+}. Cell Calcium 11:547-556.

Williamson, J.R., R.H. Cooper and J.B. Hoek. 1981. Role of calcium in the hormonal regulation of liver metabolism. Biochimica et Biophysica Acta 639:243-295.

Wong, E.C.C., D.B. Sacks, J.P. Laurino and J.M. McDonald. 1988. Characteristics of calmodulin phosphorylation by the insulin receptor kinase. Endocrinology 123,No.4:1830-1836.

APPENDIX A

BACKGROUND

The insulin receptor is a large membrane protein usually greater than 350 kDa in size (reviewed in: Olefsky, 1990; Roth et al., 1992; Kahn et al., 1993). It is a tetramer consisting of two alpha and two beta subunits which are disulfide linked. The insulin receptor has an N-terminal signal sequence which directs synthesis to the endoplasmic reticulum via the SRP/SRP Receptor pathway (reviewed in Olefsky, 1990). Cleavage of the signal sequence in the ER results in a completed proreceptor which is approximately 180 kDa in size. In the ER and Golgi the proreceptor is disulfide linked and cleaved at an ARG LYS ARG ARG site to produce an alpha-beta dimer (Salzman et al., 1984; Olson et al., 1986; Olson et al., 1987). Two dimers are further disulfide linked to produce a complete tetramer. Without any post-translational modifications the alpha and beta subunits are 82 500 and 69 700 Da respectively (reviewed in Olefsky, 1990). These subunits undergo extensive modifications including glycosylation, sulfation, acetylation and acylation in the ER and Golgi to produce approximately 130 000 Da alpha and 95 000 Da beta subunits (Hedo et al., 1987; Collier and Gordon, 1991; Hayes et al., 1991). Post-translational modifications, in particular N-linked glycosylation, has been implicated in the transmembrane signalling mechanism of the insulin receptor (Leconte et al., 1992)

The alpha subunit is entirely extracellular and contains the ligand (insulin) binding domain (reviewed in Yip, 1992). From mutational studies several regions have been implicated in insulin binding;

1. The cysteine rich region at the c terminus (Yip et al., 1988, 1991; Rafaeloff et al., 1989)
2. Phenalalanine 89 (Wedekind et al., 1989; Waugh et al., 1989; [DeMeyts et al., 1990])

Further studies implicate both carboxy and amino terminal residues of the alpha subunit in insulin binding to the receptor (Anderson et al., 1992; Fabry et al., 1992; [Schumacher et al, 1991] and 1993). While there are two possible insulin binding sites on the $\alpha 2\beta 2$ tetramer there is some contravorsy as to whether there is only one high affinity site per receptor with a lower affinity second site or whether both are occupied (Pang and Shafer, 1984; reviewed in Yip et al., 1992; Yip and Jack, 1992; Shoelson et al., 1993)

Each beta subunit has several important functional domains including;

1. Two consensus internalization sequences, NPXT (presumed ß-turns) on the cytoplasmic side of the transmembrane domain (Backer et al., 1990; Theis et al., 1990; reviewed in McLain, 1992).
2. A tyrosine kinase domain with a consensus ATP binding site (GXGXXGG...20-25aa..K) (Ullrich et al., 1985).
3. Several tyrosine phosphorylation sites (1163, 1162, 1158 and some minor sites at C term) (reviewed in Rosen et al., 1987).
4. A single transmembrane domain (reviewed in Olefsky, 1990)

The above regions have all been shown to be involved in the transmembrane signalling mechanism (reviewed in Olefsky, 1990). It has been suggested that the transmembrane domain plays a passive role in signalling. Mutational studies and domain swap experiments suggest that the residues in the transmembrane region are not specifically required for the receptor to transmit a signal (Fratteli et al., 1991). A study comparing the membrane spanning residues of 16 integral membane proteins and 11 receptors, including the insulin receptor, revealed no significant sequence differences between the two groups (Brandl et al., 1988). This suggests that the receptor transmembrane domain is functioning primarily as an anchoring domain and that other regions are responsible for transmitting the signal upon insulin binding. These results are in contrast to results from more recent mutational studies which suggest a more active role for the transmembrane domain (Yamada et al., 1992; Longo et al., 1992; reviewed in Murray-Rust, 1993)

The insulin receptor primary sequence shares homology with other tyrosine kinase active receptors and oncogenes (Evans and Graham in Membrane Structure and Function In Focus series D. Rickwood Ed. 1989; Ullrich and Schlessinger, 1990). While the extracellular domain is relatively unique, the cytoplasmic domain is highly homologous to a variety of proteins all bearing a tyrosine kinase domain (Ullrich et al., 1985; Ullrich and Schlessinger, 1990). These include a group of tyrosine kinase receptors such as EGFR PDGFR CSF-1R and IGF1R as well as the src and ros families of oncogenes (reviewed in Ullrich and Schlessinger, 1990).

While the ligands that bind to the tyrosine kinase receptors differ considerably there is evidence for a common early response to ligand binding. This is consistent with the above sequence comparisons in which the ligand binding regions are unique while the intracellular regions responsible for signalling are conserved to a greater extent. Within seconds of ligand binding to the receptor several tyrosine

residues on the cytoplasmic domain of the beta subunit are phosphorylated. There is a concomitant activation of the tyrosine kinase. This may suggest common or similar transmembrane signalling pathways. While the early events in signalling are common to a number of receptors, the downstream events vary greatly for each receptor (reviewed in Ullrich and Schlessinger, 1990).

There are several well characterized cellular changes that can be used as indicators of insulin binding and of activation of the receptor. Phosphorylation of the beta subunit on several tyrosine residues is the earliest indicator of binding and signal transduction (Tavare et al., 1991). For this reason it is often used to assay for insulin receptor function. Phosphorylations on Tyr 1158, 1162 and 1163 appear to be the most important for acute insulin action and phosphorylation of all three sites appears to be necessary for full kinase activation and for acute insulin action (examples: Ellis et al., 1986; Morgan and Roth., 1987; Wilden et al 1992). Phosphorylation of these tyrosines occurs sequentially with phosphorylation at 1158 and 1162 occuring earliest and 1163 being phosphorylated subsequent to these residues (Dickens et al., 1992). Furthermore, phosphorylation of the two ß subunits in the tetramer appears to occur assymetrically (Lee et al., 1993). There is evidence that while phosphorylation is an early event involved in the acute response to insulin it may not be required for all insulin actions in the cell (Simpson and Hedo, 1984; Debant et al., 1988; Debois et al., 1992; Sung et al., 1992; reviewed in Sung, 1992). Phosphorylation was initially believed to be autophosphorylation but recent evidence from EGF receptor and insulin receptor studies suggest an intermolecular reaction between adjacent receptors which may act to amplify the signal (Honegger et al., 1989; Shoelson et al., 1991; Hayes et al., 1991).

Several studies have also shown that the insulin receptor and EGF receptor are, in their activated state, aggregates on the cell surface (Fugita-Yamaguchi et al., 1989; Kubar and Van Obberghen, 1989; reviewed in Ballotti et al., 1992). The observation that the aggregated receptors are highly phosphorylated and have higher kinase activity suggested a role for receptor aggregation in signal transduction (Fugita-Yamaguchi et al., 1989; Kubar et al., 1989). Mutations in Tyr1162 and Tyr1163 result in a loss of some receptor functions. Several of the lost functions can be restored by crosslinking the receptors. This result provides further evidence for the importance of receptor aggregation in receptor activation and for kinase independent signalling mediated by the insulin receptor (Debant et al., 1989).

The exact mechanism or event responsible for transmembrane signalling is not clearly defined. There is evidence, using conformation dependent antibodies, that suggests binding of insulin leads to conformational changes in

the intracellular domain of the ß-subunit and that these conformational changes are distinct from, and possibly precede, those which occur upon autophosphorylation and activation of the kinase activity (Baron et al., 1992). Binding of insulin may cause extracellular conformational changes which may be transmitted either by subsequent aggregation of the receptors (as proposed in Kubar et al., 1989), or directly throughout the receptor transmembrane domain.

Considerable work has been done in identifying the second messengers involved in propagating the signal transmitted by the receptor. While many proteins are phosphorylated in response to insulin stimulation, of cells in culture, many of these are serine/threonine phosphorylations. Few direct substrates for the tyrosine kinase function of the receptor have been characterized (reviewed in Rosen, 1987; Roth et al., 1992).

Recently a protein of approximate molecular mass 180,000 (pp185 or IRS-1) has been found to be tyrosine phosphorylated in response to insulin stimulation within minutes of activation of the insulin receptor kinase (Del Vecchio and Pilch, 1989). IRS-1 is phophorylated on tyrosyl residues and was found to be a direct substrate of the insulin receptor tyrosine kinase (Rothenberg et al., 1991; Saad et al., 1992; Sun et al., 1992;). IRS-1 contains a consensus YMXM sequence which is posphorylated by the insulin receptor (Shoelson et al., 1992). Initial characterization of IRS-1 suggested it was a microsomal protein but recent evidence suggests it may be associated with the cytoskeleton (Okamoto et al., 1991). PI3 kinase activation in response to insulin has been found to be medited via interactions of the SH2 domain of the 85 KDa alpha subunit of PI3 kinase to the tyrosine phosphorylated IRS-1, thus formining a ternary signalling complex with the activated insulin receptor (Cohen et al., 1990; Backer et al., 1992; Hadri et al., 1992; Sung and Golfine, 1992; Yonezawa et al., 1992; Myers et al., 1992; Folli et al., 1992; Lavan et al., 1992; Backer et al., 1992 and 1993). Interstingly IRS-1 appears to be a common substrate for both the insulin receptor and IGF-1 receptors tyrosine kinases and is responsible for coupling activation of both receptors to PI3 kinase activation (Yamamoto et al., 1992; Myers et al., 1993).

ERK's, or MAP kinases (MAPK), are another set of proteins found to be activated via tyrosine and serine phosphorylation in response to growth factors and peptide hormones (Boulton et al., 1991a 1991b; Lee et al., 1992; reviewed in Pelech and Sangera, 1992; Nishida and Gotoh, 1993). These are a family of serine/threonine kinases that are each activated by a different set of extracellular signals. While early work suggested that these could act as adaptors for receptors, converting the tyrosine kinase function to serine/threonine kinase, more recent studies do not support this hypothesis. ERK's require both tyrosine and serine/threonine

phosphorylation for maximal activation of their kinase, suggesting they act as a point of integration of signals. This is supported by the identification of MAP kinase kinase (MAPKK) which is a dual specificity kinase, i.e. it recognizes and phosporylates threonine and tyrosine residues which are in close proximity on the MAP kinase (reviewed in Pelech and Sangera, 1992; Nishida and Gotoh, 1993). MAPKK is itself activated by another kinase which interacts with activated receptors thus completing the phosphorylation cascade. Map kinase activation in response to insulin stimulation has been reported 3T3-L1 adipocytes and in intact rat liver and is thought to play a role in insulin activation of glucose transport (Ciaraldi and Maisel, 1989; Tobe et al, 1992; Inoue et al., 1993)

As well as IRS-1 and MAPK there is work being done on other protein mediators of the insulin signal. Evidence for the role of G proteins has been reported and appears to involve the association of the insulin receptor with G proteins of molecular weight 41 and 67 kDa (Jo et al., 1992). From the above study and studies using GTP-gammaS it appears that G proteins associated with the receptor may be involved in modulatng the kinase activity of the receptor (Russ et al., 1993). pp120/ecto-ATPase, a liver specific protein, appears also to be a substrate for the insulin receptor kinase (Margolis et al., 1990; Najjar et al., 1993 and references within). A summary of other putative substrates of the insulin receptor kinase are described in a review by Roth et al., 1992.

Along with protein mediators of the insulin response, there is growing evidence for increased levels of other molecules that may act as second messengers. Diacylglycerol and inositol-glycan are both found in increased levels after insulin stimulation of cells (reviewed in Walaas and Walaas, 1988; Suzuki et al., 1991). Generation of these molecules in response to insulin requires the kinase activity of the insulin receptor. Furthermore the increase in diacylglycerol is found to be dependent on phosphorylation of insulin receptor Tyr 1163 and 1162 (Cherqui et al., 1990; Suuki et al., 1992). Phospholipase Cγ has not been found to be activated in response to insulin and so is probably not responsible for the observed insulin induced increase in inositol-glycan (reviewed in Walaas and Walaas, 1988; Nishibe et al., 1990; reviewed in Cockcroft and Thomas, 1992). Instead there is a novel phoshpholipase C responsible for insulin mediated inositol-glycan production (Walaas and Walaas, 1988; Saltiel and Cuatrecasas, 1986). The production of DAG in response to insulin suggests protein kinase C (PKC) may be stimulated in response to insulin but it is not clear what role PKC plays, if any in insulin action (Heindenreich et al., 1990 and refernces within; Caro et al., 1992; Chin et al., 1993; Welsh et al., 1993).

Minutes after insulin binding several measureable changes occur in the cell (the following intracellular events are reviewed in: Moore, 1983; Goldfine, 1987; Housley, 1989; Denton, 1990; Olefsky, 1990; Roth et al., 1992). Some of these are as follows;

1. Internalization of the insulin/receptor complex. Internalization requires two NPXY consensus sequences on the ß subunit. Studies have shown that internalization is not necessary for activation or deactivation of the insulin receptor or subsequent cellular events. Receptor phosphorylation is not necessary for internalization. Proteolytic cleavage of the ß-subunit upon internalization may be involved in downregulation of the receptor (Knutson, 1991)
2. Mobilization of insulin regulated glucose transporters to the cell surface resulting in an increase in glucose uptake (reviewed in Joost and Weber 1989; Czech et al., 1992).
3. Changes in enzymatic activities. The effects of insulin on several metabolic enzymes are well characterized i.e. phosphorylase kinase, phosphorylase, glycogen synthase, pyruvate dehydrogenase etc.
4. Increased transcription of some genes and regulation of RNA and DNA synthesis.
5. Increased ion transport.
6. Increased serine/threonine phosphorylation of insulin receptor ß-subunit (possible downregulatory mechanism or mitogenic signalling).
7. There are also some long term growth factor effects of insulin stimulation such as increased lipid, protein and nucleic acid synthesis. The mitogenic effects of insulin are not dependent on Tyr 1163 and Tyr 1162 phosphorylation and may be mediated through a different pathway than the early cellular responses to insulin. C terminal regions of the ß subunit appear to be involved in the mitogenic effects of insulin.

APPENDIX A REFERENCES

Andersen, A.S., T. Kjeldsen, F.C. Wiberg, H. Vissing, L. Schaffer, J.S. Rasmussen, P. De Meyts and N.P. Moller. Identification of determinants that confer ligand specificity on the insulin receptor. JBC 267,No.19:13681-13686.

Backer, J.M., C.R. Kahn and M.F. White. 1989. Tyrosine phosphorylation of the insulin receptor is not required for receptor internalization: studies in 2,4-dinitrophenol-treated cells. PNAS USA 86:3209-3213.

Backer, J.M., C.R. Kahn, D.A. Cahill, A. Ullrich and M.F. White. 1990. Receptor-mediated internalization of insulin requires a 12-amino acid sequence in the juxtamembrane region of the insulin receptor ß-subunit. JBC 265,No.27:16450-16454.

Backer, J.M., G.G. Schroeder, C.R. Kahn, M.G. Myers Jr., P.A. Wilden, D.A. Cahill and M.F. White. 1992. Insulin stimulation of phosphatidylinositol 3-kinase activity maps to insulin receptor regions required for endogenous substrate phosphorylation. JBC 267,No.2:1367-1374.

Backer, J.M., M.G. Myers Jr., S.E. Shoelson, D.J. Chin, X.J. Sun, M. Miralpeix, P. Hu, B. Margolis, E.Y. Skolnik, J. Schlessinger and M.F. White. 1992. Phosphatidylinositol 3'-kinase is activated by association with IRS-1 during insulin stimulation. EMBO J. 11,No.9:3469-3479.

Backer, J.M., S.E. Shoelson, M.A. Weiss, Q.X. Hua, R.B. Cheatham, E. Haring, D.C. Cahill and M.F. White. 1992. The insulin receptor juxtamembrane region contains two independent tyrosine/beta-turn internalization signals. J. Cell Biology 118,No.4:831-839.

Ballotti, R., V. Baron, N. Gautier, P. Hainaut, J.C. Scimeca, J. Dolais-Kitabji, R. Lammers, J. Schlessinger, A. Ullrich and E. Van Obberghen. 1992. Activation and regulation of the insulin receptor kinase. Diabete et Metabolisme 18:98-103.

Boulton, T.G., J.S. Gregory and M.H. Cobb. 1991. Purification and properties of extracelluar signal-regulated kinase 1, an insulin-stimulated microtubule-associated protein 2 kinase. Biochemistry 30,No.1:278-286.

Boulton, T.G., S.H. Nye, D.J. Robbins, N.Y. Ip, E.Radziejewska, S.D. Morgenbesser, R.A. DePinho, N. Panayotatos, M.H. Cobb and G.D. Yancopoulos. 1991. ERKs: a family of protein-serine/threonine kinases that are activated and tyrosine phosphorylated in response to insulin and NGF. Cell 65:663-675.

Brandi, C.J., R.B. Deber, L.C. Hsu, G.A. Woolley, X.K. Young and C.M. Deber. 1988. Evidence for similar function of transmembrane segments in receptor and membrane-anchored proteins. Biopolymers 27:1171-1182.

Cherqui, G., C. Reynet, M. Caron, B. Melin, D. Wicek, E. Clauser, J. Capeau and J. Picard. 1990. Insulin receptor tyrosine residues 1162 and 1163 control insulin stimulation of myristoyl-diacylglycerol generation and subsequent activation of glucose transport. JBC 265,No.34:21254-21261.

Chin, J.E., M. Dickens, J.M. Tavare and R.A. Roth. 1993. Overexpression of protein kinase C isoenzymes alpha, beta I, gamma, and epsilon in cells overexpressing the insulin receptor. Effects on receptor phosphorylation and signalling. JBC 268,No.9:6338-6347.

Ciaraldi, T.P. and A. Maisel. 1989. Role of guanine nucleotide regulatory proteins in insulin stimulation of glucose transport in rat adipocytes - influence of bacterial toxins. Biochem. J. 264:389-396.

Cockcroft, S. and G.M.H. Thomas. 1992. Review article - inositol-lipid-specific phospholipase C isoenzymes and their differential regulation by receptors. Biochem. J. 288:1-14.

Cohen, B., M. Yoakim, H. Piwnica-Worms, T.M. Roberts and B.S. Schaffhausen. 1990. Tyrosine phosphorylation is a signal for the trafficking of pp85, and 85-kDa phosphorylated polypeptide associated with phosphatidylinositol kinase activity. PNAS USA 87:4458-4462.

Collier, E. and P. Gordon. 1991. O-linked oligosaccharides on insulin receptor. Diabetes 40:197-203.

Czech, M.P., B.M. Clancy, A. Pessino, C.-W. Woon and S.A. Harrison. 1992. Complex regulation of simple sugar transport in insulin-responsive cells. TIBS 17:197-200.

Debant, A., E. Clauser, G. Pnzio, C. Filloux, C. Auzan, J.-O. Contreres and B. Rossi. 1988. Replacement of insulin receptor tyrosine residues 1162 and 1163 does not alter the mitogenic effect of the hormone. PNAS USA 85:8032-8036.

Debant, A., G. Ponzio, E. Clauser, J.O. Contreres and B. Rossi. 1989. Receptor cross-linking restores an insulin metabolic effect altered by mutation on tyrosine 1162 and tyrosine 1163. Biochemistry 28:14-17.

Del Vecchio, R.L. and P.F. Pilch. 1989. Insulin stimulates the tyrosine phosphorylation of a 165 kDa protein that is associated with microsomal membranes of rat adipocytes. BBA 989:41-46.

Denton, R.M.. 1990. Insulin signalling - search for the missing links. Nature 348:286-287.

Desbois, C., J. Capeau, I. Hainault, D. Wicek, C. Reynet, D. Veissiere, M. Caron, J. Picard, M. Guerre-Millo and G. Cherqui. 1992. Differential role of insulin receptor autophosphorylation sites 1162 and 1163 in the long-term insulin stimulation of glucose transport, glycogenesis, and protein synthesis. JBC 267,No.19:13488-13497.

Dickens, M. and J.M. Tavare. 1992. Analysis of the order of autophosphorylation of human isulin receptor tyrosines 1158, 1162 and 1163. Biochemical and Biophysical Research Communications 186,No.1:244-250.

Ellis, L., E. Clauser, D.O. Morgan, M. Edery, R.A. Roth and W.J. Rutter. 1986. Replacement of insulin receptor tyrosine residues 1162 and 1163 compromises insulin-stimulated kinase activity and uptake of 2-deoxyglucose. Cell 45:721-732.

Evans, W.H. and J.M. Graham. 1989. Membrane structure and function. Ed. D. Rickwood. IRL Press, Oxford.

Fabry, M., E. Schaefer, L. Ellis, E. Kojro, F. Fahrenholz and D. Brandenburg. 1992. Detection of a new hormone contact site within the insulin receptor ectodomain by the use of a novel photoreactive insulin. JBC 267,No.13:8950-8956.

Frattali, A.L., J.L. Treadway and J.E. Pessin. 1991. Evidence supporting a passive role for the insulin receptor transmembrane domain in insulin-dependent signal transduction. JBC 266,No.15:9829-9834.

Fujita-Yamaguchi, Y., J.T. Harmon and S. Kathuria. 1989. Radiation inactivation experiments predict that a large aggregate form of the insulin receptor is a highly active tyrosine-specific protein kinase. Biochemistry 28:4556-4563.

Goldfine, I.D.. 1987. The insulin receptor: molecular biology and transmembrane signalling. Endocrine Reviews 8,No.3:235-255.

Gottschalk, W.K., R. Lammers and A. Ullrich. 1992. The carboxy terminal 110 amino acid portion of the insulin receptor is important for insulin signalling to pyruvate dehydrogenase. BBRC 189,No.2:906-911.

Hadari, Y.R., A.U. Tzahar, O. Nadiv, P. Rothenberg, C.T. Roberts, D. LeRoith, Y. Yarden and Y. Zick. 1992. Insulin and insuliomimetic agents induce activation of phosphatidylinositol 3'-kinase upon its association with pp185 (IRS-1) in intact rat livers. JBC 267,No.25:17483-17486.

Hayes, G.R., L.D. Lydon and D.H. Lockwood. 1991. Intermolecular phosphorylation of insulin receptor as possible mechanism for amplification of binding signal. Diabetes 40:300-303.

Hayes, G.R., J.N. Livingston and D.H. Lockwood. 1991. Mapping of carbohydrate sites on the human insulin receptor. BBRC 174,No.2:735-741.

Hedo, J.A., E. Collier andA. Watkinson. 1987. Myristyl and palmityl acylation of the insulin receptor. JBC 262,No.3:954-957.

Heidenreich, K.A., S.P. Toledo, L.L. Brunton, M.J. Watson, S. Daniel-Issakani and B. Strulovici. 1990. Insulin stimulates the activity of a novel protein kinase C, PKC-ϵ, in cultured fetal chick neurons. JBC 265,No.25:15076-15082.

Honegger, A.M., R.M. Kris, A. Ullrich and J. Schlessinger. 1989. Evidence that autophosphorylation of solubilized receptors for epidermal growth factor is mediated by intermolecular cross-phosphorylation. PNAS USA 86:925-929.

Houslay, M.D. and K. Siddle. 1989. Molecular basis of insulin receptor function. British Medical Bulletin 45,No.1:264-284.

Inoue, G., H. Kuzuya, T. Hayashi, M. Okamoto, Y. Yoshimasa, A. Kosaki, S. Kono, M. Okamoto, I. Maeda, M. Kubota and H. Imura. 1993. Effects of ML-9 on isnsulin stimulation of glucose transport in 3T3-L1 adipocytes. JBC 268,No.7:5272-5278.

Jo, H., B.Y. Cha, H.W. Davis and J.M. McDonald. 1992. Identification, partial purification, and characterization of two guanosine triphosphate-binding proteins associated with insulin receptors. Endocrinology 131,No.6:2855-2862.

Joost, H.G. and T.M. Weber. 1989. Review - The regulation of glucose transport in insulin-sensitive cells. Diabetologia 32:831-838.

Kahn, C.R., M.F. White, S.E. Shoelson, J.M. Backer, E. Araki, B. Cheatham, P. Csermely, F. Folli, B.J. Goldstein and P. Huertas. 1993. The insulin receptor and its substrate: molecular determinants of early events in insulin action. Recent Progress in Hormone Research 48:291-339.

Knutson, V. P.. 1991. Proteolytic processing of the insulin receptor ß subunit is associated with insulin-induced receptor down-regulation. JBC 266,No.24:15656-15662.

Kubar, J. and E. Van Obberghen. 1989. Oligomeric states of the insulin receptor: binding and autophosphorylation properties. Biochemistry 28:1086-1093.

Lavan, B.E., M.R. Kuhne, C.W. Garner, D. Anderson, M. Reedijk, T. Pawson and G.E. Lienhard. 1992. The association of insulin-elicited phosphotyrosine proteins with src homology 2 domains. JBC 267,No.16:11631-11636.

Leconte, I., C. Auzan, A. Debant, B. Rossi and E. Clauser. 1992. N-linked oligosaccharide chains of the insulin receptor beta-subunit are essential for transmembrane signalling. JBC 267,No.24:17415-17423.

Lee, J., T. O'Hare, P.F. Pilch and S.E. Shoelson. 1993. Insulin receptor autophosphorylation occurs asymmetrically. JBC 268,No.6:4092-4098.

Lee, R., M.H. Cobb and P.J. Blackshear. 1992. Evidence that extracellualr signal-regulated kinases are the insulin-activated Raf-1 kinase kinases. JBC 267,No.2:1088-1092.

Longo, N., R.C. Shuster, L.D. Griffin, S.D. Langley and L.J. Elsas. 1992. Activation of insulin receptor signalling by a single amino acid substitution in the transmembrane domain. JBC 267,No.18:12416-12419.

Margolis, R.N., M.J. Schell, S.I. Taylor and A.L. Hubbard. 1990. Hepatocyte plasma membrane ecto-ATPase (pp120/HA4) is a substrate for tyrosine kinase activity of the insulin receptor. BBRC. 166,No.2:562-566.

McClain, D.A.. 1990. Endocytosis of insulin receptors is not required for activation or deactivation of the hormone response. JBC 265,No.34:21363-21367.

McClain, D.A.. 1992. Mechanism and role of insulin receptor endocytosis. American Journal of the Medical Sciences 304,No.3:192-201.

Moore, R.D.. 1983. Effects of insulin upon ion transport. BBA 737:1-49.

Morgan, D.O. and R.A. Roth. 1987. Acute insulin action requires insulin receptor kinase activity: introduction of an inhibitory monoclonal antibody into mammalian cells blocks the rapid effects of insulin. PNAS USA 84:41-45.

Murray-Rust, J.. 1993. Do specific interactions between transmembrane helices play a part in signalling transduction? Exploration with the insulin receptor. Bioessays 15,No.1:61-62.

Myers, M.G. Jr., J.M. Backer, X.J. Sun, S. Shoelson, P. Hu, J. Schlessinger, M. Yoakim, B. Schaffhausen and M.F. White. IRS-1 activates phosphatidylinositol 3'-kinase by associating with src homology 2 domains of p85. PNAS USA 89,No.21:10350-10354.

Myers, M.G. Jr., X.J. Sun, B. Cheatham, B.R. Jachna, E.M. Glasheen, J.M. Backer and M.F. White. 1993. IRS-1 is a common element in insulin and insulin-like growth factor-I signalling to the phosphatidylinositol 3'-kinase. Endocrinology 132,No.4:1421-1430.

Najjar, S.M., D. Accili, N. Philippe, J. Jernberg, R. Margolis and S.I. Taylor. 1993. pp120/ecto-ATPase, an endogenous substrate of the insulin receptor tyrosine kinase, is expressed as two variably spliced isoforms. JBC 268,No.2:1201-1206.

Nishibe, S., M.I. Wahl, P.B. Wedegaertner, J.J. Kim, S.G. Rhee and G. Carpenter. 1990. Selectivity of phospholipase C phosphorylation by the epidermal frowth factor receptor, the insulin receptor, and their cytoplasmic domains. PNAS USA Biochemistry 87:424-428.

Nishida, E. and Y. Gotoh. 1993. The MAP kinase cascade is essential for diverse signal transduction pathways. TIBS April:128-131.

Okamoto, M., A. Karasik, M.F. White and C.R. Kahn. 1991. Coordinate phosphorylation of insulin-receptor kinase and its 175,000-M_r endogenous substrate in rat hepatocytes. Diabetes 40:66-72.

Olefsky, J.M.. 1990. Perspectives in diabetes - the insulin receptor - a multifuntional protein. Diabetes 39:1009-1016.

Olson, T.S. and M.D. Lane. 1987. Post-translational acquisition of insulin binding activity by the insulin proreceptor. Correlation to recogniton by autoimmune antibody. JBC 262,No.14:6816-6822.

Olson, T.S., M.J. Bamberger and M.D. Lane. 1988. Post-translational changes in tertiary and quaternary structure of the insulin proreceptor. Correlation with acquisition of function. JBC 266,No.13:7342-7351.

Pang, D.T. and J.A. Shafer. 1984. Evidence that insulin receptor from human placenta has a high affinity for only one molecule of insulin. JBC 259,No.13:8589-8596.

Pelech, S.L. and J.S. Sanghera. 1992. Mitogen-activated protein kinases: versatile transducers for cell signalling. TIBS June:233-238.

Rafaeloff, R., R. Patel, C. Yip, I.D. Goldfine and D.M. Hawley. 1989. Mutation of the high cysteine region of the human insulin receptor α-subunit increases insulin receptor binding affinity and transmembrane signalling. JBC 264,No.27:15900-15904.

Rosen, O.M.. 1987. After insulin binds. Science 237:1452-1458.

Roth, R.A., B. Zhang, J.E. Chin and K. Kovacina. 1992. Substrates and signalling complexes: the tortured path to insulin action. Journal of Cellular Biochemistry 48,No.1:12-18.

Rothenberg, P.L., W.S. Lane, A. Karasik, J. Backer, M. White and C.R. Kahn. 1991. Purification and partial sequence analysis of pp185, the major cellular substrate of the insulin receptor tyrosine kinase. JBC 266,No.13:8302-8311.

Russ, M., H. Reinauer and J. Eckel. 1993. Regulation of cardiac insulin receptor function by guanosine nucleotides. FEBS Letters 317,No.1-2:72-76.

Saad, M.J., E. Araki, M. Miralpeix, P.L. Rothenberg, M.F. White and C.R. Kahn. 1992. Regulation of insulin receptor substrate-1 in liver and muscle of animal models of insulin resistance. Journal of Clinical Investigation 90,No.5:1839-1849.

Salzman, A., C.F. Wan and C.S. Rubin. 1984. Biogenesis, transit, and functional properties of the insulin proreceptor and modified insulin receptors in 3T3-L1 adipocytes. Use of monensin to probe proreceptor cleavage and generate altered receptor subunits. Biochemistry 23:6555-6565.

Schumacher, R., M.A. Soos, J. Schlessinger, D. Brandenburg, K. Siddle and A. Ullrich. 1993. Signaling-competent receptor chimeras allow mapping of major insulin receptor binding domain determinants. JBC 268,No.2:1087-1094.

Shoelson, S.E., M.Boni-Schnetzler, P.F. Pilch and C.R. Kahn. 1991. Autophosphorylation within insulin receptor ß-subunits can occur as an intramolecular process. Biochemistry 30:7740-7746.

Shoelson, S.E., S. Chatterjee, M. Chaudhuri and M.F. White. 1992. YMXM motifs of IRS-1 define substrate specificity of the insulin receptor kinase. PNAS USA 89:2027-2031.

Shoelson, S.E., J. Lee, C.S. Lynch, J.M. Backer and P.F. Pilch. 1993. BpaB25 insulins. Photoactivatable analogues that quantitatively cross-link, radiolabel, and activate the insulin receptor. JBC 268,No.6:4085-4091.

Simpson, I.A. and J. A. Hedo. 1984. Insulin receptor phosphorylation may not be a prerequisite for acute insulin action. Science 223:1301-1303.

Smith, R.M., T. Sasaoka, N. Shah, Y. Takata, J. Kusari, J.M. Olefsky and L. Jarett. 1993. A truncated human insulin receptor missing the COOH-terminal 365 amino acid residues does not undergo insulin-mediated receptor migration or aggregation. Endocrinology 132,No.4:1453-1462.

Sun, X.J., M. Miralpeix, M.G. Myers Jr., E.M. Glasheen, J.M. Backer, C.R. Kahn and M.F. White. 1992. Expression and function of IRS-1 in insulin signal transmission. JBC 267,No.31:22662-22672.

Sung, C.K.. 1992. Insulin receptor signalling through non-tyrosine kinase pathways: evidence from anti-receptor antibodies and insulin receptor mutants. Journal of Cellular Biochemistry 48,No.1:26-32.

Sung, C.K. and I.D. Goldfine. 1992. Phosphatidylinositol-3-kinase is a non-tyrosine phosphorylated member of the insulin receptor signalling complex. BBRC 189,No.2:1024-1030.

Sung, C.K., X.-L. Han, A. Brunetti, B. Maddux, R. Yamamoto-Honda and I.D. Goldfine. 1992. Regulation of biological functions by an insulin receptor monoclonal antibody in insulin receptor ß-subunit mutants. Biochemistry 31,No.1:168-174.

Suzuki, S., K. Sugawara, Y. Satoh and T. Toyota. 1991. Insulin stimulates the generation of two putative insulin mediators, inositol-glycan and diacylglycerol in BC3H-1 myocytes. JBC 266,No.13:8115-8121.

Suzuki, S., Y. Taneda, S. Hirai, R. Yamamoto-Honda and T. Toyota. 1992. Mutated insulin receptor Val996 reduces insulin-dependent generation of inositol glycan and diacylglycerol. Diabetes 41,No.11:1373-1379.

Tavare, J.M., B. Clack and L. Ellis. 1991. Two-dimensional phosphopeptide analysis of the autophosphorylation cascade of a soluble insulin receptor tyrosine kinase. JCB 266,No.3:1390-1395.

Thies, R.S., N.J. Webster and D.A. McClain. 1990. A domain of the insulin receptor required for endocytosis in rat fibroblsts. JBC 265,No.17:10132-10137.

Tobe, K., T. Kadowaki, K. Hara, Y. Gotoh, H. Kosako, S. Matsuda, H. Tamemoto, K. Ueki, Y. Akanuma, E. Nishida and Y. Yazaki. 1992. Sequential activation of MAP kinase activator, MAP kinases, and S6 peptide kinase in intact rat liver following insulin injection. JBC 267,No.29:21089-21097.

Ullrich, A., J.R. Bell, E.Y. Chen, R. Herrera, L.M. Petruzzelli, T.J. Dull, A. Gray, L. Coussens, Y.-C. Liao, M. Tsubokawa, A. Mason, P.H. Seeburg, C. Grunfeld, O.M. Rosen and J. Ramachandran. 1985. Human insulin receptor and its relationship to tyrosine kinase family of oncogenes. Nature 313:756-761.

Ullrich, A. and J. Schlessinger. 1990. Signal transduction by receptors with tyrosine kinase activity. Cell 61:203-212.

Walaas, O. and S. I. Walaas. 1988. Second messengers identified for insulin? TIPS 9:151-152.

Waugh, S.M., E.E. DiBella and P.F. Pilch. 1989. Isolation of a proteolytically derived domain of the insulin receptor containing the major site of cross-linking/binding. Biochemistry 28:3448-3455.

Wedekind, F., K. Baer-Pontzen, S. Bala-Mohan, D. Choli, H. Zahn and D. Brandenburg. 1989. Hormone binding site of the insulin receptor - analysis using photoaffinity-mediated advin complexing. Biol. Chem. Hoppe-Seyler 370:251-258.

Welsh, G.I. and C.G. Proud. 1993. Evidence for a role for protein kinase C in the stimulation of protein synthesis by insulin in swiss 3T3 fibroblasts. FEBS Letters 316,No.3:241-246.

Wilden, P.A., C.R. Kahn, K. Siddle and M.F. White. 1992. Insulin receptor kinase domain autophosphorylation regulates receptor enzymatic function. JBC 267,No.23:16660-16668.

Yamada, K., E. Goncalves, C.R. Kahn and S.E. Shoelson. 1992. Substition of the insulin receptor transmembrane domain with the c-neu/erbB2 transmembrane domain constitutively activates the insulin receptor kinase in vitro. JBC 267,No.18:12452-12461.

Yamamoto, K., D. Altschuler, E. Wood, K. Horlick, S. Jacobs and E.G. Lapetina. 1992. Association of phosphorylated insulin-like growth factor-I receptor with the SH2 domains of phosphatidylinositol 3-kinase p85. JBC 267,No.16:11337-11343.

Yip, C.C., H. Hsu, R.H. Patel, D.M. Hawley, B.A. Maddux and I.A. Goldfine. 1988. Localization of the insulin-binding site to the cysteine-rich region of the insulin receptor α-subunit. BBRC 157,No.1:321-329.

Yip, C.C., C. Grunfeld and I.D. Goldfine. 1991. Identification and characterization of the ligand-binding domain of insulin receptor by use of an anti-peptide antiserum against amino acid sequence 241-251 of the α subunit. Biochemistry 30:695-701.

Yip, C.C.. 1992. The insulin-binding domain of insulin receptor is encoded by exon 2 and exon 3. Journal of Cellular Biochemistry 48,No.1:19-25.

Yip, C.C. and E. Jack. 1992. Insulin receptors are bivalent as demonstrated by photoaffinity labelling. JBC 267,No.19:13131-13134.

Yonezawa, K., H. Ueda, K. Hara, K. Nishida, A. Ando, A. Chavanieu, H. Matsura, K. Shii, K. Yokono, Y. Fukui, B. Calas, F. Grigorescu, R. Dhand, I. Gout, M. Otsu, M.D. Waterfield and M. Kasuga. 1992. Insulin-dependent formation of a complex containing an 85-kDa subunit of phosphatidylinositol 3-kinase and tyrosine-phosphorylated insulin receptor substrate. JBC 267,No.36:25958-25965.

Yonezawa, K., K. Yokono, K. Shii, W. Ogawa, A. Ando, K. Hara, S. Baba, Y. Kaburagi, R. Yamamoto-Honda, K. Momomura, T. Kadowaki and M. Kasuga. 1992. In vitro association of phosphatidylinositol 3-kinase activity with the activated insulin receptor tyrosine kinase. JBC 267,No.1:440-446.

APPENDIX B

APPB-i
ABSTRACT

We have previously observed that SRPRα and preprolactin are not translated with equal efficiency even when the two coding regions are flanked by the same 5′ and 3′ UTRs (unpublished data). Therefore, the extent to which the coding region is involved in translational efficiency was examined to determine if it may play a significant role in expression of the insulin receptor. While we have shown that sequences in the coding region can significantly inhibit translation it remains to be determined whether improvement can be achieved by changing these sequences. The implications of these results for expressing the insulin receptor and other cloned genes in oocytes are discussed.

APPB-ii
INTRODUCTION

The effect of the coding region on translational efficiency is not clearly defined, but there are a growing number of examples of how the coding region affects the ability to translate a given transcript both in cell free lysates and *in vivo* (reviewed in Kozak, 1987 and 1991; Baim and Sherman, 1988; Wolin and Walters, 1988 and 1989; Rabinovich and Kreinin, 1991; Kim et al., 1991; Krasheninnokov et al., 1991; Doohan and Samual, 1992; Tate and Brown, 1992; Kim and Hollingsworth, 1992).

One mechanism by which the coding region controls expression is the translational pause (Wolin and Walters, 1988 and 1989; Kim et al., 1991; Kim and Holligsworth, 1992). Experimentally, using a novel nuclease protection assay, ribosomes have been shown to "pause" and stack over a 29 nt stretch at 2 positions on preprolactin RNA, at GGC codons, in both wheat germ and reticulocyte lysate translation reactions (Wolin and Walters, 1988). This same assay was used in an *in vivo* study to show pause sites in spinach chloroplast mRNA (Kim and Hollingsworth, 1992). While pause sites have been identified, the extent to which they affect total synthesis is unknown. While it is clear that the ribosomes stop at these sites and thereby decrease the rate of elongation, the sequences responsible for signalling pausing of ribosomes on internal sites of an mRNA have not been clearly defined. Furthermore, the mechanism(s) involved in ribosome pausing is as yet unknown.

In a more general study, the translation of total mitochondrial polypeptides was followed over time (Rabinovich and Kreinin, 1991). The translation patterns of the major proteins being synthesized fit into 3 groups according to their rates of synthesis over time. Some of the mitochondrial proteins also displayed pausing of the ribosomes within the coding region of the RNA (Rabinovich and Kreinin, 1991). The same pattern of a non-uniform distribution of ribosomes was also demonstrated for translation of chloroplast membrane protein mRNA, rabbit globin mRNA and reoviral S1 mRNA (Kim et al., 1991; Krasheninnkov et al., 1991; Doohan and Samual, 1992). All of these studies support a model in which elongation occurs at different rates along the length of an RNA and this results in a non-uniform distribution of ribosomes (Rabinovich and Kreinin, 1991). The rate of translation initiation and elongation is also affected by the nucleotide sequence immediately 3′ of the initiation site (Liebhaber et al., 1992). These studies clearly demonstrate that the coding region of an RNA can be involved in controlling translation and therefore gene expression.

Even with optimized untranslated regions the SRP receptor α subunit was one of the poorest expressing clones tested in the 5′ UTR and 3′ UTR studies (Mina Falcone, unpublished data). It was therefore a good candidate for examining the putative effects of the coding region on translational efficiency. We have identified a region of SRP receptor alpha subunit which inhibits translation both in reticulocyte lysate and microinjected Xenopus oocytes. The effects of deleting this region on translational efficiency of SRPRα are presented here, along with experiments to more precisely define the sequence involved in inhibition of translation.

TABLE 1

CONSTRUCTS

MADE BY OR REF.	5'UTR	CODING REGION	3'UTR	MAC PLASMID
D.FALCONE	UTR	SRPRalpha - full length	ENDOGENOUS	191
D.W.ANDREWS	"	SRPRalpha - nt 1-232 deleted	"	3
J.YOUNG	"	SRPRalpha - nt 1-114 deleted	"	458
J.YOUNG	"	SRPRalpha - nt 114-237 deleted	"	459
D.W.ANDREWS	"	SRPRalpha - nt 1-84 deleted	"	55
A.VASSIL./ D.W.ANDREWS	"	SRPRalpha - nt 469-752 deleted	"	456
A.VASSIL./ D.W.ANDREWS	"	SRPRalpha - nt 1186-1623 deleted	"	457
A.VASSIL.	"	SRPRalpha - nt 1-232/469-752 deleted	"	489
A.VASSIL.	"	glycoGLOBIN(N terminal 70nt) SRPRalpha (nt 528-735) protein A	ENDOG. prot.A	518
F.JANIAK	"	glycoGLOBIN(N terminal 70nt) protein A	"	486

TABLE 1
CONSTRUCTS
Table one summarizes the 5′ UTR, coding region and 3′ UTR for each construct. Each construct is referenced to the researcher responsible for constructing this version of the prolactin or SRPRα clones.

APPB-iii
RESULTS AND DISCUSSION

EFFECTS OF CODING REGION ON TRANSLATIONAL EFFICIENCY IN RETICULOCYTE LYSATE (RRL) AND WHEAT GERM EXTRACT (WG)

Experiments done previously in this laboratory suggested a role for the coding region in translational efficiency in RRL and WG extract reactions (D. Falcone, unpublished data). The results from this study are presented in Figure 1a, 1b and 1c. Translation of a variety of coding regions shows a consistent pattern of expression which is independent of the 5′ and 3′ UTRs (D. Falcone unpublished data used to generate Figure 1a panels KD, UTK and UTR). For example, prolactin transcript is consistently translated better than SRPRα. The only variable that correlates with translational efficiency in these experiments was the coding region, suggesting that the coding region plays a role in the translational efficiency of a given transcript. In these experiments the 3′ UTR was removed by linearizing the plasmids after the translation termination codon. While this eliminates differences in the 3′ context of the coding regions, we previously showed that the 3′ UTR does not affect translational efficiency or RNA stability in RRL translations (chapter III-2).

The coding regions were of a variety of lengths ranging from 142 to 638 amino acids. It was possible that the differences in translation were simply related to the length of the coding region translated. If this correlation was correct the insulin proreceptor, which is 180,000 Da in size (Ullrich et al., 1985), would be very poorly translated in spite of the optimized UTR'S. The relative protein synthesis in both wheat germ and RRL was determined by densitometric scanning of the fluorograms and correction for the number of methionines in each coding region (D. Falcone, unpublished data). This was plotted against the length of the coding region (Figure 1b). No correlation between the length of coding region and level of protein synthesis was observed. For example, PrP and protein A are approximately the same size and yet in RRL protein A is translated 10 fold better than PrP (Figure 1b compare translation of constructs indicated by <).

Because the genetic code is degenerate, in most cases there is more than one codon, and therefore more than one tRNA for an amino acid. The codon usage in a given species varies considerably and the availability of tRNA molecules and amino acyl tRNA synthetases for a particular codon may reflect the codon usage (For review see Shields, 1990; and codon usage table for several hundred species given in Wada et al., 1992). Studies in which mammalian proteins were expressed in bacteria or yeast show that when expressing cloned genes from other

species, synthesis may be impaired if there are a large number of rare codons present (e.g. Mandrup et al., 1991; Adams et al., 1992; Demoulder et al., 1992). Furthermore, even within a given species codon usage has been correlated with the level of expression and mRNA translation rate for various genes (e.g. Sharp et al., 1988; Sharp and Devine, 1989; Sorenson et al., 1989; Karlin et al., 1990). Although the RRL and WG translation reactions are supplemented with exogenous tRNA, amino acyl tRNA synthetases may still be limmiting. Analysis of the rare codon content of the coding regions for the 5 rarest codons in rabbit (source of the reticulocyte lysate) is shown in Figure 1c. The content of a particular rare codon does not correlate with synthesis (compare CGA: Prolactin - 20/1000, cSRC - 4/1000).

Together these results suggest that some other feature of the coding region influences the rate of translation.

FIGURE 1
EFFECT OF THE CODING REGION ON TRANSLATIONAL EFFICIENCY IN CELL-FREE TRANSLATIONS

FIGURE 1a

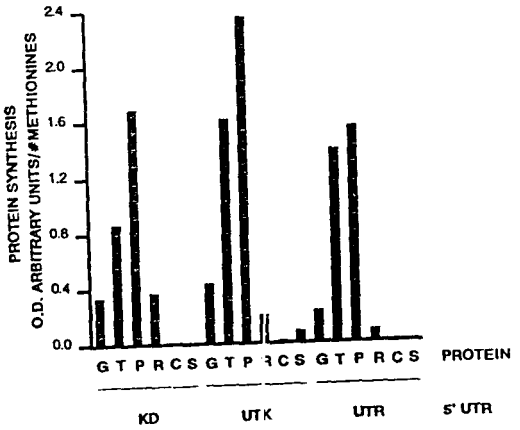


FIGURE 1b

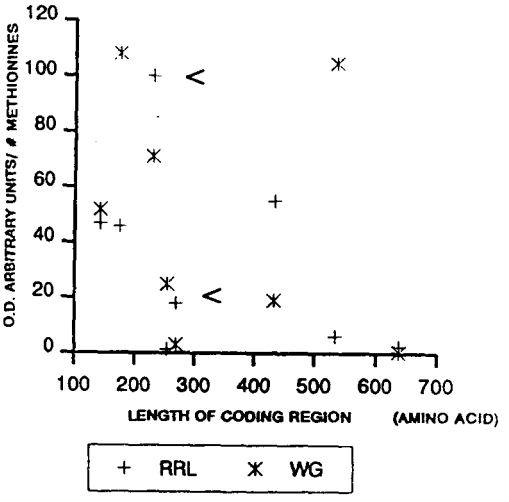


FIGURE 1c

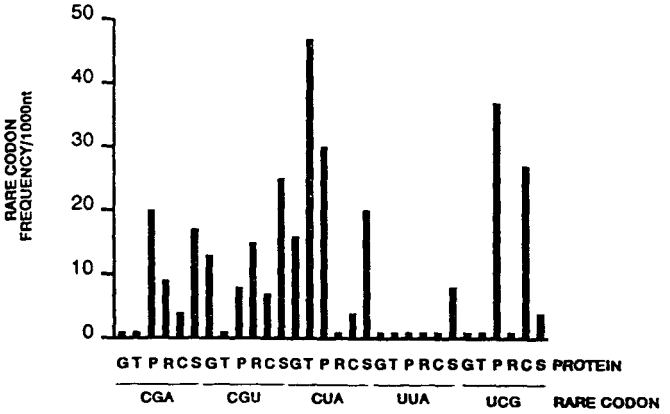


FIGURE 1a
EFFECT OF THE CODING REGION ON TRANSLATIONAL EFFICIENCY IN RRL
In vitro synthesized transcripts were normalized by fluorometry and translated in RRL for 1 hour. Translation products were separated by SDS-PAGE and the gels fluorographed, dried and exposed to film. Protein synthesis was quantified by densitometric scanning of the fluorograms. The results are plotted as protein synthesis (O.D. in arbitrary units- corrected for the number of methionines in the coding region) vs the 5' UTR and the coding region [G-globin, T-truncated bovine prolactin, P-bovine prolactin, R-prion related protein, C-cSRC, S-SRD4(SRPR deletion mutant)]. Data provided by MINA FALCONE.

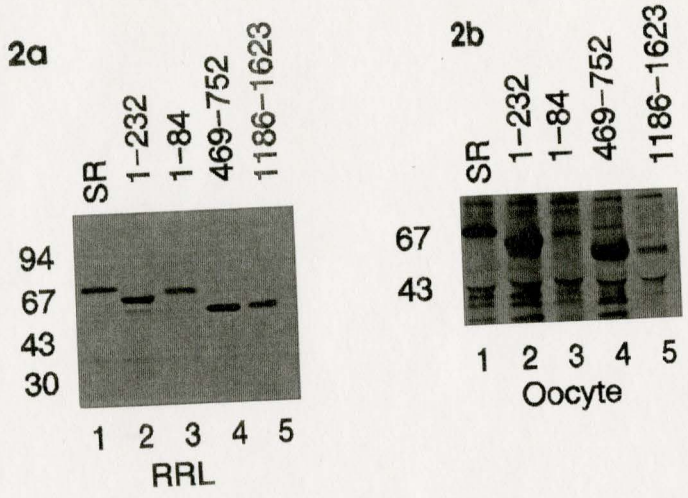
FIGURE 1b
EFFECT OF CODING REGION LENGTH ON TRANSLATIONAL EFFICIENCY IN RRL AND WG
In vitro synthesized transcript was normalized by fluorometry and translated for 1 hour in RRL or WG. Translation products were separated by SDS-page and protein synthesis quantified as in Figure 1a. The results are plotted as protein synthesis (O.D. in arbitrary units corrected for the number of methionines) vs length of coding region. CODING REGIONS (abbrev. as in 1a) AND LENGTH - G (142aa), T (174aa), P (229aa), R (254aa), C (533aa), ATIII (432aa), SRPRa (638aa), Protein A (269aa). Data provided by MINA FALCONE.

FIGURE 1c
CODON USAGE
The coding sequences of the proteins used for the experiment described in Figure 1a were analyzed for the incidence of 5 rare rabbit codons (<5/1000). The results are plotted as frequency/1000nt vs coding region and rare codon. Abbreviations are as in Figure 1a.

TRANSLATIONAL EFFICIENCY OF SRPRα DELETION MUTANTS

SRPRα was one of the most poorly expressed of the coding regions studied. Therefore it was selected for further study. A variety of deletion mutants were available from previous studies on SRPRα function (Table 1). Expression of the deletion mutants was tested in RRL and in Xenopus oocytes in an attempt to identify regions responsible for inhibiting translation. Two regions of SRPRα were identified which, when deleted, increased translation of SRPRα in both RRL and Xenopus oocytes (Figure 2a and 2b compare SRPRα, 1-232 and 469-752 NOTE: deletion mutants are indicated by the nucleotide numbers deleted in the construct. Nucleotides are numbered from the A residue of the initiating methionines). Deletion of another region, nt 1186-1623, similar in size to 469-752, did not result in increased translation compared to full length SRPRα (NOTE: deletion of nt 1186-1623 removes 5 methionine residues and so quantitative comparrisson between this mutant and the other mutants requires correction of the densitometric data for methionine number, Figure 2c). This is consistent with the results in figure 1b in which the length of coding region per se does not decrease translation, i.e. decreasing the length of the coding region per se in mutant 469-752 is not responsible for the observed increase in translation (2a, 2b and 2c). The mutant in which both inhibitory regions were deleted translated better than either of the single deletion mutants (Figure 2c 1-232/469-752). Results from densitometric scanning of fluorograms from several RRL experiments were normalized to full length SRPRα and the results plotted as synthesis vs coding region (Figure 2c). The 1-232 region did not improve translation to the same extent consistently, resulting in the largest error of the single deletion mutants.

TRANSLATIONAL EFFICIENCY OF SRPRα DELETION MUTANTS IN RRL AND XENOPUS OOCYTES



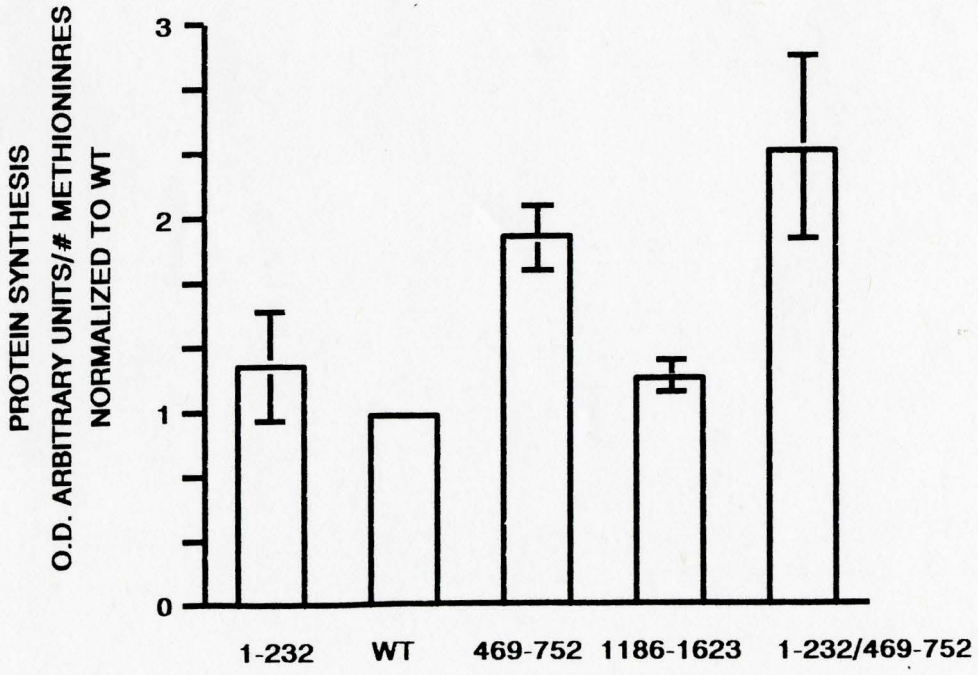


FIGURE 2a-c
TRANSLATIONAL EFFICIENCY OF SEVERAL SRPRα DELETION MUTANTS IN RRL AND XENOPUS OOCYTES
For both the RRL and oocyte experiments *in vitro* synthesized transcript was normalized by fluorometry. RRL translations and treatment of translation products were as described in 1a. 2a is a representative autoradiogram of results obtained from RRL translations. Results of densitometric scanning of fluorograms from several such experiments are shown in 2c. Results are plotted as protein synthesis (corrected for number of methionines and normalized to WT) vs SRPRα mutant [full length SRPRα is shown as WT, nucleotides deleted is given for the mutants and (numbered from A of the initiating AUG)]. Figure 2b is representative of the results obtained in Xenopus oocytes. Oocytes were microinjected with normalized transcript:^{35} (1:1 mixture) and incubated for 6 hours. SRPRα (full length and deletion mutant) translation products were immunoprecipitated, separated by SDS-PAGE and the gels fluorographed, dried and exposed to film.

In making such extensive deletions in the SRPRα molecule there is the possibility the observed increase in translation represents changes in the stability of the transcript in RRL or oocytes. Functional RNA stability was tested by adding transcript to unlabelled RRL reactions or injecting transcript without label into oocytes and incubating for 30 minutes and 3 hours respectively. ^{35}S methionine was then added after the incubation and samples incubated another 30 minutes (RRL) and 5 hours (oocytes). The relative translational efficiency was not affected suggesting that no one transcript was more or less stable during translation and that the rate of translation was responsible for the observed differences (DNS).

The rate of initiation of full length SRPRα was compared to both 1-232 and 469-742 deletion mutants (table 2). Both mutants had higher rates of initiation than full length SRPRα (table 2).

TABLE 2
RATE OF TRANSLATION INITIATION OF SRPRα MUTANTS

TABLE 2

CONSTRUCT	INIT.RATE	MAC PLASMID
FULL LENGTH	499	3
1-232	1909	191
469-752	1348	456
1-232/469-752	3141	489

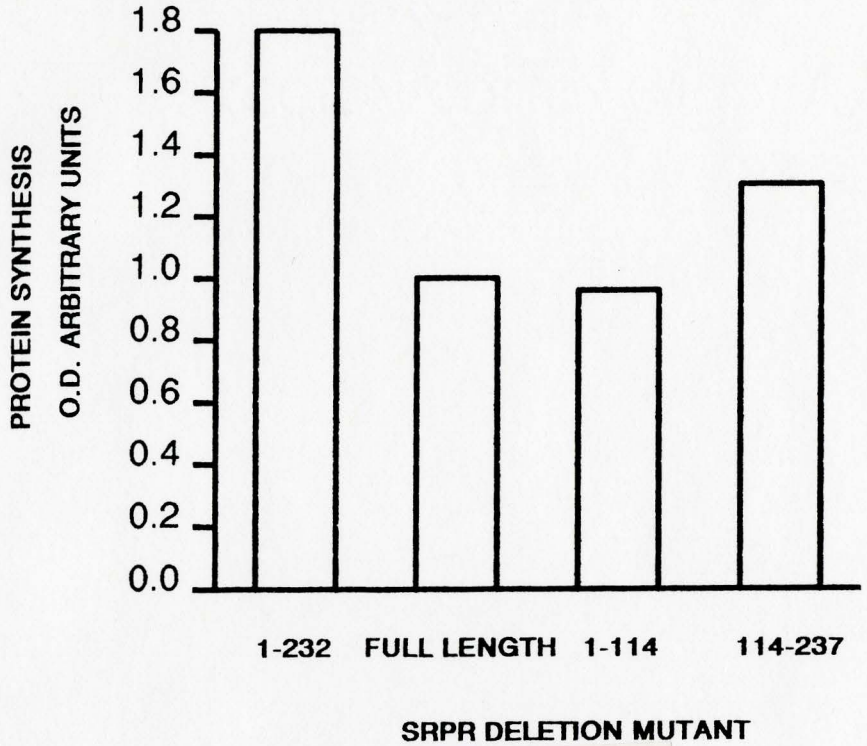
TABLE 2
RATE OF INITIATION OF SRPRα MUTANTS
Cumulative initiation was measured by removing aliquots of RRL translations at 0, 3, 6, 9, 15, 20, 25 minutes and adding them to 4mM 7-methylguanosine and 0.1mM aurin tricarboxylic acid to block new initiations. Translation was continued for 60 minutes to ensure complete synthesis of molecules initiated prior to addition of inhibitors. Results of densitometric scanning of the full length products (O.D. in arbitrary units) were plotted against the time of addition of initiation inhibitors to give a plot of cumulative initiations. The slope of the resulting line gave the cumulative initiations/minute. The rate of translation initiation is presented as cumulative initiations (protein synthesis O.D. in arbitrary units)/time (min).

The distribution of rare codons over the coding region does not correlate with the inhibition of translation. The frequency of rare codons in both of the inhibitory regions (1-232 and 469-742) is lower than the incidence in full length SRPRα. Therefore it is unlikely that differences in codon usage account for the relative translational efficiency of these regions.

To define more precisely the region responsible for inhibition in the 1-232 region smaller deletions were analyzed. These deletions were non-overlapping and together covered the entire 1-232 region. The translational efficiency of these deletion mutants in RRL was not significantly higher than full length SRPRα (Figure 3a and 3b). Deletion mutant 114-237 was translated slightly better than full length but not to the extent of the mutant with entire deletion (Figure 3a and 3b compare 114-237 and 1-232).

FIGURE 3
EFFECT OF NON-OVERLAPPING DELETIONS IN THE 1-232 REGION OF SRPRα ON TRANSLATIONAL EFFICIENCY IN RRL.

3a



3b

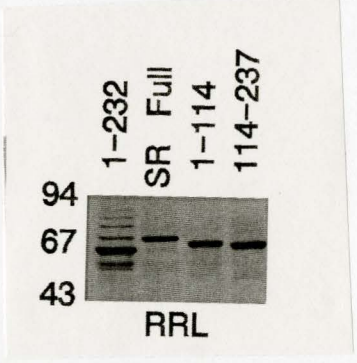


FIGURE 3
EFFECT OF NON-OVERLAPPING DELETIONS IN THE 1-232 REGION OF SRPRα ON TRANSLATIONAL EFFICIENCY IN RRL.
In vitro synthesized transcript was normalized by fluorometry andtranslated for 1 hour in RRL. Translation products were separated by SDS-PAGE and the gels fluorographed, dried and exposed to film. Figure 3b is representative of the results obtained. In 3a the results shown in 3b were quantified by densitometry and the results plotted as protein synthesis (O.D. in arbitrary units normalized to full length SRPRα) vs SRPRα construct.

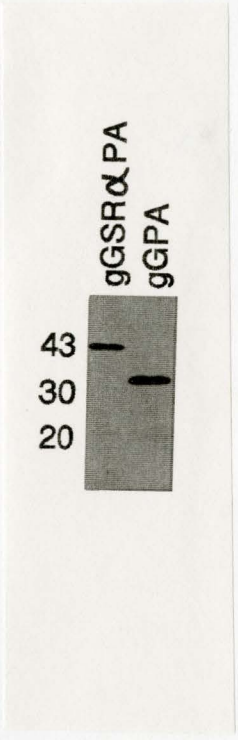
These results suggest that the increased translation of the 1-232 deletion mutant was not due to the removal of an inhibitory region but likely the result of increased translation initiation due to changing the region immediately 3′ of the translation start site. A number of studies have shown that the region surrounding the initiation site can affect the translational efficiency of a protein (Sharp et al., 1988; Gross et al., 1990; Liebhaber et al., 1992; reviewed in Kozak, 1991). In addition, Liebhaber et al. (1992) showed that secondary structure downstream of the initiating AUG could inhibit translation. This inhibition was dependent on the secondary structure being close to the initiating AUG (Liebhaber et al., 1992). In an earlier study, in which secondary structure was introduced between the coding region and the 3′ UTR translation was not inhibited (Lingelbach and Dobberstein, 1988). These studies suggest that secondary structure around the initiating AUG inhibits translation by inhibiting initiation and not elongation. Given that the 1-232 region changes the region immediately 3′ of the start site it is possible that the observed effect on translation is due to changes in secondary structure. Analysis of the first 50 nucleotides after the start site of full length SRPRα and the deletion mutants showed that 1-232 has a 44% G/C content while the others are all about 56% G/C content (see Appendix 2 for data). The difference in the free energy, and possibly secondary structure, resulting from fewer G/C residues 3′ of the start site, while not dramatic, may explain the observed differences in translational efficiency. This is also consistent with the finding that the rate of initiation of the 1-232 deletion mutant was higher than full length SRPRα (table 2).

Deleting the region from nucleotide 469-752 consistently increased translation 2-3 fold over full length SRPRα (Figure 2c). The increase in synthesis upon deletion of this region was also higher than the 1-232 region. Furthermore the 469-752 deletion mutant is more interesting in that the inhibiting region appears to occur after 1/3 of the SRPRα molecule has been translated. Removal of this region (deletion mutant 469-752) also increases the rate of initiation compared to full length SRPRα. Ribosomes have been shown to stack 5′ of pause sites during translation and in doing so may inhibit the rate at which new ribosomes can initiate translation (Wolin and Walters, 1988)). Alternatively sequences in the 469-752 region may inhibit translation by interacting with other regions of SRPRα RNA. While it is possible that secondary structure is involved in the observed inhibition, evidence from other studies has shown that secondary structure per se is not sufficient to inhibit the movement of ribosomes along an RNA molecule (Lingelbach and Dobberstein, 1988). If this region is a general inhibitor

of translation the effect should be transferable to an unrelated coding region. A passive passenger routinely used in this laboratory for targeting studies is gPA [a glycoglobin /protein A fusion protein](Janiak et al., submitted). To determine whether the 469-752 region can inhibit translation independent of the rest of the coding region, nucleotides 528-735 of SRPRα were cloned into gPA to produce gGSRαPA. The translational efficiency of this construct was 2 fold less than gPA (Figure 4a and 4b). The inhibition of translation by this region in gPA is consistent with the observed 2 fold increase in translation when it is removed from SRPRα suggesting a sequence in the 528-735 region can inhibit translation independent of the rest of the SRPRα coding region. Consistent with this result, mapping of ribosome pause sites in SRPRα has identified a pause in this region (J. Young, unpublished data). These results suggest that sequences in the 469-752 region of SRPRα are sufficient for inhibiting translation. However, further studies are required to determine more precisely the sequences involved in inhibiting translation in SRPRα. Furthermore, the 469-752 region may not contain the only coding sequence involved in regulating translation of SRPRα. In Figure 1a the translation of SRD4, a deletion mutant of SRPRα, is 30 fold lower than preprolactin, the highest expressed coding region. Both the SRD4 and preprolactin constructs have the same 5′ UTR, suggesting the diffences observed are due to the effects of the coding region on translation. Deletion of region 469-752 improves translation only 2-3 fold, suggesting there are other sequences in the coding region of SRPRα involved in translational efficiency. When the effects of these sequences are combined they may affect translation to even a greater extent than the 5′ UTR.

FIGURE 4a and 4b
EFFECT OF INSERTING SRPRα nt 528-735 (gGSRPA) INTO GLYCOGLOBIN/PROTEIN (gGPA) ON TRANSLATIONAL EFFICIENCY IN RRL

4a



4b

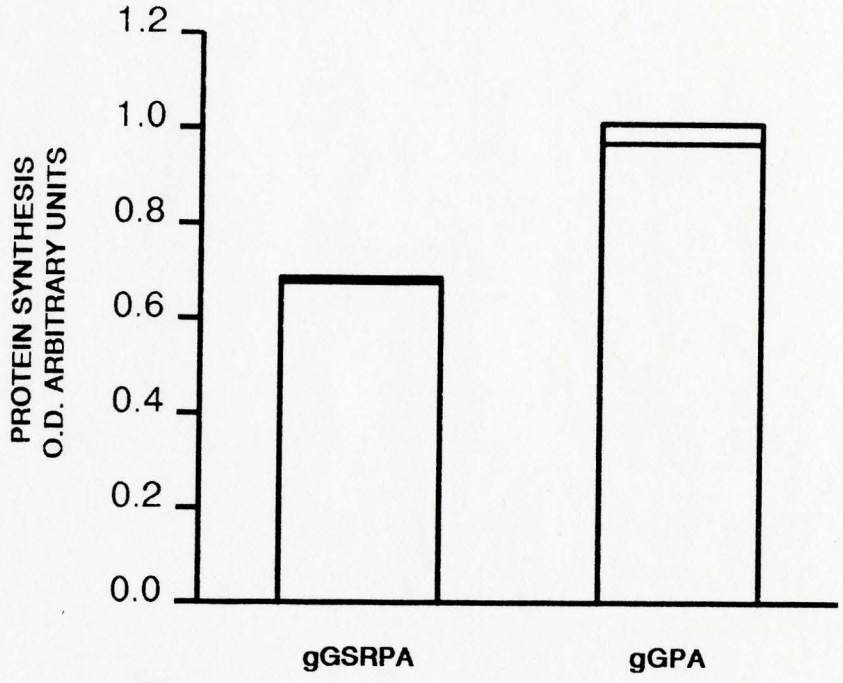


FIGURE 4
EFFECT OF INSERTING SRPRα nt 528-735 (gGSRPA) INTO GLYCOGLOBIN/PROTEIN (gGPA) ON TRANSLATIONAL EFFICIENCY IN RRL
In vitro synthesized transcript was normalized by fluorometry and translated for 1 hour in RRL. Translation products were separated on SDS-PAGE, the gels fluorographed, dried and exposed to film. A typical result is shown in Figure 4a. Figure 4b shows the result of densitometric scanning of the fluorograms from 2 experiments done in duplicate (normalized to gGPA).

APPB-iv
CONCLUSION

There is growing evidence in the literature that the coding region of mRNA molecules can be involved in the control of gene expression. The observations described above are consistent with previous studies which demonstrated that coding sequences in a variety of mRNA species affect translational efficiency and that ribosome distribution on an mRNA is non-uniform during translation (Rabinovich and Kreinin 1991). Further characterization of these regions and the precise sequences involved will allow elucidation of the mechanisms by which they affect translation. As coding sequences which affect translation are identified and characterized, the significance of the coding region in control of protein expression will be fully understood. Furthermore, these studies will determine the relative importance of the coding region in translation in general. In the case of SRPRα the coding region was found to affect translation to a similar extent as the 5′ UTR. Replacement of the endogenous 5′ UTR and start site of SRPRα with the UTK leader results in a 4-5 fold increase in translation (III-1 Figure 3). Removal of the 469-752 region improved translation 2-3 fold over full length SRPRα. While this is only one example, the results make it clear that translational efficiency can be as dependent on the coding region as well as the 5′ UTR. For this reason when attempting to express the insulin receptor in Xenopus oocytes we must also consider the effects of the coding region on translational efficiency. If each inhibitory region identified decreases translation by a factor of 2, as does the SRPRα469-752 region, then the combined effects of the entire coding region may affect translation considerably, especially in Xenopus oocytes where the effects of the coding region were more pronounced (compare Figures 2a and 2b).

The mechanisms by which the coding region affects translation is not understood at this time and therefore, there is no *a priori* basis for changes in coding sequence that could improve translation. Until these mechanisms are better understood, optimizing the 5′ and 3′ regions will be the only reliable method of improving translation through manipulation of the DNA sequence. Deletion analysis, the experimental approach taken to identify inhibitory regions in the SRPRα, may be too difficult for larger molecules such as the insulin receptor. There are likely many regions involved in translational efficiency and removal of just one may not result in as significant a change in insulin receptor expression as seen in SRPRα. The gGPA construct provides a convenient method to screen for inhibitory regions in the insulin receptor. Instead of deleting regions and looking for increased insulin receptor expression, regions of the insulin receptor can be cloned into gPA and tested for translation

inhibition. The smaller size and fewer restriction enzyme sites in gPA make further analysis of inhibitory regions easier. Furthermore, it would not be feasible to map translational pause sites in insulin receptor mRNA by the primer extension assay described by Wolin and Walters (1988) because of the number of primers necessary to cover the entire molecule. If an inhibitory region is found by using gGPA constructs with insulin receptor sequences, then the pause could be easily mapped in the gG-IR-PA construct using the same gG primer for all the constructs with different insulin receptor regions. Once the inhibitory sequences are precisely mapped it may be possible to change the DNA sequence to remove inhibition without changing the amino acid sequence of the insulin receptor.

The results presented here establish that the coding region plays a significant role in translational efficiency. Furthermore, the approach taken can be used in the future to identify inhibitory sequences in the insulin receptor and other cloned genes.

APPENDIX B
REFERENCES

Adams, T.E., B. MacIntosh, M.R. Brandon, P. Wordsworth and N.K. Puri. 1992. Production of methionyl-minus ovine growth hormone in Escherichia coli and one-step purification. Gene 122:371-375.

Baim, S.B. and F. Sherman. 1988. mRNA structures influencing translation in the yeast sccharomyces cerevisiae. Molecular and Cellular Biology 8,No.4:1591-1601.

Demolder, J., W. Fiers and R. Contreras. 1992. Efficient synthesis of secreted murine interleukin-2 by Saccharomyces cerevisiae: influence of 3'-untranslated regions and codon usage. Gene 111:207-213.

Doohan, J.P. and C.E. Samuel. 1992. Biosynthesis of reovirus-specified polypeptides; ribosome pausing during the translation of reovirus S1 mRNA. Virology 186No.2:409-425.

Gross, G., C. Mielke, I. Hollatz, H. Blocker and R. Frank. 1990. RNA primary sequence or secondary structure in the translational initiation region controls expression of two variant interferon-ß genes in Escherichia coli. Journal of Biological Chemistry 265,No.29:17627-17636.

Karlin, S., E.E. Blaisdell and G.A. Schachtel. 1990. Contrasts in codon usage of latent versus productive genes of Epstein-Barr virus: data and hypothesis. Journal of Virology 64,No.9:4264-4273.

Kim, J., P.G. Klein and J.E. Mullet. 1991. Ribosomes pause at specific sites during synthesis of membrane-bound chloroplast reaction center protein D1. Journal of Biological Chemistry 266,No.23:14931-14938.

Kim, J. and M.J. Hollingsworth. 1992. Localization of *in vivo* ribosome pause sites. Analytical Biochemistry 206:183-188.

Kozak., M.. 1987. An analysis of 5'-noncoding sequences from 699 vertebrate messenger RNAs. Nucleic Acids Research 15,No.20:8125-8148.

Kozak, M.. 1991. An analysis of vertebrate mRNA sequences: intimations of translational control. Journal of Cell Biology 115,No.4:887-903.

Krashenninikov, I.A., A.A. Komar and I.A. Adzhubei. 1991. Nonuniform size distribution of nascent globin peptides, evidence for pause localization sites, and a cotranslational protein-folding model. Journal of Protein Chemistry 10,No.5:445-453.

Liebhaber, S.A., F. Cash and S.S. Eshleman. 1992. Translation inhibition by an mRNA coding region secondary structure is determined by its proximity to the AUG initiation codon. Journal of Molecular Biology 226:609-621.

Lingelbach, K. and B. Dobberstein. 1988. An extended RNA/RNA duplex structure within the coding region of mRNA does not block translational elongation. Nucleic Acids Research 16,No.8:3405-3414.

Mandrup, S., P. Hojrup, K. Kristiansen and J. Knudsen. 1991. Gene synthesis, expression in Escherichia coli, purification and characterization of the recombinant bovine acyl-CoA-binding protein. Biochemical Journal 276:817-823.

Rabinovich, Y.M. and M.O. Kreinin. 1991. Evidence for non-uniform translation of individual polypeptides in rat liver mitochondria. Biochimica et Biophysica Acta 1089,No.2:193-196.

Sharp, P.M., E. Cowe, D.G. Higgins, K.H. Wolfe and F. Wright. 1988. Codon usage patterns in Escherichia coli, Bacillus subtilis, Saccharomyces cerevisiae, Schizosaccharomyces pombe, Drosophila melonogaster and Homo sapiens; a review of the considerable within-species diversity. Nucleic Acids Research 16:8207-8211.

Sharp, P.M. and K.M. Devine. 1989. Codon usage and gene expression level in Dictyostelium discoideum: highly expressed genes do 'prefer' optional codons. Nucleic Acids Research 17,No.13:5029-5039.

Shields, D.C.. 1990. Switches in species-specific codon preferences: the influence of mutation. Journal of Molecular Evolution 31,No.2:71-80.

Sorensen, M.A., C.G. Kurland and S. Pedersen. 1989. Codon usage determines translation rate in Escherichia coli. Journal of Molecular Biology 207:365-377.

Tate, W.P. and C.M. Brown. 1992. Translational termination: "stop" for protein synthesis or "pause" for regulation of gene expression. Biochemistry 31,No.9:2443-2450.

Wada, K., Y. Wada, F. Ishibashi, T. Gojobori and T. Ikemura. 1992. Codon usage tabulated from the GenBank genetic sequence data. Nucleic Acids Research 20:2111-2118.

Wolin, S.L. and P. Walter. 1988. Ribosome pausing and stacking during translation of a eukaryotic mRNA. EMBO 7,No.11:3559-3569.

Wolin, S.L. and P. Walter. 1989. Signal recognition particle mediates a transient elongation arrest of preprolactin inreticulocyte lysate. Journal of Cell Biology 109:2617-2622.