



Large scale synthesis and purification of iron responsive element RNA for crystallographic studies of its interactions with iron regulatory proteins 1 & 2
by Brian John Eilers

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in
Biological Sciences
Montana State University
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Abstract:

Iron homeostasis is important to organisms in order to prevent various diseases, such as anemia and hemochromatosis, a disease state caused by elevated iron levels that leads to increased levels of reactive oxygen species and death due to stroke, heart attack, diabetes, cirrhosis of the liver or liver cancer, usually by age 50. In addition, misregulation of iron has been associated with many diseases of the central nervous system including Alzheimer's and Parkinson's. Thus, greater insight into the molecular mechanisms of iron transport and homeostasis could lead to strategies to treat or prevent these diseases. Iron regulatory proteins 1&2 are known to play a central role in the maintenance of iron homeostasis. IRPs respond to cellular iron levels by binding to iron responsive elements present in the mRNA corresponding to proteins involved in the transport, use and storage of iron. This interaction serves to appropriately up or down regulate translation of these proteins. A major goal of the laboratory is to obtain the crystallographic structures of the IRP-2/IRE complex. We have worked to express mg amounts of rat IRP-2, and to synthesize mg amounts of the rat ferritin IRE. A mutant IRP-2, IRP-2- Δ DD was created and initial expression trials indicated that it expressed at 0.5mg/L in a *Saccharomyces cerevisiae* expression system. The IRE was produced by enzymatic synthesis using T-7 RNA polymerase. Two strategies were used. The first was a simple run-off transcription reaction. Large scale synthesis of run-off IRE can yield up to 3 mg of RNA per 60 mL reaction. The second method incorporated 5' and 3' flanking hammerhead ribozymes (HHR) in order to produce a homogenous transcript. Initial small scale HHR cleavage experiments indicated that homogenous IRE could be obtained from the HHR containing RNA. In both cases, the T7 transcription reactions were purified by phenol/chloroform extraction, ethanol/isopropanol precipitation, and by size exclusion chromatography. Size exclusion columns effectively removed the majority of the unincorporated NTPs, while a Mono Q column was used to separate IRE from the HHR. Bandshift assays indicated that the run-off IRE could bind to IRPs and was therefore active.

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ELEMENT RNA FOR CRYSTALLOGRAPHIC STUDIES OF ITS INTERACTIONS
WITH IRON REGULATORY PROTEINS 1 & 2

By

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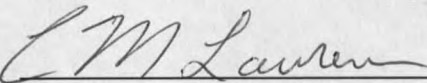
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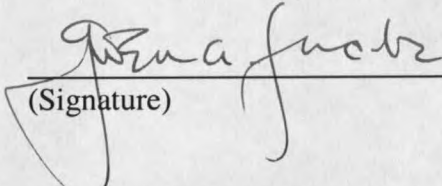
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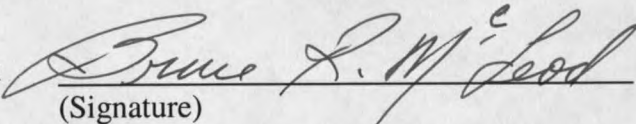
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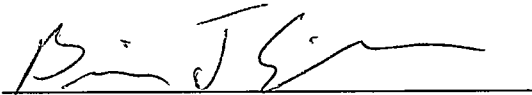
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ABSTRACT

Iron homeostasis is important to organisms in order to prevent various diseases, such as anemia and hemochromatosis, a disease state caused by elevated iron levels that leads to increased levels of reactive oxygen species and death due to stroke, heart attack, diabetes, cirrhosis of the liver or liver cancer, usually by age 50. In addition, misregulation of iron has been associated with many diseases of the central nervous system including Alzheimer's and Parkinson's. Thus, greater insight into the molecular mechanisms of iron transport and homeostasis could lead to strategies to treat or prevent these diseases. Iron regulatory proteins 1&2 are known to play a central role in the maintenance of iron homeostasis. IRPs respond to cellular iron levels by binding to iron responsive elements present in the mRNA corresponding to proteins involved in the transport, use and storage of iron. This interaction serves to appropriately up or down regulate translation of these proteins. A major goal of the laboratory is to obtain the crystallographic structures of the IRP-2/IRE complex. We have worked to express mg amounts of rat IRP-2, and to synthesize mg amounts of the rat ferritin IRE. A mutant IRP-2, IRP-2- Δ DD was created and initial expression trials indicated that it expressed at 0.5mg/L in a *Saccharomyces cerevisiae* expression system. The IRE was produced by enzymatic synthesis using T-7 RNA polymerase. Two strategies were used. The first was a simple run-off transcription reaction. Large scale synthesis of run-off IRE can yield up to 3 mg of RNA per 60 mL reaction. The second method incorporated 5' and 3' flanking hammerhead ribozymes (HHR) in order to produce a homogenous transcript. Initial small scale HHR cleavage experiments indicated that homogenous IRE could be obtained from the HHR containing RNA. In both cases, the T7 transcription reactions were purified by phenol/chloroform extraction, ethanol/isopropanol precipitation, and by size exclusion chromatography. Size exclusion columns effectively removed the majority of the unincorporated NTPs, while a Mono Q column was used to separate IRE from the HHR. Bandshift assays indicated that the run-off IRE could bind to IRPs and was therefore active.

CHAPTER 1

Introduction

Iron Homeostasis

Regulation in Cellular Iron Metabolism

When iron is present as heme, iron-sulfur clusters, or in a direct relation with proteins it has a key role in an extensive number of crucial cellular functions, such as oxygen transport, mitochondrial energy metabolism, electron transport, deoxynucleotide synthesis, or detoxification (1). The free iron (non-protein bound) is generally thought to catalyze the formation of highly reactive free radicals, which are known to damage membranes, proteins and DNA (1). Thus, it is extremely important for both the cells and the organism to maintain iron homeostasis to ensure a healthy supply of iron, but at the same time to prevent excess accumulation of iron. Anemia is usually induced by either a nutritional deprivation or malabsorption in whole organisms that results from an insufficient uptake of iron (1). The opposite occurs in a genetic disorder called hemochromatosis, where iron overload occurs by pathologically increasing the amount of iron uptake. Hemochromatosis will lead to permanent cell and tissue damage because the extracellular iron binding capacity of transferrin as well as the intracellular iron storage capacity of ferritin has exceeded safe limits (1). Disease states associated with hereditary hemochromatosis include heart attack, stroke, diabetes, cirrhosis of the liver, and liver cancer with death usually occurring by age 50. In addition, misregulation of iron is also

implicated in diseases of the central nervous system, including Parkinson's, Alzheimer's, multiple system atrophy, and neuronal brain iron accumulation type 1.

Intracellular iron levels control the expression of key proteins in the iron metabolism of vertebrate cells at the transcriptional level. Specific mRNA-protein interactions in the cytoplasm mediate this regulation. Some mRNAs form specific hairpin structures known as iron responsive elements (IREs) (1). These IREs are recognized by iron-regulatory proteins (IRPs), which have been formerly referred to as IRE-binding protein (IREBP), iron regulatory factor (IRF), or ferritin repressor protein (FRP) (1). Two IRPs have been identified, IRP-1 and IRP-2, that control the rate of mRNA translation or stability (1). Both IRP-1 and IRP-2 demonstrate an ability to bind IREs under conditions of iron deprivation, but when the iron supply to cells is increased they become posttranslationally inactivated (IRP-1) or degraded (IRP-2) (Table 1) (1).

Gene	Low iron: IRP active	High iron: IRP inactive
Single IRE in 5' UTR	Translation inhibited	Translation not inhibited
Ferritin H-chain and L-chain	Iron storage low	Iron storage increased
Ferroportin	Iron storage?	Iron storage
Erythroid 5-ALA-synthase	Porphyrin synthesis diminished?	Porphyrin synthesis normal
Mitochondrial aconitase	?	?
Mitochondrial succinate dehydrogenase subunit b*	?	?
Multiple IREs in 3' UTR	mRNA degradation inhibited	Rapid mRNA degradation
Transferrin receptor	Iron uptake increased	Iron uptake low
DMT-1	Iron transport?	Iron transport?
dCytb	?	?
β -amyloid precursor	?	?

Table 1: Physiological effects of IRE-IRP interactions.

*Present in *Drosophila melanogaster*, but not evident in man.

The posttranscriptional (translational) control mechanisms resulting from IRE/IRP interactions have provided information about cellular iron homeostasis and the maintenance of free cellular iron at the junction between iron uptake, iron storage, and iron incorporation into proteins. Since IREs are present in a variety of mRNAs that function in either of these pathways by encoding the proper protein, the IRE/IRP interactions affect every major aspect of iron metabolism.

Mechanism of Ferritin Translational Control

IREs were first discovered in the 5' untranslated regions (UTR) of ferritin H- and L-chain mRNAs (2). These IREs were found to mediate inhibition of ferritin mRNA translation in iron-deprived cells, but when cellular iron is abundant ferritin synthesis is not halted, this leads to an increase in the iron storage capacity of the cell (2). The physiological significance is that this results in a feedback regulation whereby a free iron pool controls the formation of its own storage sites. This regulation is supported by the fact that inhibition of ferritin mRNA translation (*in vitro*) depends directly on IRP-1 binding to the 5' IREs in the ferritin H- and L-chain mRNA (3).

In the 1960s, iron regulation of ferritin expression was thought to be under translational control. Activation of ferritin synthesis in iron-treated rats was shown to be insensitive to transcriptional inhibitors and associated with a shift in ferritin mRNA from translationally inactive messenger ribonucleoprotein particles to polyribosomes (4). This suggested that a protein was binding to the 5' UTR of ferritin mRNA and suppressed its translation in iron depleted cells. During the 1980s, it was determined that the regulation

of ferritin translation was controlled by IRP-1 binding to an IRE in the 5' UTRs of the ferritin H- and L-chain mRNAs (2). "The IRE is necessary for the posttranscriptional regulation of ferritin expression by iron and suffices to confer IRP-mediated translational control to reporter mRNAs in transfected cells" (1). Polyadenylation of the mRNA template is not required, at least *in vitro*, for IRP-mediated translational repression (5). This indicates that the binding of IRP-1 or IRP-2 to the IRE is sufficient to regulate translation without requiring additional translation factors. Non-IRE sequences on either side of the IRE may affect the structure and function of the IRE, and pre- and posttranslational mechanisms have been shown, *in vivo*, to regulate ferritin expression (6). The IRE/IRP complex inhibits mRNA translation by preventing the small ribosomal subunit (the 43S translation preinitiation complex) from binding to the mRNA (7). The 43S preinitiation complex binds the 5' UTR of the ferritin mRNA, and since the ferritin IRE is located < 40 nucleotides from the 5' end of the mRNA they are both competing for the same site (6). Therefore, the IRE/IRP complex is acting like a steric inhibitor of the 43S preinitiation complex binding (Fig. 1 and Fig. 2)

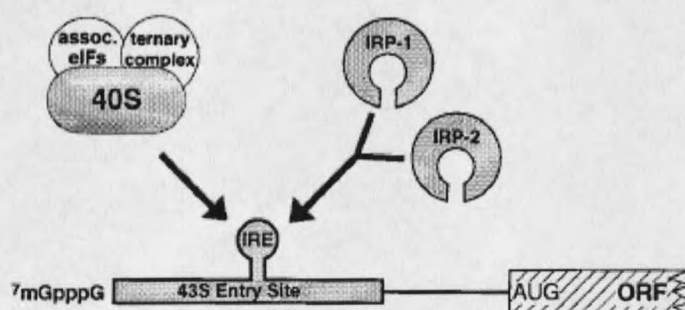


Fig. 1: Illustration showing how IRPs could prevent the ribosomal components from binding to ferritin mRNA (1).

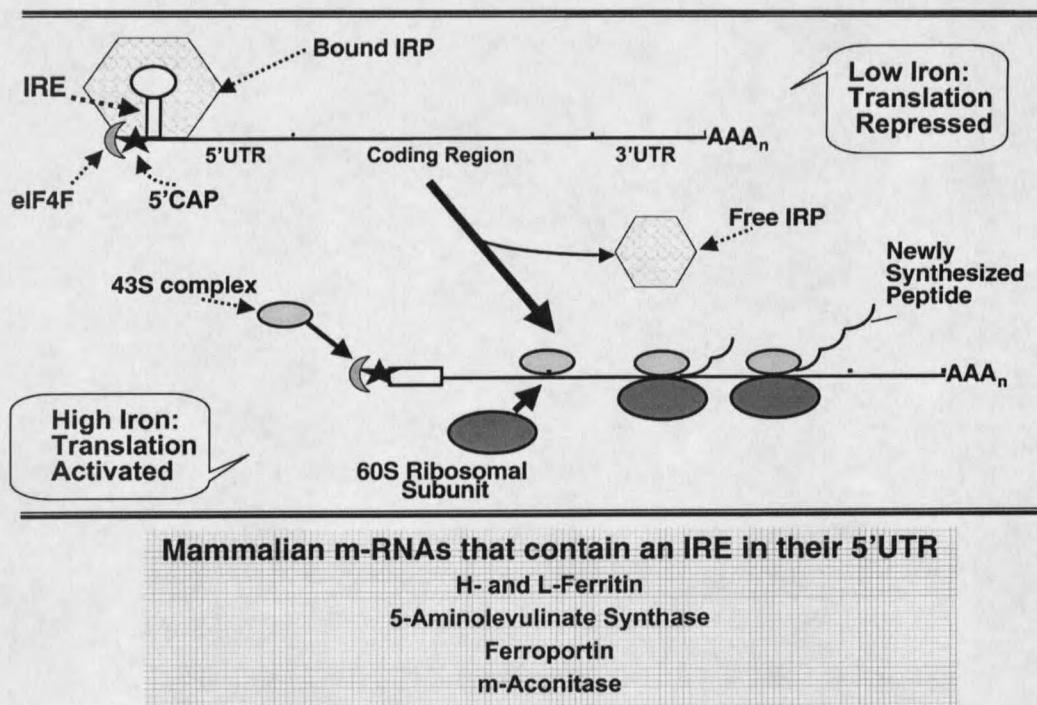


Fig. 2: Interactions of IRP with Iron and IRE containing mRNA.

Mechanism of Transferrin Receptor Translational Control

After discovering the IREs in the ferritin mRNAs, five similar motifs were identified within the 3' UTR of transferrin receptor (TfR) mRNA (8). The specific location of these IREs agreed with two regions of about 200 bases each that are known to give differential stability to the TfR mRNA depending upon the cellular iron levels (9). Interaction between IRP-1 and the TfR IREs was demonstrated in cells that were treated with an iron chelator this caused stabilization of TfR mRNA and increased protein expression (10). The opposite was seen when iron was added to the cells, the extra iron inhibited IRE binding to IRP-1 (within 2 hours) and degraded TfR mRNA (1). Through analysis of numerous deletions and mutations in the regulatory regions it appeared that

IRP-1 protected TfR mRNA once it was bound (1). Therefore when cells are iron deficient more TfR is produced in order to absorb more iron through endocytosis of transferrin. Just like iron storage, iron uptake is adjusted by a feedback control loop in which a free iron pool controls its own size.

Differential expression of TfR was first reported in the early 1980s when proliferating cells responded to variations in iron availability (11). Once the TfR cDNAs were isolated, experiments in cultured cells showed an increase or a decrease in TfR mRNA levels in the presence of iron chelators and iron salts respectively (12). Initially TfR regulation was believed to be under transcriptional control, but early experiments didn't show this (13). It wasn't until the 3' UTR of the TfR mRNA were deleted that translational control became evident (9). With the 3' UTR deleted, TfR expression increased and was unregulated in cultured cells. "Deletion mapping of the regulatory sequences identified two necessary areas of about 200 nucleotides each, separated by some 250 nucleotides" (1). These two regulatory sequences (not adjacent to the 3' UTRs) showed high sequence identity between human and rat mRNAs (94%) and between human and chicken mRNAs (89%) (10). The conservation in sequence in these regions was higher than that of the coding regions themselves. Since the regulatory regions of TfR mRNA are more complex than that of ferritin mRNA, its structure can't be predicted with certainty, but the RNA stabilizing and destabilizing elements have been found. Through the use of *in vivo* footprinting experiments the IREs are believed to adopt the hairpin conformation, and *in vitro* binding experiments showed that at least four IREs are available for IRP binding (14). When iron levels are low in the cell the binding activity of

IRE for IRP correlates with the stabilization of TfR mRNA. Through mutational studies it was determined that if the IRE was mutated so that it could not bind IRP, the instability of the TfR mRNA would increase (15). In addition, mutations or deletions in the destabilizing regions would lead to a stable TfR mRNA (8,15). When the iron levels increase in the cell the IRPs become inactive and the IRE/IRP complex dissociates, this causes the destabilizing regions to be exposed (Fig. 3) (16). It is not known if the destabilizing regions (once exposed) undergo a conformational change before the mRNA is degraded. The actual mechanism of RNA degradation and the ribonucleases involved remains unsolved for now.

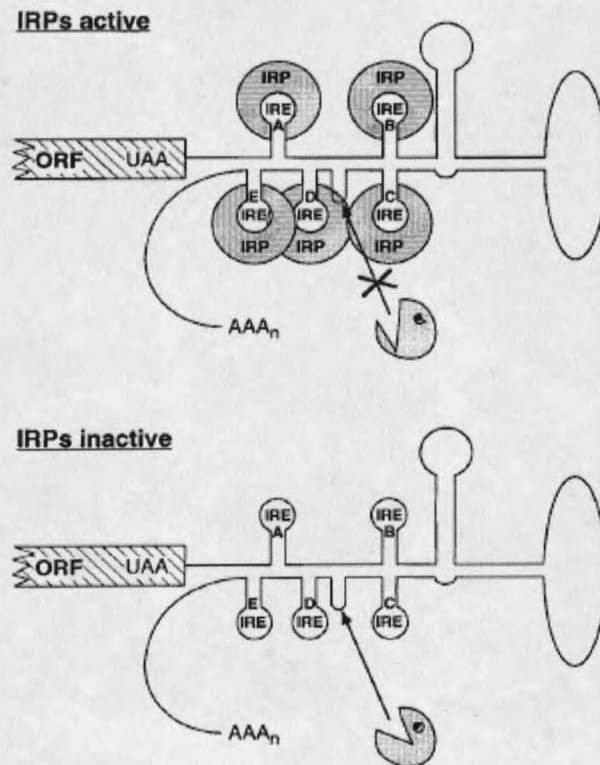


Fig 3: IRP stabilizes transferrin receptor mRNA by preventing its degradation.

An extended regulatory network that operates through IRPs appears to connect the synthesis of protoporphyrin IX (in erythroid cells) and mitochondrial iron sulfur proteins to the availability of iron (1). There are several IREs that are located in the 5' UTRs of the mRNAs that encode proteins in these pathways. These mRNAs function similarly to those of the ferritin mRNA by acting as translationally controlled elements. The IRE in 5-ALA synthase mRNA is a good example of a regulatory connection between iron availability and heme synthesis for hemoglobin, which is a major iron utilization pathway (17). Direct experimental evidence linking the inhibitory effect of activated IRPs on the synthesis of 5-ALA-synthase is still lacking. However, with IREs present in the mRNAs encoding for citric acid cycle enzymes there seems to be an unexpected connection between iron and mitochondrial energy metabolism. How and why this connection evolved remains largely unsolved, but the discovery of these new IREs confirms an earlier evolutionary origin, tracing back to arthropods and mollusks (18). The known network of IRE-regulated mRNAs may grow in the future as research progresses.

Binding of IRP-1 and IRP-2 to IREs

That the IRE is highly conserved for any given gene between species and is similar between different genes containing IREs implies that precise structural constraints in the binding of IRE to IRPs are required. The structure of the IRE consists of a stem and loop with a five base paired stem of variable sequence followed by a six nucleotide loop with a consensus 5'-CAGUGN-3' (19). Beneath the paired stem there is an unpaired cytosine that results in a small asymmetrical bulge (19). In some IREs this

bulge is drawn as a single unpaired C nucleotide, while in others the C nucleotide and two other nucleotides oppose one free nucleotide. The C bulge could give a specific bend to the IRE structure. Below the C bulge lies another base paired region that appears to give stability to the IRE hairpin (Fig. 4) (1). Recently the NMR structure of a 30 nucleotide IRE with a single C bulge has been determined (20). The loop samples a wide range of conformations, while the C bulge appears to be unstructured, and stems appear well defined as expected.

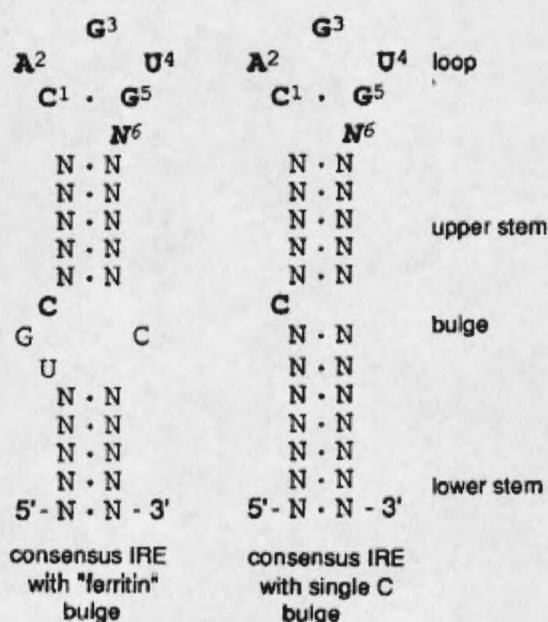


Fig. 4: Consensus IRE sequences, N represents any nucleic acid. The bulge and the loop are conserved between species, while the stems differ between

Several IRE/IRP-1 binding experiments have been done to elucidate the structure and sequence constraints of the IRE (1). Wild-type IREs have a high affinity of binding ($K_d \approx 10\text{-}30\text{ pM}$) to IRP-1, but when nucleotides in the loop and bulge region are deleted

the binding affinity is severely impaired or destroyed (21). Point mutations disrupting the upper stem were found to be nonfunctional; however compensatory mutations restoring the base pairing were tolerated (21). Nucleotide exchanges in the first five nucleotides of the loop, or within the bulge nucleotides of the IRE usually result in significant decrease in binding to IRP-1 (21). These types of mutations can dramatically affect iron regulation *in vivo*, as in the case of dominant familial hyperferritinemia that leads to early-onset cataract (22). In hyperferritinemia, the ferritin L-chain genes have an A² to G² mutation in the IRE loop (see Fig 4 for nomenclature and location); this lowers the affinity for IRP causing excessive ferritin synthesis in the affected individuals.

In SELEX experiment, a pool of 16,384 IRE variants was selected in order to find the best IRP-1 binding sequences (23). The variants were mutated randomly in the six nucleotide loop and C bulge. The consensus sequence selected from the top picks corresponded precisely with the phylogenetically conserved IRE, but a number of single and double mutations were tolerated and showed a 2 to 10 fold decrease in affinities (1). These experiments provided strong evidence that the loop is likely to be structured between nucleotides one and five (1). Some of the double mutations at the loop, specifically C¹ and G⁵ altered to U¹A⁵ and to G¹C⁵ (see Fig 4 for nomenclature and location), showed affinities comparable to wild-type IRE (28). NMR analysis of the IRE structure suggested that there are intraloop base pair interactions (20). While, a SELEX approach and a direct screening method yielded IRE mutants and double mutants that specifically bind to IRP-1 and IRP-2 respectively (23). Differences in affinity between

IRP-1 and IRP-2 for the various IREs allow subtle regulation of the various messages involved in iron transport and homeostasis.

Posttranslational Regulation of IRE-Binding Activity

IRP-1 and IRP-2 were first discovered when they interacted with IREs in rat liver cytoplasmic extracts, they have since been found in most cell lines and tissues (24). Both IRP-1 and IRP-2 have strong affinities for the wild-type IREs. They are active when iron levels are low in the body, but lose their affinity for the IRE when iron levels are high (25). Through the uses of western blots and gel retardation assays it was determined that IRP-1 is ubiquitous in all vertebrate tissues (25). IRP-1 expression varies between cells lines from 50,000 (mouse) and 100,000 (human) molecules per cell, with only 10% actively binding IRE, but with the addition of an iron chelator IRP-1 activity can reach 100% (1). This would indicate an all-or none event, but this is not the case. IRP regulation depends on the equilibrium between IRE/IRP complex and free IRP. This allows the cell to have the flexibility to adapt to changes in the supply of iron, and to respond to iron requirements (e.g. cell proliferation).

The total amount of IRP-1 in the cell doesn't have to change in order to alter the activity of IRP-1. Through various experiments it was determined that all of the IRP-1 whether it was active or not, could be converted into the active IRE binding form by adding 2% 2-mercaptoethanol (26). This meant that an estimate of the total amount of IRP-1 present could be obtained, and this provided the first solid evidence that changes in IRP-1 activity resulted from an iron-induced posttranslational modification. In order to

explore the iron-induced posttranslational modification, IRP-1 was purified. IRP-1 consists of 889 amino acids with a predicted molecular weight of 98 kDa, exists as a monomer in the cytoplasm, and is homologous to mitochondrial and bacterial aconitases (14). Aconitases are iron sulfur proteins that interconvert citrate to isocitrate, so it was predicted that IRP-1 might display some aconitase activity as well (27). By adding ferrous iron and sulfide to iron depleted cells, IRP-1 converted into a cytoplasmic aconitase by binding a 4Fe-4S cluster (27). The 4Fe-4S cluster binds to IRP-1 at three cysteine residues; the residues are located at positions homologous to other aconitases (27). When the 4Fe-4S cluster is present, IRP-1 cannot actively bind IRE and dissociates from mRNA. However, when the 4Fe-4S cluster is not present the apoprotein (iron deficient form) can actively bind IRE (27). The ability of IRP-1 to convert between two different forms illustrates how the cellular iron sensing mechanism can control the interactions between IRP-1 and IREs. By mutating the three cysteines necessary to bind the 4Fe-4S cluster to serine, IRP-1 reverts back to the apo-form and loses its aconitase activity (28). Since the structure of IRP-1 is unknown, the crystal structure of mitochondrial aconitase can give clues to its possible structure. Presumably, IRP-1 is composed of four domains with a deep cleft forming between domains 1-3 and domain 4, with domain 4 connected to domains 1-3 by a flexible linker (Fig. 5) (1). From the aconitase structure and various UV crosslinking studies, the IRE binding site appears to involve several domains that cover the length of the IRE stem loop (29). The actual mechanism regulating the interconversion of IRP-1 and cytosolic aconitase by cellular iron remains largely unsolved, as do the details of the interaction between IRPs and IREs.

Also, the IRP-1 gene knockouts in mice show no discernable phenotype (BioIron 2003), but mice with IRP-2 gene knockouts show various neural disorders (30). Ultimately, the crystal structure of the IRP-1/IRE complex will help to answer these questions.

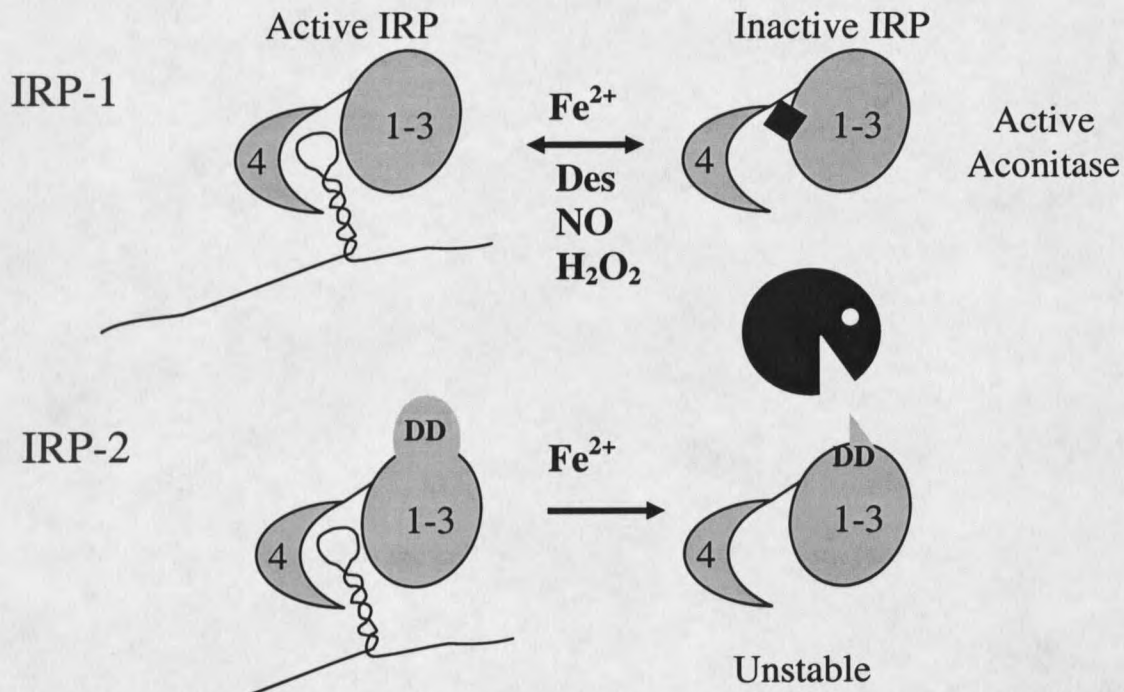


Fig. 5: Regulation of IRP-1 and IRP-2. In iron replete cells, IRP assembles a cubane Fe-S cluster that is liganded via Cys 437, 503 and 506. In iron replete cells, IRP-2 is targeted for degradation via the degradation domain whereas IRP-1 is stable and active as a cytoplasmic aconitase.

Even less is known about IRP-2 than IRP-1. IRP-2 is expressed in a variety of tissues but it is far less abundant than IRP-1, from gel retardation assays IRP-2 is strongly expressed in the intestine and brain (30). When looking at the relative transcription levels of the IRPs it was determined that IRP-2 had higher levels in the brain and IRP-1 had higher levels in the heart and liver (30). Once the full length cDNA of IRP-2 was cloned it gave great insight into its function. Human IRP-2 is 57% identical and 79%

similar in amino acid sequence to human IRP-1 (25). Purified IRP-2 is slightly larger than IRP-1 with a predicted molecular weight of 105 kDa, due to the presence of a 73 amino acid sequence (31). The 73 amino acid sequence or degradation domain is not located in IRP-1, but highly conserved in IRP-2 between species (25). IRP-2 contains the necessary cysteines residues to bind a 4Fe-4S cluster, but it does not have aconitase activity (32). It remains unclear if IRP-2 can accommodate a 4Fe-4S cluster. Unlike IRP-1, IRP-2 is proteolytically degraded in iron depleted cells when iron is added, due to the unique 73 amino acid sequence. When the 73 amino acid sequence is deleted from IRP-2, the protein does not go through proteolytic degradation (31). However, when IRP-1 has the 73 amino acid sequence inserted it goes through the iron dependent degradation, implying that the degradation domain is both necessary and sufficient to catalyze iron mediated degradation (31). A multiple-sequence alignment was performed on human IRP-1, human IRP-2, and pig mitochondrial aconitase (Fig 6). The alignment also indicates the domains, and critical residues that are conserved.

IRP-2 degradation is controlled by cellular iron levels and degradation appears to occur in the proteasome because it is prevented when specific proteasome inhibitors are used. IRP-2 becomes oxidatively modified and ubiquitinated *in vivo* and is degraded by the proteasome (33). It was determined that when IRP-2 is exposed to iron, oxygen, and DTT it was the degradation domain that became oxidatively modified (33). Using mass spectroscopy it was determined that the cysteine residues in the degradation domain were being converted to a product 2 atomic mass units less than the cysteine (33).

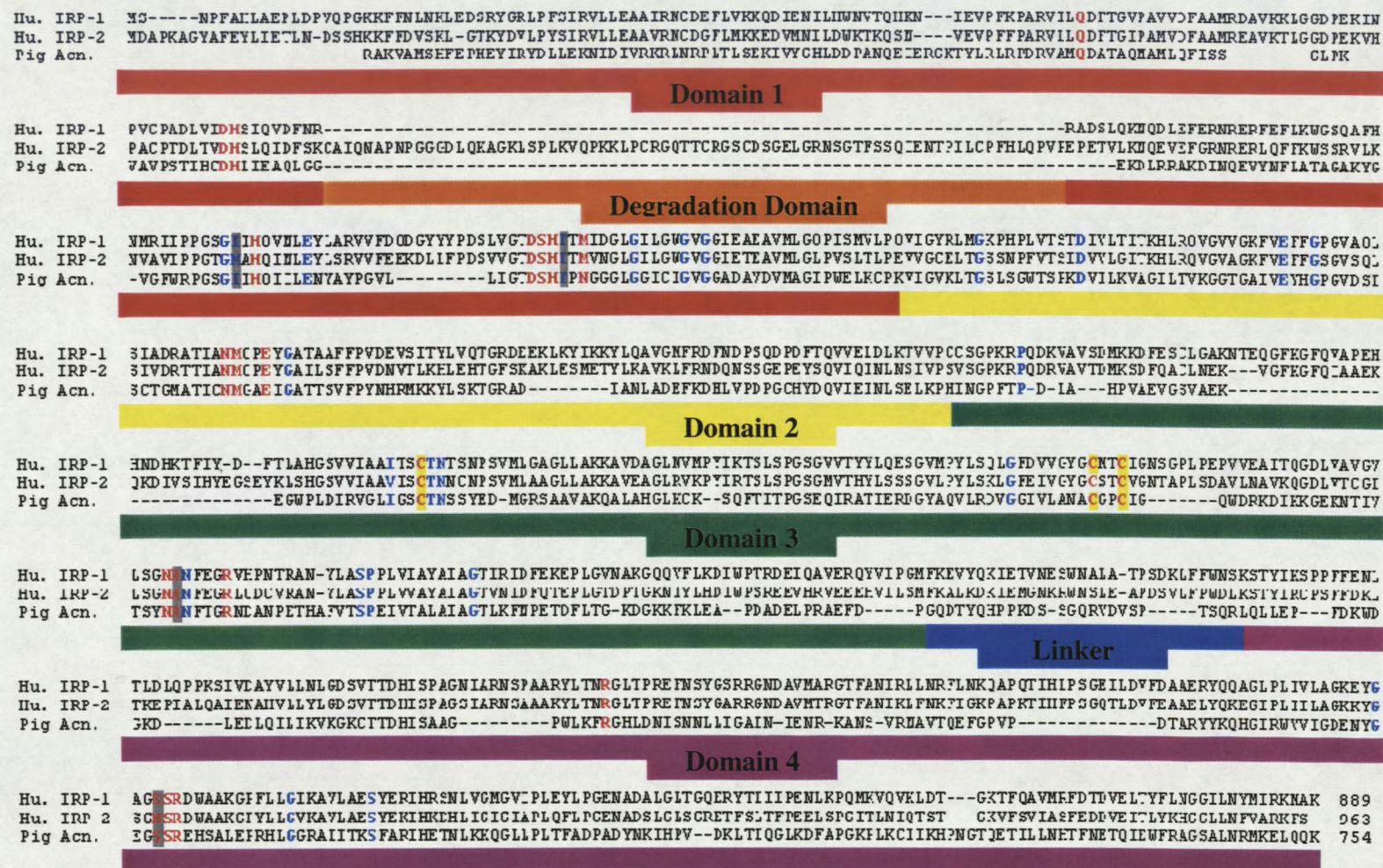


Fig. 6: A multiple-sequence alignment of Human IRP-1, Human IRP-2, and Pig Aconitase is shown. Aconitase active-site residues defined in crystallographic studies are indicated in red, additional active-site residues conserved in all members of the Fe-S isomerase family are indicated in blue, residues highlighted in grey indicate aconitase active-site residues that are not conserved in IRP-2, residues highlighted in yellow indicated residues critical for binding the 4Fe-4S cluster.

The loss of the cysteine residue required only micromolar concentrations of iron, with 50 micromolar iron reducing the number of cysteines by one (It is interesting to note, however, that iron concentrations within the labile iron pool ("free iron") are believed to be on the order of 1 micromolar, 50 fold less than that of the iron concentrations used in this study (33). Thus the physiological significance of this study is uncertain.) In addition to the cleavage of the cysteine residues, it was determined that two lysine residues were also susceptible to hydrolysis, which indicates that they are also near the iron-binding site. By mutating the first cysteine to an alanine, the rate of oxidative modification decreased by over 50% and by mutating the second cysteine the rate is decreased to greater than 80% (33). The initial step in the oxidative modification was hypothesized to convert cysteine to dehydrocysteine, which is 2 atomic mass units less than cysteine; subsequently the dehydrocysteine is converted into aminomalonic acid (33). The conversion of the cysteine residues to acidic aminomalonate could induce conformational changes in the degradation domain that might be important in the recognition of IRP-2 for ubiquitination and degradation by the proteosome (33). The first step in marking IRP-2 for proteolytic degradation is hypothesized to be binding of ferric iron that is reduced by a cysteine to the ferrous state (33). This then allows molecular oxygen to bind. Abstraction of a second hydrogen would lead to dehydrocysteine and a ferric hydroperoxy or ferryl moiety that could further oxidize the dehydrocysteine (33). The ferric ion is then released to bind to another IRP-2 molecule and catalyze another oxidation cycle.

It appears that heme also plays some part in the degradation of IRP-2 by binding to the 73 amino acid sequence and causing instability (1). Experiments indicated that heme, rather than free iron, could also catalyze degradation of IRP-2 (34). The experiments determined that while free iron can degrade 80% of the IRP-2, the presence of inhibitors showed that the heme was not just a source for free iron (34). This suggested that the iron must first be incorporated into the heme or heme like compound before it can degrade the IRP-2 (34). Recently, experiments showed that when IRP-2 was oxidized in the presence of heme, the IRP-2 was targeted for ubiquitination by the RING finger protein HOIL-1 (34). Ubiquitination is carried out by a series of reactions that is catalyzed by the E1, E2, and E3 family of enzymes, more specifically; the E3 RING finger ligases target specific recognition signals (34). It is clear from experimental data that the degradation domain has evolved into an efficient iron sensor. It should also be noted that mouse IRP-2 knockouts overexpress ferritin and develop progressive neurodegenerative diseases (35). These mice develop progressive tremors, proximal muscle weakness and ataxia. Experiments indicate that the neurodegeneration in the mice is caused by excess iron in certain areas of the brain due to the misregulation of ferritin synthesis (35). Iron accumulation has been implicated in the progression of many human neurodegenerative diseases such as Parkinson's disease, Alzheimer's disease, multiple system atrophy, and neuronal brain iron accumulation type 1 (35). Therefore, it is important to determine the mechanisms of IRP-1 and IRP-2 regulation as they might aid in the design of potential therapeutics for these neural diseases. Obtaining the crystal

structure of IRP-2/IRE complex will provide structural insight into its role in iron metabolism, and for the design of specific drugs.

CHAPTER 2

Cloning and Protein Expression

Expression of IRP-2 Degradation Domain Deletion MutantsIntroduction

IRP-2 contains a 73 amino acid sequence or degradation domain that is not present in IRP-1. The degradation domain, as its name implies, targets IRP-2 for degradation when iron levels in the cell are high. While the exact mechanism for how the degradation domain signals degradation is not presently known, there are several hypotheses; they include iron catalyzed oxidation of cysteine that are then recognized by a specific ubiquitin ligase complex; binding of heme by the degradation domain and subsequent recognition by a ubiquitin ligase; or other signals for degradation that could involve phosphorylation. Removal of the degradation domain from IRP-2 could conceivably stabilize IRP-2, leading to increased yield of over expressed IRP-2. In addition, the degradation domain deletion mutant might be more amenable to crystallization since the degradation domain may contain intrinsically disordered residues. A major goal of the lab is to obtain crystallographic structures of IRP-2; if the degradation domain were disordered this might interfere with the crystallization process. Therefore, we decided to create two mutants of IRP-2, one where the domain is deleted completely, and another in which the domain is replaced with two glycine residues. The rationale for adding of two glycine residues is to compensate for any flexibility that was lost when the degradation domain was deleted.

From the literature on IRPs, it was determined that IRP expression in bacteria yielded mostly non-soluble protein in the form of inclusion bodies, while baculovirus and yeast expression systems yielded some soluble IRP-2 protein. In order to obtain crystallographic structures of protein, the protein must be soluble. Therefore, the laboratory decided to use a baculovirus expression system from *Gibco BRL*, and a yeast expression system from *Invitrogen* in order to obtain soluble IRP-2 minus the degradation domain.

Construction of Degradation Deletion Mutant

Construction of these two mutants was performed with site directed mutagenesis by overlap extension (36, 37). Site directed mutagenesis by overlap extension involves the generation of DNA fragments in one round of PCR that can be fused together in another round of PCR (36). Two sets of primers are used, primers A and D allow the whole gene to be amplified (outside primers), while primers C and B have complementary sequences to each other, they contain the desired changes, and anneal to sites within the gene (36). In the first round of PCR, primer A anneals to the 5' end of the gene, while primer B anneals to sequence right before the degradation domain (36). In another PCR reaction primer D anneals to the 3' end of the gene while primer C anneals to the sequence right in behind the degradation domain (36). These PCRs will generate two fragments AB and CD; fragment AB contains the sequence right before the degradation domain, while CD contains the sequence right after the degradation domain (36). In the final round of PCR, the AB fragment and the CD fragment will anneal to each other because the B and C primers contain complementary sequences, once the two

fragments are annealed the sequence can be extended and amplified using primers A and D (Fig. 7) (36).

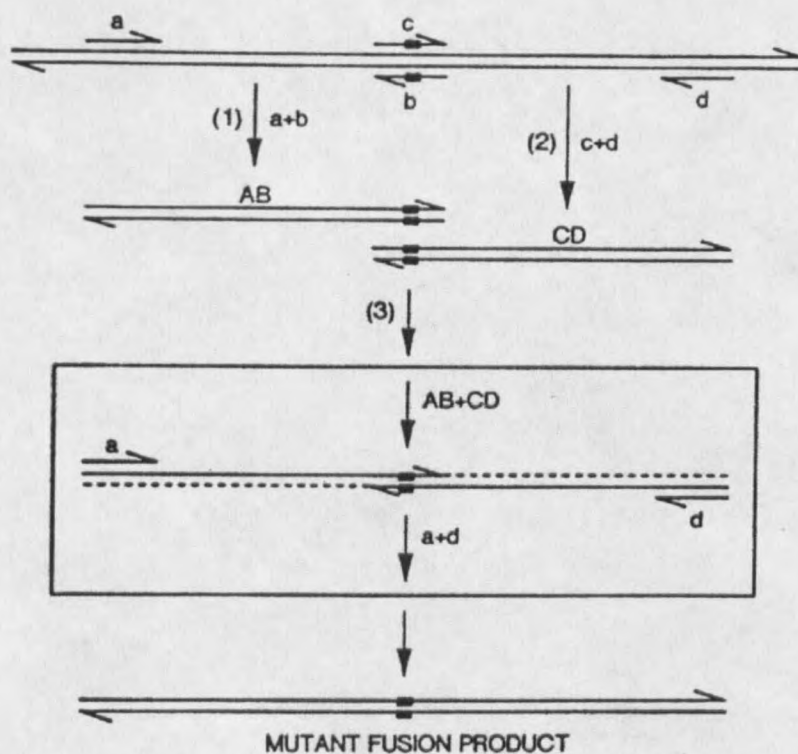


Fig. 7: Schematic diagram of site directed mutagenesis by overlap extension. The DNA and primers are represented by lines with arrows indicating the 5' to 3' direction. The black box represents a site that can have a mutated, deleted, or inserted product.

Elizabeth Leibold (University of Utah) graciously provided the lab with the rat IRP-2 gene (in the pHT B2 YES vector). Since, the pHT B2 YES vector is a yeast expression vector the IRP-2 gene was recloned into an *E. Coli* expression vector pUC19. The IRP-2 gene was now in two vectors pHT B2 YES and pUC19.

George Gauss (Montana State University) helped to setup the experimental procedure needed to perform site-directed mutagenesis. To create the degradation domain deletion mutant (without extra glycine residues) the AB and CD fragments must be generated. The PCR reaction mixture used to amplify the AB fragment was as follows; 1 ng of template DNA (pHT B2 YES, IRP-2 gene from rat), 0.5 μ M primer A: GHG 120(5' CCGACAGGATCCACCATGGACTCCCCAAGTG C 3'), 0.5 μ M primer B: BJE 102 (5' aacaccgtctcaggttcTTTACTGAAGTCAATCT 3'), 0.2 μ M dNTPs, 0.03 U/ μ l Taq polymerase, 0.00006 U/ μ l Vent polymerase, 50mM Tris pH 9.2, 16mM $(\text{NH}_4)_2\text{SO}_4$, 1.75 mM MgCl_2 , 0.1% Triton X-100. The reaction mixture was placed in a thermocycler with the following parameters; [initial 94°C 120 seconds], 94°C 20 seconds, 55°C 30 seconds, 72°C 60 seconds repeated 35 times, 72°C 5min, and 4°C hold. The PCR reaction mixture used to amplify the CD fragment was as follows; 1 ng of template DNA (pHT B2 YES, IRP-2 gene from rat), 0.5 μ M primer C: BJE 101 (5' gaacctgagacggtgttAAAAAATC 3'), 0.5 μ M primer D: BJE 201 (5' AATTTCACAGCTT TAAGGTA 3'), 0.2 μ M dNTPs, 0.03 U/ μ l Taq polymerase, 0.00006 U/ μ l Vent polymerase, 50mM Tris pH 9.2, 16mM $(\text{NH}_4)_2\text{SO}_4$, 1.75 mM MgCl_2 , 0.1% Triton X-100. The reaction mixture was placed in a thermocycler with the following parameters; [initial 94°C 120 seconds], 94°C 20 seconds, 55°C 30 seconds, 72°C 60 seconds repeated 35 times, 72°C 5min, and 4°C hold. PCR reactions were run out on a 1% agarose gel to check for correct fragment size (Fig. 8).

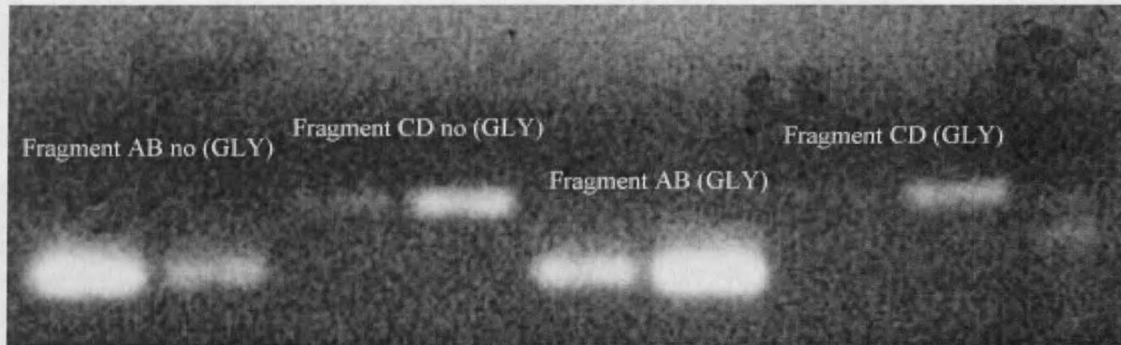


Fig. 8: Amplification of AB and CD fragments by PCR. PCR reactions were run out on a 1% agarose gel for 45 min. at 100V. The gel was ethidium stained and analyzed under

The same reaction conditions that were used to create the AB and CD fragments are also used to create the AD fragment. The only exceptions are: primer A: GHG 120 (0.5 μ M), primer D: BJE 201 (0.5 μ M), 100ng of fragment AB (gel purified), and 100ng of fragment CD (gel purified). The two fragments anneal together and act as the template DNA because the ends of the BJE 101 and BJE 102 primers base pair together (small case lettering). The thermocycling was under the same conditions as the AB and CD fragments except that 72°C extension time was increased from 60 seconds to 120 seconds. The PCR reactions were run out on a 1% agarose gel to check for correct fragment size (Fig. 9).

The same procedure that was used to create the degradation domain deletion mutant (without glycine residues) is used to make the degradation domain with the two glycine residues, except primer B would be BJE 104 (5' gtctcaggttctcctccTTTACTGAA GTCAATCT 3') and primer C would be BJE 103 (5' ggaggagaacctgagacGGTGTTAAA AAATCAAG 3'). The result of these PCR experiments lead to obtaining amplified IRP-2 gene with deletion of the degradation domain with or without glycine residues added.

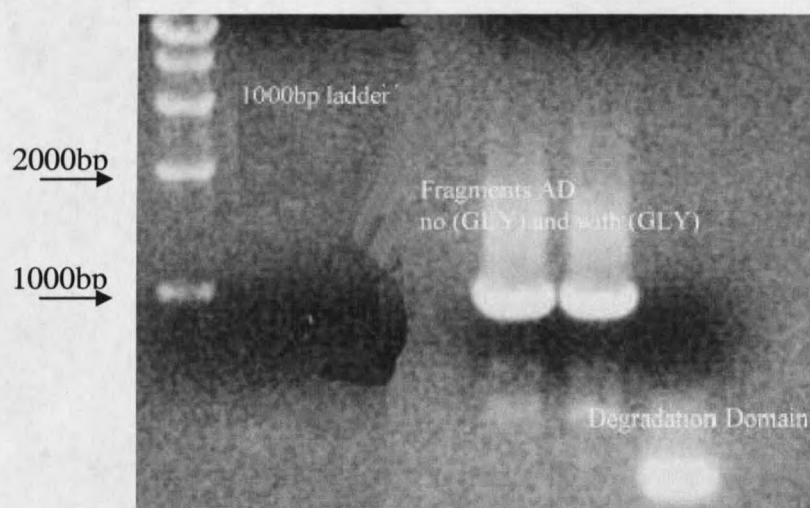


Fig. 9: PCR amplified IRP-2 fragments AD with and without extra glycine residues. PCR reactions were run out on a 1% agarose gel for 45 min. at 100V. The gel was ethidium stained and analyzed under a UV light.

Once the degradation domain was deleted from the IRP-2 gene, it was then possible to subclone the gene using traditional methods. The primer on the far 3' end was engineered to include a natural enzyme restriction site (Avr II) located ≈ 100 bases behind the degradation domain, and the primer on the 5' end was already close to a natural restriction site (Bgl II) located ≈ 370 bases from 5' end, this meant that the newly cloned portion of IRP-2 could be ligated into the original IRP-2 gene. This was done by using Avr II and Bgl II to cut pUC19 (with IRP-2 gene), and the AD fragments (created by PCR). The digested products were gel purified and then ligated together to create the IRP-2 gene lacking the degradation domain (IRP-2- Δ DD) and the IRP-2 gene lacking the degradation domain plus two glycines (IRP-2- Δ DD-Gly).

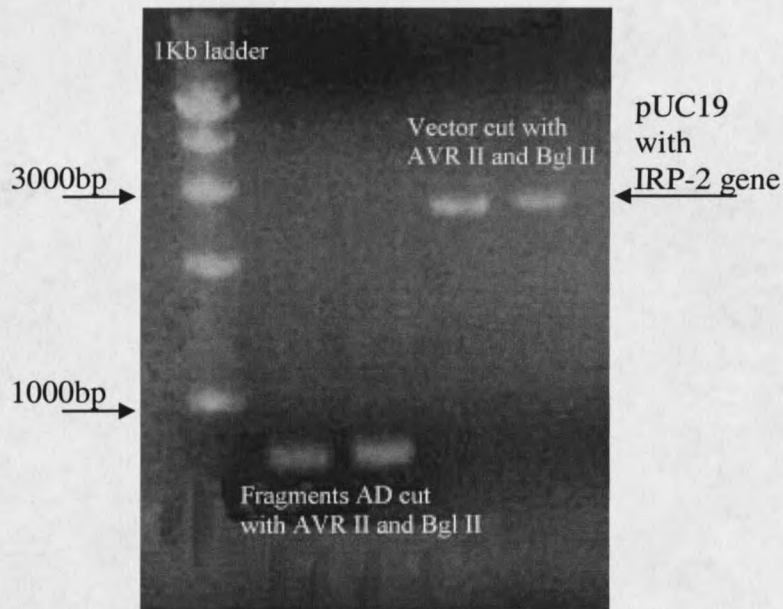


Fig. 10: IRP-2- Δ DD and IRP-2- Δ DD-Gly (fragments AD) cut with Avr II and Bgl II. DNA ladder starts at 5kb.

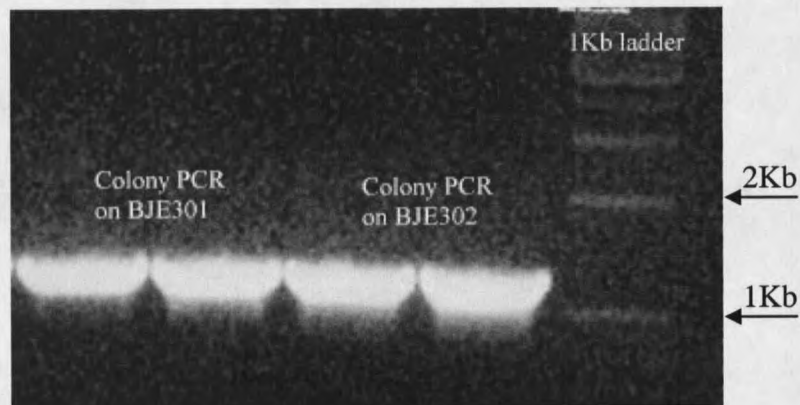


Fig. 11: PCR of *E. coli* colonies to check IRP-2- Δ DD (BJE 301), and IRP-2- Δ DD-Gly (BJE 302) for the degradation domain deletion.

E. coli (XL-1 blue strain) were transformed with the ligation products, and plated out on LB selection media with antibiotic resistance (ampicillan $100\mu\text{g}/\mu\text{l}$). Plates were

incubated overnight at 37°C, and colonies were picked to use for DNA minipreps. The miniprep DNA was then used for sequencing reactions. Sequencing Reactions were sent out to Iowa State University Sequencing Facility for analysis. Only the newly cloned portion of gene needed to be re-sequenced (≈ 800 bp) versus re-sequencing the entire gene (≈ 2800 bp). Two clones without the degradation domain were now generated; IRP-2- Δ DD (BJE 301), and IRP-2- Δ DD-Gly (BJE 302).

Subcloning IRP-2- Δ DD constructs into Baculovirus expression vectors

The subcloning procedure was devised by George Gauss (Montana State University). The vectors IRP-2- Δ DD (BJE 301), and IRP-2- Δ DD-Gly (BJE 302) along with the pFastBac1 vector (*Gibco BRL*) were digested with Bam HI and Xba I. The appropriate bands were cut out of an agarose gel and ligated together. This generated mutant IRP-2 genes that were now in the pFastBac1 vector IRP-2- Δ DD (BJE 303), and IRP-2- Δ DD-Gly (BJE 304).

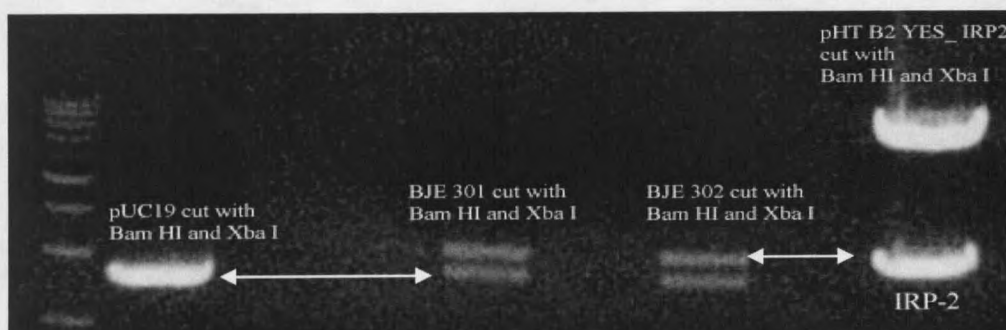


Fig. 12: Restriction digest of IRP-2- Δ DD (BJE 301), and IRP-2- Δ DD-Gly (BJE 302) for insertion into the pFastBac1 vector. Top band of BJE 301 and BJE 302 is the IRP-2 gene with the degradation domain deleted, while the bottom band is vector pUC19.

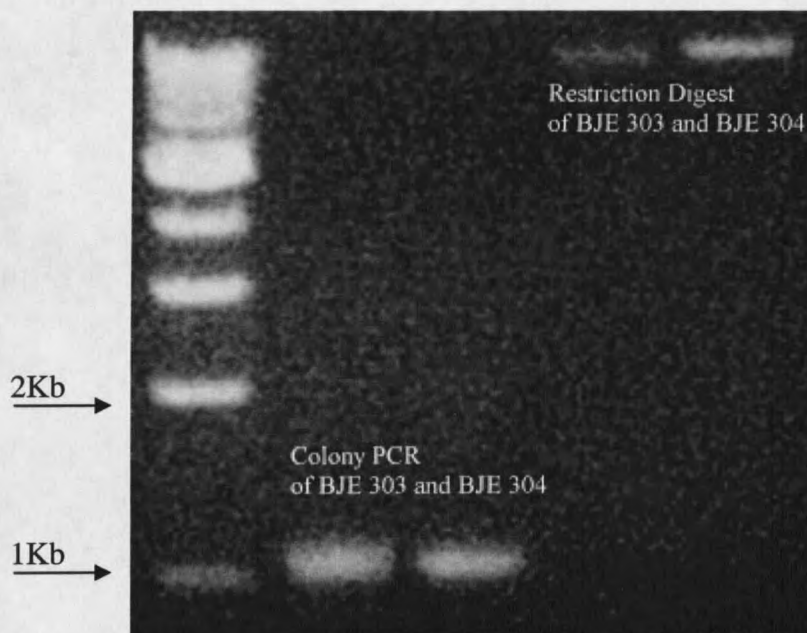


Fig. 13: Colony PCR and Restriction Digest to check for insertion of IRP-2 degradation domain deletion genes into pFastBac1 vector.

Using standard protocols found in the BAC-to-BAC baculovirus expression systems manual from *Gibco BRL*, the pFastBac 1 vector was transformed into competent DH10Bac *E. coli* cells that contain the baculovirus genome (Bacmid), resulting in transposition of the IRP-2 gene from pFastBac1 into the Bacmid. The cells are then plated out under antibiotic resistance. Colonies containing the IRP-2- Δ DD inserts will be white, while colonies that do not undergo transposition retain the β -galactosidase gene and appear blue. One white colony was picked and underwent a mini-prep for high molecular weight DNA. The DNA was then used to transfect Sf9 insect cells and virus was grown for 72 hours (Fig. 14). The primary viral stock was centrifuged to pellet the cells, and the cells underwent a Western blot to test for IRP-2 expression (Fig. 15).

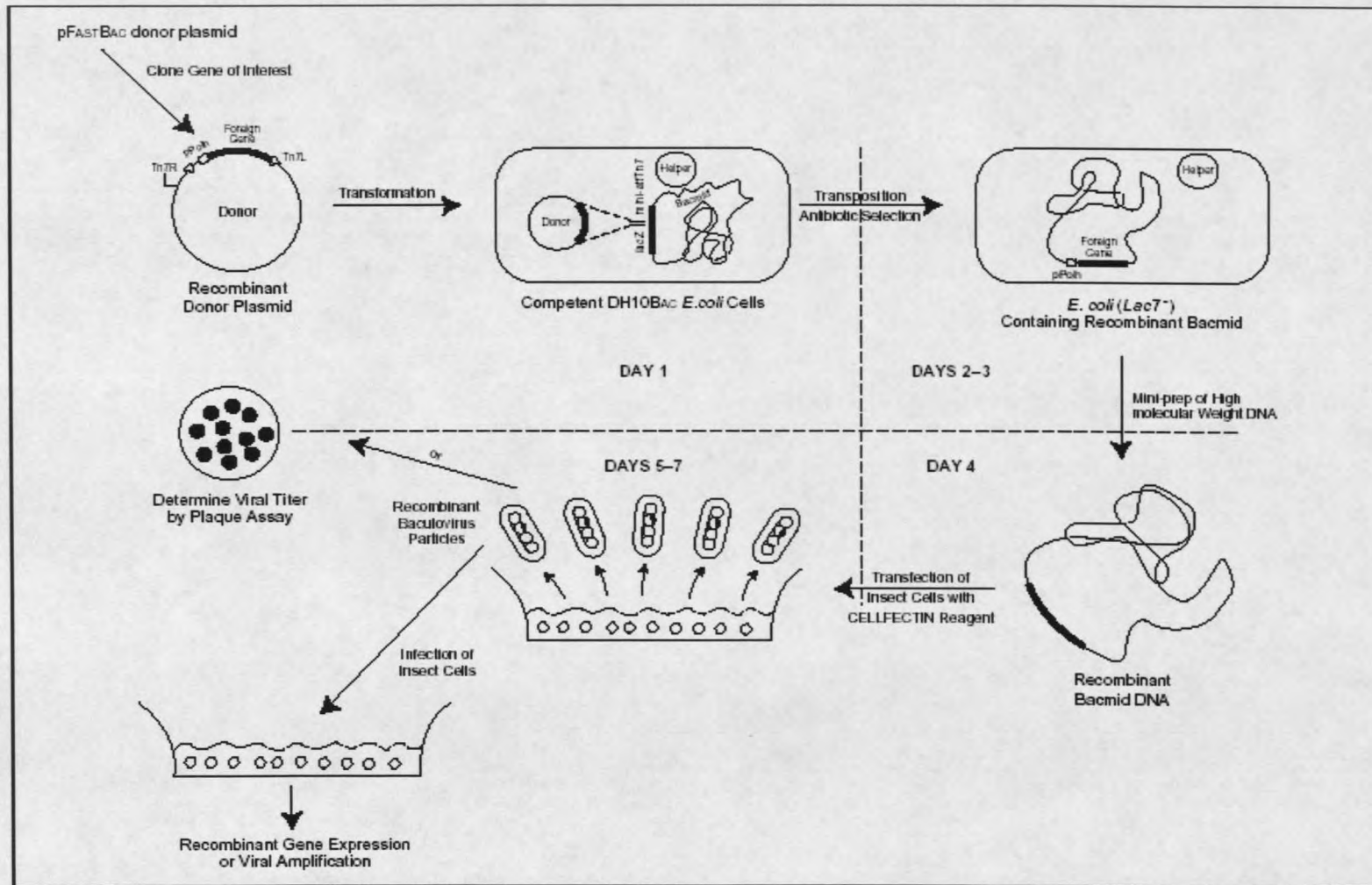


Fig. 14: The procedure for the BAC-to BAC baculovirus expression system. The pFast Bac-1 plasmid with gene of interest gets transposed into the baculovirus genome (Bacmid). Purified bacmid is used to infect insect cells and produce virus. The viral stock is then used to infect insect cells and express protein

The initial results from the expression study showed that the mutant IRPs expressed at the same level as wild-type IRP-2 in baculovirus (0.5mg/L, George Gauss). From this result, it was decided that IRP-2 expression in baculovirus should be put on hold.

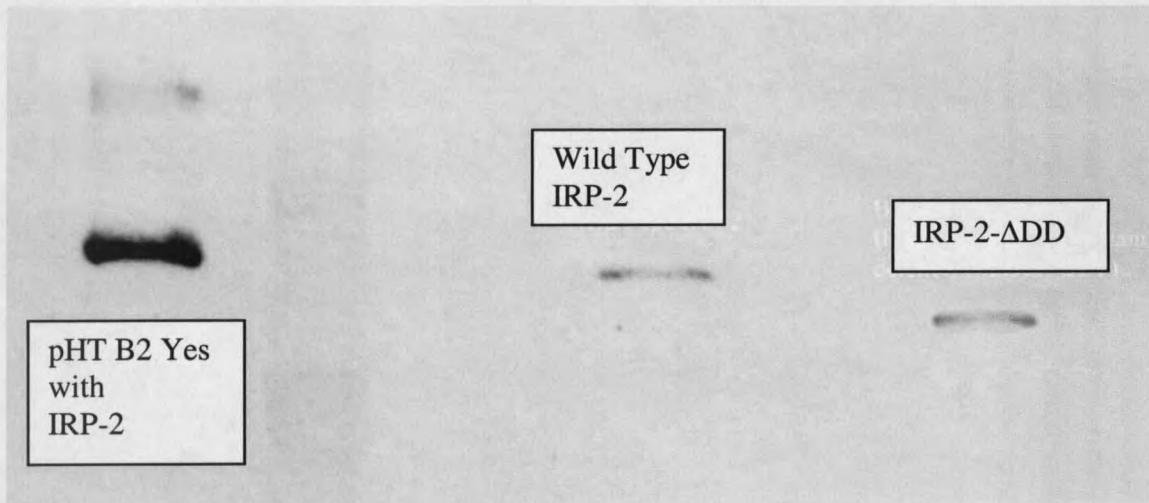


Fig. 15: Western blot of pelleted cells from primary viral stock. α -His antibody used

Gateway Cloning and Yeast Expression

The Lawrence lab has now made the switch from traditional cloning methods to the Gateway Cloning System by *Invitrogen*. Therefore, George Gauss (Lawrence Lab, Montana State University) cloned the IRP-2- Δ DD (BJE 301) and IRP-2- Δ DD-Gly (BJE 302) vectors into the Gateway Entry Vector.

The entry vector allows the user to transfer the desired gene of interest into any expression vector in the Gateway system (*Invitrogen*). In the case of IRP-2 degradation mutants, a yeast expression vector (pDest52) was used because native IRP-1 and IRP-2 were expressing at sufficient levels (1-3mg/L), and yeast culture is significantly easier than protein expression using the baculovirus system. IRP-2- Δ DD and IRP-2- Δ DD-Gly

were moved into pDest 52 using the Gateway site specific recombination system. A yeast cell line was transformed with each of the pDest52-IRPADD vectors following the EasyComp Yeast kit by *Invitrogen*. The procedure involves mixing the DNA, yeast competent cells, and Easy Comp Solution III (*Invitrogen*) in a microfuge tube and incubating it at 37°C for one hour. The cells were then plated on –Ura plates containing dextrose and incubated at 30°C for 2-3 days. A colony was picked and grown in 50 ml starter culture containing dextrose. Then 25 ml of the overnight culture was added to expression media expression containing galactose rather than dextrose. The yeast cultures then underwent expression for 24 hours. From previous experiments on IRP-1 and IRP-2 in yeast it was determined that 24 hours was sufficient in order to see expression of protein. The yeast cultures were centrifuged to pellet the cells, and cell pellets were frozen at -20° C. Some additional things to consider for yeast expression are:

1. All work should be done in a sterile hood to avoid contamination of the yeast
2. Raffinose can also be used instead of dextrose for overnight cultures
3. Galactose, glucose, and dextrose can be autoclaved
4. Raffinose must be filter sterilized
5. Overnight starter cultures may be spun down to remove sugars before adding the cells to the expression media
6. After 48 hours yeast cells become difficult to break open.
7. Yeast cells break open best when using a bead beater

The yeast cell pellets were then thawed on ice, and resuspended in lysis buffer (50 mM Tris-HCl pH 8.0, 300 mM NaCl, 5 mM imidazole, 2.5 mM 2-ME, 0.1 mM EDTA pH 8.0, and 0.1 mM PMSF). The resuspended cells, 60 grams of dry glass beads, and additional lysis buffer were added to the bead beater container. The cells were broken open by grinding for 30 seconds and allowed to cool for 60 seconds, this was repeated 5 times. The lysate was then separated from the beads, and centrifuged at 4°C for 20 minutes at 17k rpm. The lysate was then loaded onto a gravity flow Ni-NTA column (3ml of resin). The column was then washed 3 times with 3 ml of lysis buffer, and then washed 2 times with 3 ml lysis buffer plus 20 mM imidazole. The protein was eluted from the column in 3 ml of lysis buffer plus 250 mM imidazole. The fractions were then run out on a 10% SDS-PAGE gel for 45 min at 225 V.

Analysis of the gels indicated that the IRP-2- Δ DD construct had expressed protein at the correct molecular weight (97 kDa) that eluted off the Ni-NTA column (Fig.16). The IRP-2- Δ DD-Gly construct showed protein expression in the cell pellet, but no protein was seen in the elution fractions. According to Bradford assays, IRP-2- Δ DD expressed at 0.5mg/L. George Gauss and Philippe Bénas were able to express wild-type IRP-2 at 1mg/L and IRP-1 at 2-3mg/L respectively in yeast cells (*saccharomyces cerevisiae*).

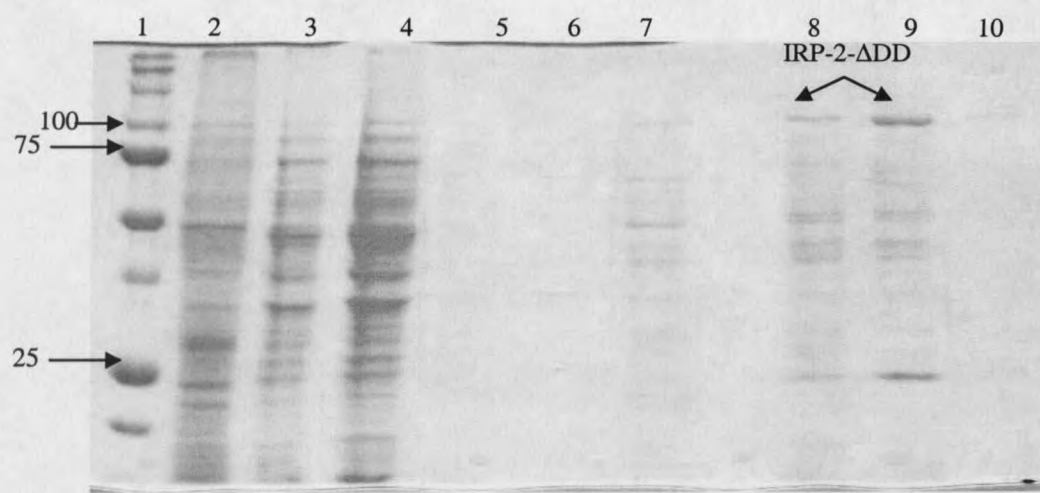


Fig. 16: IRP-2- Δ DD expression trial. Lane 1: protein standards, Lane 2: cell pellet, Lane 3 Ni-NTA column flowthrough, Lanes 4-7 column washes, Lane 8: first elution, Lane 9: second elution, Lane 10: third elution. Fractions were then run out on a 10% SDS-PAGE gel for 45 min at 225 V.

Results

The expression levels of wild-type IRP-2 were disappointing at 0.5 mg/L the IRP-2 mutants in baculovirus (10 mg amounts needed for crystallization), but there was hope that without the degradation domain IRP-2 would express better. The mutant IRP-2s didn't express any better than the wild-type IRP-2 when western blots were compared at the primary virus isolate stage. The expression of the IRP-2 mutants in baculovirus was not pursued further. Later on, George Gauss and Philippe Bénas were able to express wild-type IRP-2 (1mg/L) and IRP-1 (2-3mg/L) respectively in yeast cells (*saccharomyces cerviciae*). Preliminary expression trials of IRP-2- Δ DD indicated that it expressed at 0.5mg/L, further expression trials will need to be performed in order to determine if expression of the IRP-2 mutants should be pursued further.

CHAPTER 3

Synthesis and Purification of IRE

IRE Construction

The main goal of the Lawrence Lab is to crystallize IRP/IRE complex for crystallographic studies. Therefore, two things that are required to accomplish this goal are purified IRP and purified IRE. IREs of varying sequence are found in many genes regulated by IRP-1 and IRP-2, and these sequences also vary between species. Also, there are two major forms of IRE; the IREs for ferritin synthesis (Bulge/Loop IRE) and the IREs for transferrin receptor (c Bulge IRE) (38). Thus, there is great diversity in IRE structure leading to many possible choices in the IRE to be expressed for structural studies. Fortunately, IRP-1 shows broad specificity and binds to a variety of IREs equally well, while IRP-2 seems to be more specific (38). Therefore, a ferritin sequence of 30 nucleotides from rat was chosen because it can bind to both IRP-1 and IRP-2 (Fig. 17).

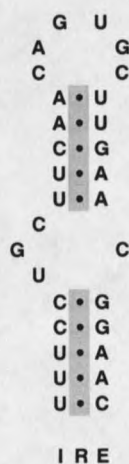


Fig. 17: Wild type ferritin IRE sequence from rat.

Two common routes for production of small RNA molecules are chemical synthesis and enzymatic synthesis. Chemical synthesis is usually done commercially; the desired sequence is simply purchased from a company such as *Dharmacon*. In contrast, enzymatic synthesis using T7 RNA polymerase is more easily done “in house”. An important consideration is the need for milligram amounts of homogenous IRE that is necessary for crystallization trials.

We initially purchased IRE from an RNA synthesis company (*Dharmacon*). The requested sequence for the IRE: (5' UUUCCUGCUUCAACAGUGCUUGAACGGAAC 3') was sent into *Dharmacon*, and for \$374 the lab received 200nmoles (0.79 mg) of IRE. This IRE was primarily used for early binding assays with IRP-2, and later used to check the size of the homemade IRE in denaturing PAGE gels. From these experiments it was determined that the IRE was not a homogeneous 30 nucleotide sequence, but consisted of at least two fragments, one at 30 nucleotides and the other at 60 nucleotides (Fig. 18).

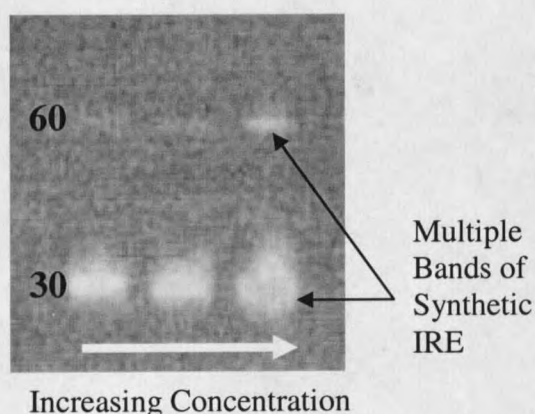


Fig. 18: Synthetic IRE appears to have multiple bands; the two most prominent are a 30mer and a 60mer. Synthetic RNA was loaded with increasing concentrations on a denaturing PAGE gel that was ethidium stained and checked with a UV light.

Considering the cost for milligram amounts of IRE and the heterogeneous mixture of IRE provided by *Dharmacon*, it was determined that we should undertake enzymatic synthesis of the IRE.

Two different strategies were used to make homemade IRE in the laboratory; both involve using *in vitro* T7 RNA polymerase transcription reactions. One is to amplify desired template by PCR or use annealed oligos containing a T7 promoter to generate a run-off 30 nucleotide sequence of the rat ferritin IRE, but this will result in heterogeneous ends (39). Typically, the T7 RNA polymerase can add anywhere from one to three extra nucleotides to the 3' end of the desired sequence, this can cause heterogeneity in the 3' end of any RNA synthesized by T7 RNA polymerase (39). The other method is to design hammerhead ribozymes, from the group one intron in *Tetrahymena thermophila*, on either side of the IRE to precisely cleave the IRE to leave homogenous ends (40).

The self-splicing pre-ribosomal RNA (rRNA) of the ciliate *Tetrahymena* was the first example of an RNA molecule that could form a catalytic active site for a series of precise biochemical reactions (40). The ribozymes in *Tetrahymena* self-splice the introns away from the rRNA allowing the exons to join together and be translated (41). The discovery of ribozymes increased the credibility of the RNA world hypothesis, where RNA served both as the genetic material and the principal cellular enzyme (41). Descendants from this proposed RNA-dominated world occur in the form of natural ribozymes present in organisms ranging from bacteria to humans (41).

Hammerhead ribozymes (HHR) are the smallest of the naturally occurring ribozymes (~40 nucleotides), and recent experiments show that the hammerhead motif is

the most efficient self-cleaving sequences that can be isolated from randomized pools of RNA (41). The HHR catalyses site-specific cleavage of one of its own phosphodiester bonds via nucleophilic attack of the adjacent 2'-oxygen at the scissile phosphate (Fig. 19) (41). Initial studies revealed a requirement for a divalent metal ion for catalysis (Mg^{2+}) to stabilize the active HHR structure, but its direct role in RNA cleavage is unknown (41).

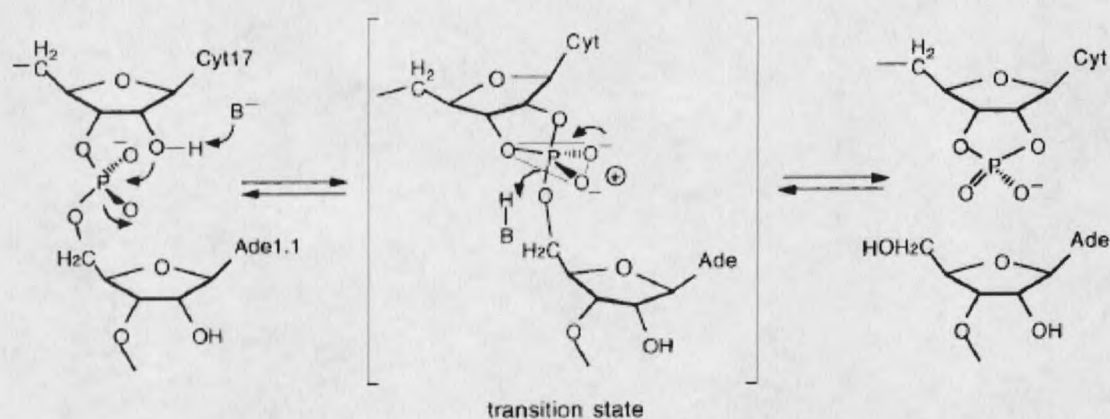


Fig. 19: The proposed HHR cleavage mechanism. The 2'-oxygen atom attacks the 3'-phosphorus group with expulsion of the 5'-oxygen atom proceeding via the trigonal bipyramidal transition state. The reaction could be accelerated by removal of the proton from the 2'-oxygen atom by a base (shown as B^-) and protonation of the departing oxyanion (by B-H).

The first strategy (run-off IRE transcripts) will generate some heterogeneity in 3' ends of the IRE, but it should not affect IRP binding since the extra nucleotides are distant from the more critical hexa loop and loop/bulge region of the IRE. However, the extra nucleotides may interfere with crystal packing if they protrude too far out of the complex. The second strategy utilizes HHR to yield homogenous IRE with defined 5'- and 3'-termini. Upon addition of Mg^{2+} , the HHR catalyzes precise cleavage reactions excising the IRE (Fig. 20).

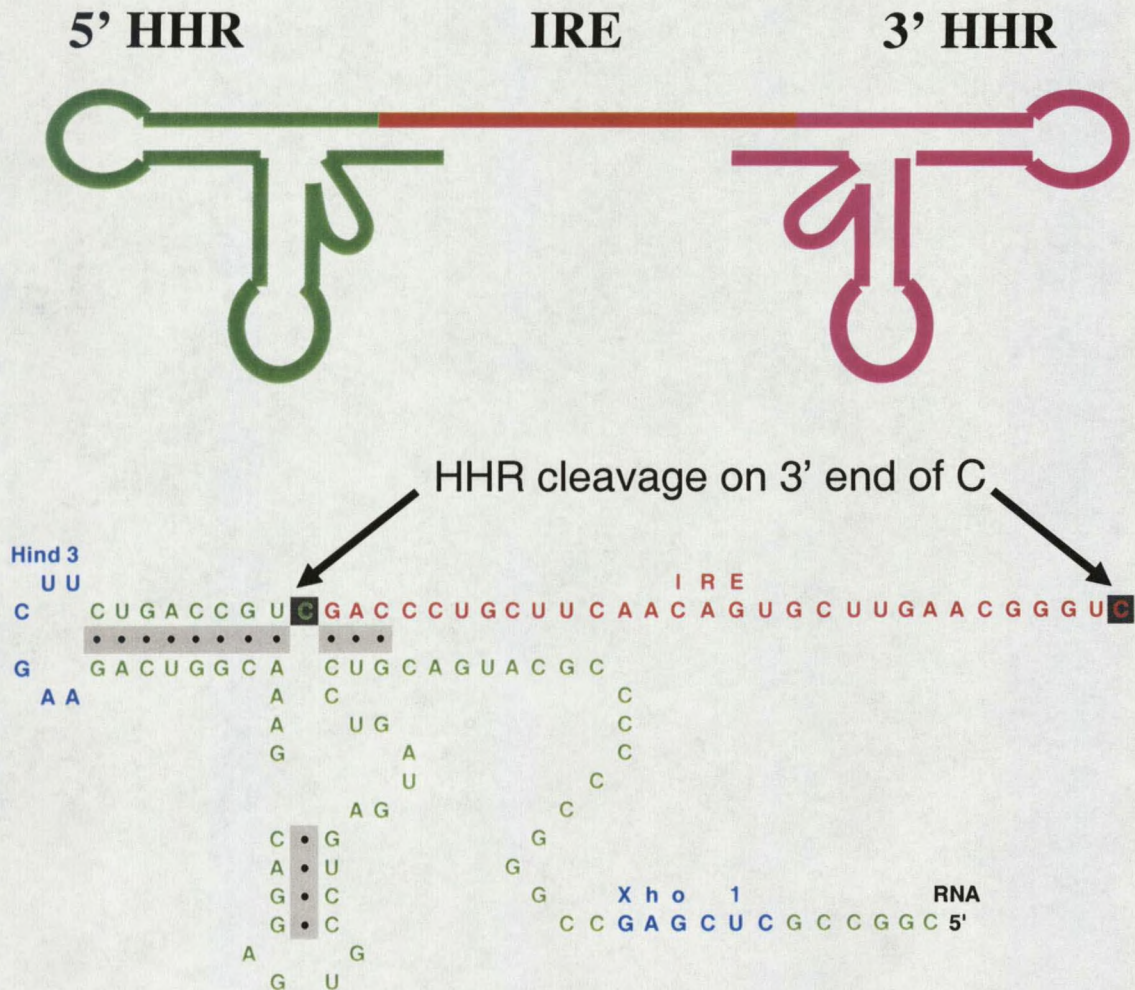


Fig. 20: The HHR-IRE-HHR construct. Visual representation of what the construct looks like (above), as well as the sequence of the 5' HHR. When the 3' and 5' HHR fold in the presence of Mg^{2+} they will precisely cleave away leaving the IRE on the 3' end of the cytosine (black box). Red text indicates IRE sequence, green text indicates 5' HHR sequence, blue text indicates restriction sites, and grey boxes indicate base pairing.

Philippe Bénas (Montana State University) designed the sequence for two HHR (Fig. 20) and then cloned the sequence for two HHRs separated by a Sal I cloning site into the pBluescript vector, which was named pBPBE-3. The cloned sequence for rat H-

ferritin IRE was inserted into the Sal I site. The Sal I site allows flexibility to change the IRE.

For HHR cleavage, it was necessary to modify the IRE lower stem sequence to allow base pairing with the HHR (Fig. 21). This sequence change should not affect IRP-2 binding because of the introduced compensatory sequence changes to maintain full base pairing of the lower stem.

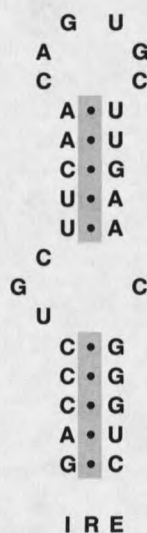


Fig. 21: Mutant ferritin IRE that varies from the wild type ferritin IRE in the lower stem to allow base pairing with the HHR.

By using a combination of the two strategies (PCR amplification of desired template, HHR) there are many different possible reactions that can be run to synthesize a variety of RNAs (Fig 22). By using different primer combinations with and without the pBPBE-3 plasmid a variety of template DNA can be made such as: wild-type IRE, a mutant IRE, 5' HHR-IRE-HHR 3', IRE-HHR 3', and plasmid sequence-5' HHR-IRE-HHR 3'-plasmid sequence.

Because the pBPBE-3 was designed to include restriction sites within the HHR-IRE-HHR sequence, ferritin IRE can be changed to a transferrin receptor IRE, other IREs, or different HHR sequences can easily be swapped in allowing further choices in IRE and HHR constructs.

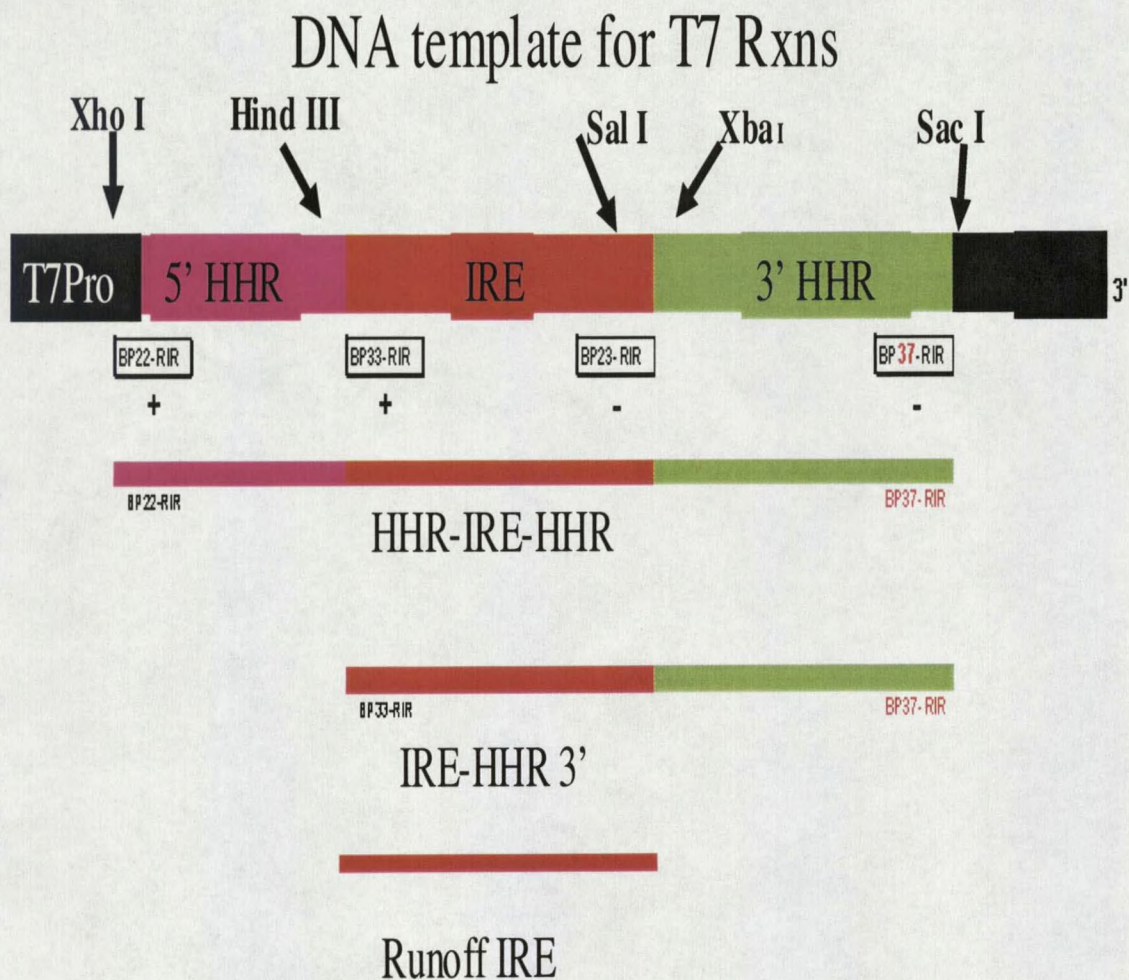


Fig. 22: The pBPBE-3 vector and various primers can be used to generate a variety of RNA fragments including: 5'HHR-IRE-HHR3', IRE-HHR3', and run-off IRE (wild type and mutant). Restriction sites are also shown.

RNase Control

RNases are a family of enzymes that degrade RNA rapidly by cleaving the RNA at specific sites, without RNases the amounts of mRNA in an organism would accumulate and cause problems with the protein expression levels in the organism. However, it is essential to inhibit these RNases if you would like to work with them in the laboratory environment because the RNases will degrade the RNA before any meaningful experiments can be completed. Controlling and monitoring RNase activity are essential techniques that must be learned in order to work with RNA. Common sources for RNase contamination and ways to control it are (42):

1. "Fingerases"; bodily fluids, and skin cells are often referred to as "fingerases"
2. Tips and tubes; autoclaving alone will not destroy all RNase activity; these enzymes are very robust and can refold upon cooling to room temperature to regain partial activity.
3. Water and buffer; DEPC-treated water is the most common method used to inactivate RNases.
4. Laboratory surfaces; benchtops, glassware, metalware, plasticware, and other surfaces that are exposed to the environment; glassware and metalware can be baked overnight at 450°F to inactivate RNases.
5. Endogenous RNases; all tissue samples contain endogenous RNases.
6. RNA samples; small amounts of RNases may co-purify with isolated RNA.

7. Plasmid DNA; DNA used for *in vitro* transcription and other reactions can introduce RNase contamination, a phenol/chloroform extraction can be used on the DNA to help eliminate RNases.
8. RNA storage; to preserve isolated RNA for long-term storage a salt/alcohol precipitation is essential; typically the nucleic acid is stored as a precipitate in solution.
9. Chemical cleavage; RNA molecules can also undergo strand scission when heated in the presence of divalent cations such as Mg^{2+} , Ca^{2+} , and Pb^{2+} at $>80^{\circ}C$ for 5 minutes or more.
10. Enzymes; both commercially purchased and laboratory prepared enzymes.

RNases can be inhibited through a variety of methods, some work better than others, while others are easier to use. Strongly acidic ($pH < 4$) or basic solutions ($pH > 9$) will denature the RNases and are useful to clean pH electrodes, UV-cells, and purification columns. Hydrogen peroxide (H_2O_2) at a concentration of 3% (or more) for ten minutes causes an irreversible inhibition of the RNase and is easily removed with several washes of RNase-Free water; the use of H_2O_2 is practical for cleaning glassware. The addition of 5mM EDTA will cause reversible inhibition by chelation of magnesium (Mg^{2+}), magnesium is an essential component for optimal RNase activity (and for folding of the HHR), it is useful for the long term storage of RNA but EDTA can be difficult to remove completely. DEPC at 0.05% causes irreversible inhibition it is commonly used to treat water and to clean glassware, but it can be difficult to remove and can react with

nucleic acids (causes the aromatic rings to open). Denaturing agents and detergents are also used but are difficult to remove later on. Commonly used denaturing agents are; 10% SDS, Urea (3M), and Guanadinium chloride (6 M). Commonly used detergents are 1% Triton X-100, and 1% Tween 20. There are also many commercially available RNase eliminators to clean glassware; RNase Zap (contents unknown) by *Ambion* is frequently used in our lab. RNase Zap is easy to use, apply a few drops to the desired piece of glassware, wipe it down with a lint free paper towel, and wash it thoroughly with RNase-Free water. A non-chemical alternative to inhibiting RNases wash the glassware with five volumes of water and then bake the glassware at 450°F for six to eight hours or more. Autoclaving the glassware will not destroy RNases. Metal instruments can be cleaned in a similar way, by heating the metal instrument to high temp with a Bunsen burner and rinsing it with RNase Free water. RNase activity also needs to be controlled at the reaction stage for RNA transcription by adding specific RNase inhibitors (RNasins). 1 Unit/ml RNasin inhibits RNase A, while 1 Unit/ml SUPERase•In (*Ambion*) inhibits RNase A, RNase T1, and RNase C. Ethanol (95%-100%) was used to clean the bench top, gloves, the outside of columns and tubing, and pipetmen. Table 2 lists some common enzymes that can modify nucleic acids (43).

Table 2: Nucleic Acid modifying enzymes

Enzyme	Family	Nuclease type	Reaction catalysed	Generates	Comments
RNase H	RNase	Endoribo	Cleaves RNA only in DNA~RNA hybrids	5'p-RNA	
RNase A	RNase	Endoribo	Cp/N	RNA-p3'	
			Up/N		
RNase T1	RNase	Endoribo	Gp/N	RNA-p3'	
RNase U2	RNase	Endoribo	Ap/N	RNA-p3'	
			(Gp/N)		
RNase C1-3	RNase	Endoribo	Cp/N	RNA-p3'	
			(Ap/N)		
			(Gp/N)		
RNase S7	DNase/RNase	Endonucl.	Np/A	(D/R)NA-p3'	
			Np/U		
RNase ϕ M	RNase	Endoribo	Np/U	RNA-p3'	
			Np/A		
			Np/G		
RNase T2	RNase	Endoribo	Np/N	RNA-p3'	Unspecific endoribonuclease
Nuclease P1	DNase/RNase	Endonucl.	Np/N	(D/R)NA-p3'	
DNase 1	DNase	Endonucl.	N/pN	DNA-p3'	Unspecific endodeoxyribonuclease
Snake venom Phosphodiesterase		Exonucl.	N/pN	RNA-OH3'	Unspecific 3' - 5' exonuclease requires RNA with free 3'OH
Spleen Phosphodiesterase		Exonucl.	Np/N	RNA-p3'	Unspecific 5' - 3' exonuclease requires RNA with free 5'OH
Alkaline Phosphatase (PAL)			p/N	5'OH-N	Removal of all terminal phosphate groups

Table 2: continued: Nucleic Acids modifying enzymes

Polynucleotide kinase (PNK)	pol		p-p-p-rA + 5'HO-poly(N) → 5'p-poly(N)	5'p-(D/R)NA	Transfer the γ-Phosphate of ATP to the 5'-OH of a nucleic acid
PNK 3' phosphatase mutant	pol		ibid	ibid	Useful for 3' end-labeling
RNA ligase	pol		RNA3'OH + pppORN → RNA~pORN	RNA~ORN	Adds 5'-phosphate donor RNA to the 3'-OH of RNA
PolyA polymerase	pol		RNA3'OH + n (d/r)ATP → RNA~(pA)n	RNA-(pA)n	Adds ATP (r or d) to the 3'-OH of RNA
Terminal deoxynucleotidyl Transferase	pol		DNA3'OH + n dNTP → DNA~(pN)n	DNA-(pN)n	Adds dNTPs at the 3' of DNA
T7 RNA polymerase (T7RNP)	pol		DNA-dep RNA pol	RNA	Uses DNA as template, rNTPs as primers
Reverse transcriptase (RT)	pol	RNase H domain	RNA-dep DNA pol DNA-dep DNA pol	DNA	Uses either DNA or RNA as template Uses either DNA or RNA as primer
Taq pol	pol		DNA-dep DNA pol	DNA	Uses DNA as template, DNA as primer
Vent pol	pol		DNA-dep DNA pol	DNA	Uses DNA as template, DNA as primer
Pyrophosphatase (PPiase)			PPi → 2 Pi	Pi	Also removes PPi groups in cap structures
Apyrase			ppp-rA → 2 Pi + p-rA	rAMP	Hydrolysis of the pyrophosphate bonds in rATP

For assembly of the IRP/IRE complex, the RNA must be correctly folded. Thus our protocols must use reagents that can be easily removed and will not disrupt the RNA secondary structure. Therefore, 30% H_2O_2 and *RNase Zap* were commonly used to clean glassware. 30% H_2O_2 is added to the glassware/plasticware with shaking for ten minutes (or let stand for thirty minutes) then remove the H_2O_2 (can be reused for at least one month before the H_2O_2 goes bad) and wash with five volumes of RNase-Free water. *RNase Zap* was only used when glassware needed to be cleaned right away, since it is much faster, wipe on and wash off (five volumes of RNase-Free water). Metal instruments were cleaned as described previously, and RNasins were used at the concentration mentioned above as well. Un-opened boxes of gloves were assumed to be RNase free, and great care was taken when putting on the gloves to ensure that potential "fingerases" didn't contaminant the exterior of the gloves. When in doubt, potential contaminated gloves were exchanged for clean gloves. Ethanol (95%) was commonly used to clean gloves and pipetmen at any stage to ensure good sterile technique and to wash off any potential RNases.

RNase-Free solutions are needed for the transcription reactions, and purification steps. The reagents necessary to make the solutions were purchased separately and kept isolated from general lab use. Only people currently working with RNA are allowed to use these reagents. RNase-Free bottles were used, and unopened Falcon tubes (50ml and 15ml tubes, assumed to be RNase-Free) to mix and store the solutions. The powdered or liquid reagents would be added directly to the bottle (without a spatula), and by placing the bottle on scale it would eliminate the use of a weigh boat or weigh paper. This

eliminated any chance of RNase contamination coming from weighing out the reagents. To adjust the pH of the solutions, an RNase free pH probe would be used, or two identical solutions would be made where one of them would be checked with a regular pH probe. By keeping track of how much acid or base that was added to the non-RNase free solution, the second solution could be made with out the use of a pH probe. To assure that the pH of the RNase-Free solution was correct, we commonly take a small aliquot of the solution and check it with a pH meter.

It is necessary at times to check solutions for RNase activity. This includes all stock solutions, transcription reactions, and subsequent purification steps, so that RNA degradation will not interfere with complex formation or crystallization. One way to do this was with a simple time course, just add some RNA to a tube containing the solution that needed to be check and remove small aliquots and run them out on a denaturing PAGE gel. The gels proved difficult to read, so a more quantitative method was needed to detect for RNases. A more effective and quantitative way to detect for RNase activity is to use *Ambion's RNaseAlert* kit. The kit contains an RNA oligo that is double labeled with fluorescent and quenching moieties, when RNases are present they open up the RNA oligo allowing the fluorescent marker to be excited at the proper wavelength of light. The excitation wavelength is 490nm, and the emission wavelength is 520nm. The procedure is simple and allows for multiple samples to be detected using a fluorescent plate reader. Sample aliquots of 45 μ L had RNA oligo added, placed into a plate reader and readings taken every five minutes for one hour (Fig. 23).

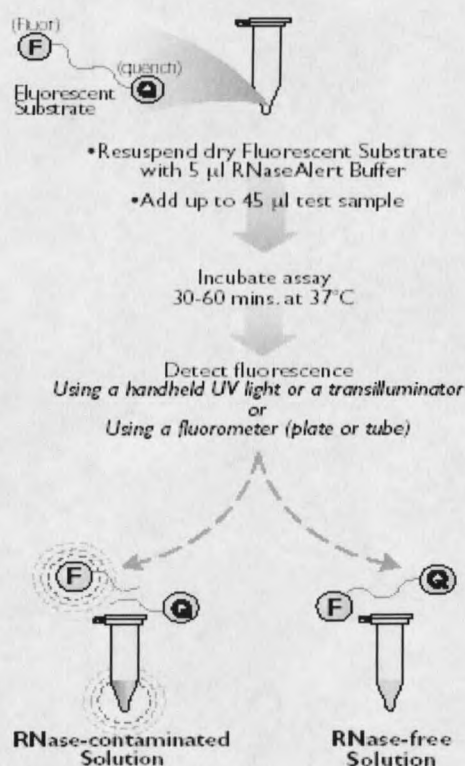


Fig.23: Ambion's RNase Alert Test kit procedure. When RNases are present the quench will be cleaved allowing the substrate to fluoresce.

A *Tecan-SAFIRE* absorbance and fluorescence plate reader (Edward Dratz, Montana State University) was used to read the samples. Experiments indicated that a 5x dilution of the RNA oligo could still generate a strong signal in the plate reader from an RNase positive control. The kit was used to check all solutions, transcription reactions, and fractions from purification steps. The kit also confirmed that our milliQ water from our water purifier was RNase-Free, so no further water treatments had to be used to ensure that our water was RNase-Free. It is important to note that if you want potential RNases to be fully active to detect with the kit, there should be 5 mM MgCl_2 and 1 mM DTT in the medium (these components are included in the RNase Alert 10 x buffer).

T7 RNA Polymerase Expression and Purification

Introduction

The interest of the lab is to eventually obtain x-ray crystallographic structures for the IRP-1/IRE and IRP-2/IRE complexes. Making these complexes will require a great deal of RNA to be produced. The most expensive of the reagents necessary for a T7 RNA polymerase reaction is the enzyme, T7 RNA polymerase (T7RNP), so it was critical to be able to make homemade T7 RNA polymerase in the lab to cut costs. Elizabeth Leibold at the University of Utah graciously donated a plasmid DNA containing the sequence for the T7 RNA polymerase, flanked by a sequence coding for a 6xHis tag, and a detailed protocol for the expression and purification of the T7 RNA polymerase.

Expression of the T7 RNA Polymerase

E. coli (BL-21 strain) were transformed with the plasmid DNA and plated on LB ampicillin (100µg/ml) plates. A colony containing the T7RNP was picked and grown in overnight starter culture at 37°C in LB media (50ml) with ampicillin (100µg/ml). The starter culture was then transferred into 2 liters of terrific broth (12 g Bacto tryptone, 24 g Bacto yeast extract, and 4 ml glycerol per 900 ml; autoclave solution, then add 100ml of filter sterilized 0.17M KH₂PO₄ and 0.72M K₂HPO₄) at 37°C and grown to an O.D.₆₀₀ of 0.4. IPTG (100µM) was added to the culture to induce protein expression. Protein expression continued for 3 hours at 37°C with shaking. The cells were then pelleted and resuspended in 30 ml of lysis buffer (50mM Tris-HCL pH 8.0, 100mM NaCl, 5mM β-

mercaptoethanol, 5% glycerol, and 1mM imidazole). Cells were lysed using a microfluidizer (Valérie Copié, Montana State University), and spun in an ultra centrifuge (45,000 RPM for 2 hours) to remove cellular debris.

Purification of the T7 RNA Polymerase

The supernatant was passed over a Ni-NTA affinity column (equilibrated with lysis buffer). The Ni-NTA resin (40 ml) was washed with lysis buffer 3x40 ml, followed by a second washing with lysis buffer plus 10mM imidazole 3x40 ml. The T7 RNA polymerase was eluted with lysis buffer plus 100mM imidazole 2x20 ml. The concentration of the T7RNP was determined by the Bradford assay. The T7 RNA polymerase was dialyzed against storage buffer 8x500 ml (10mM Tris-HCL pH 8.0, 1mM EDTA, 50% glycerol, 100mM NaCl, and 1mM DTT), exchanging the buffer approximately every two hours. A final concentration for T7RNP was obtained, and checked for RNase activity using *RNase Alert* assay. The T7 RNA polymerase was aliquoted into 1.5 ml microfuge tubes, and frozen away at -20°C. All buffers were RNase Free, as determined by the *RNase Alert* Assay.

T7 activity assay

Once the enzyme was purified, the activity of the T7RNP needed to be determined. Whenever fresh T7RNP is purified the specific activity and the total activity of the purified T7RNP should be measured, along with the concentration and the total amount of the protein. Quantification of radiolabeled transcripts produced by *in vitro* T7

transcription is used to measure the enzymatic specific activity of home-purified T7 RNA polymerase. The quantification is based on the selective precipitation of the radiolabeled transcripts on cellulose membrane by trichloroacetic acid (TCA) (44).

Like all polymerases, T7RNP uses nucleotides triphosphate (NTPs) as substrate as well as Mg^{2+} and a template DNA as cofactors to synthesize oligonucleotides. Polymerases can be classified following the nature of the neo-synthesized oligonucleotide and the nature of the template used. Based on this classification, T7RNP is a DNA-dependent RNA polymerase that uses ribonucleotides triphosphate (NTPs; N= A, U, G, C) as substrate to produce RNA by the following scheme (Fig. 24) (45):

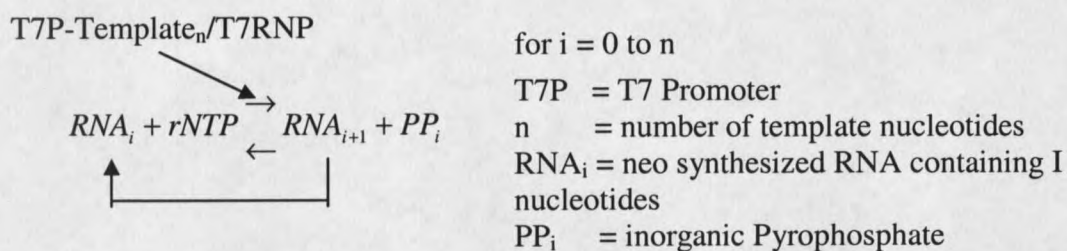


Fig. 24: T7 transcription reaction scheme

The T7RNP specific activity assay is based on the time course quantification of transcripts synthesized. This quantification is performed by selectively precipitating the oligoribonucleotides on cellulose membrane and by determining the number of radiolabeled UMP incorporated (44, 45). This approach has three major advantages:

1. The use of radioactivity provides a precise quantification applicable at analytical scale, hence minimizing the amounts of T7RNP and NTPs needed.

2. Radiolabeled transcripts of length equal or greater than 15 nt are precipitated by trichloroacetic acid (TCA) on the cellulose membrane (*Whatman 3MM* paper), whereas unincorporated NTPs are not retained.
3. An answer can be obtained within 3 hours.

In all experiments, pyrophosphatase (PPiase) was added to shift the equilibrium towards product formation; this enzyme catalyzes the hydrolysis of pyrophosphate (PP_i) into inorganic phosphate (P_i).

Methods

When preparing a working solution of radiolabeled XTP, the specific radioactivity (SA_{XTP, work}) of the given radiolabeled XTP* is required for measurements on scintillation counter efficiency (E for the given isotope). SA_{XTP, work} can be determined by the following relation (45):

$$SA_{XTP, work} \text{ (dpm/mol XTP)} = \frac{cpm_{assay}}{Y_{min} [DNA] V_{assay} E N_X} \quad \text{Eq. (1)}$$

Where cpm_{assay} represents the number of expected counts per minute (cpm), V_{assay} is the volume, $[DNA]$ is the DNA template concentration necessary to synthesize at least Y_{min} -fold more transcripts during the minimal interval time, and the full-length products containing N_X nucleosides (with X being of the same nature as the XTP* used in the reaction) (45).

For example, if the efficiency of the counter is 40% (takes also into account all quenching phenomena), if the yield at 5 min. (shortest time point) is 1.4 full-length RNA

(containing 121 U) per molecule of template DNA (present at 50 nM in the reaction), and if 2000 cpm are wanted in a 15 μ L assay, then:

$$SA_{XTP, work} = \frac{2000}{1.4 * 50.10^{-9} * 15.10^{-6} * 0.4 * 121}$$

$$= 39.3.10^{12} \text{ dpm/mol of } [\alpha\text{-}^{35}\text{S}]\text{-UTP}$$

$$\text{i.e. } SA_{XTP, work} = 39.3.10^{12} / 2.22.10^{12} = 17.7 \text{ Ci/mol}$$

The only assumption made in this relation is that all products are full-length, which is obviously wrong, but sufficient to calculate the useful isotopic dilution D_i given by:

$$D_i = SA_{XTP, work} / SA_{XTP, stock}$$

Thus, for a $[\alpha\text{-}^{35}\text{S}]\text{-UTP}$ stock solution is at $SA_{XTP, stock} = 400 \text{ Ci/mmol}$, then the isotopic dilution is: $D_i = 17.7/400.10^3 = 1/22587$.

A V_{work} μ L working stock solution of radioactive UTP, at a concentration C_{work} , can then be made according to the following equations system:

$$\begin{cases} C_{XTP, stock} * V_{XTP, stock} * SA_{XTP, stock} = C_{work} * V_{work} * SA_{XTP, work} & (1) \\ C_{XTP, stock} * V_{XTP, stock} + C_{XTP, cold} * V_{XTP, cold} = C_{work} * V_{work} & (2) \end{cases} \quad \text{Eq. (2)}$$

Where $C_{XTP, cold}$ and $V_{XTP, cold}$ are the concentration and volume of non-radioactive XTP to mix with the radiolabeled XTP (45).

$$(1) \Rightarrow V_{XTP, stock} = \frac{C_{work} * V_{work}}{C_{XTP, stock}} * \frac{SA_{XTP, work}}{SA_{XTP, stock}} = \frac{C_{work} * V_{work} * D_i}{C_{XTP, stock}} \quad \text{Eq. (3)}$$

$$(2) \Rightarrow V_{\text{XTP, cold}} = \frac{C_{\text{work}} * V_{\text{work}} - C_{\text{XTP, stock}} * V_{\text{XTP, stock}}}{C_{\text{XTP, cold}}} \quad \text{Eq. (4)}$$

For example, to prepare 22.6 μL of $[\alpha\text{-}^{35}\text{S}]\text{-UTP}$ working solution at $\text{SA}_{\text{XTP, stock}} = 17.7 \text{ Ci/mol}$ and 50 mM, using a 100 mM stock solution of non-radioactive UTP, and a stock solution of $[\alpha\text{-}^{35}\text{S}]\text{-UTP}$ at 400 Ci/mmol, with a total activity of 250 μCi in 25 μL , we have (45):

$$250.10^{-6}/400.10^3 = 625.10^{-12} \text{ mol of } [\alpha\text{-}^{35}\text{S}]\text{-UTP, i.e. 625 pmol, in 25 } \mu\text{L}$$

$$\Rightarrow C_{\text{XTP, stock}} = 625.10^{-12} / 25.10^{-6} = 25.10^{-6} \text{ M, i.e. 25 } \mu\text{M}$$

Using Eq. (3):

$$V_{\text{XTP, stock}} = \frac{50.10^{-3} * 22.6}{25.10^{-6}} * \frac{17.7}{400.10^3} = 2.0 \mu\text{L}$$

$$\text{And Eq. (4): } V_{\text{XTP, cold}} = \frac{50.10^{-3} * 22.6.10^{-6} - 25.10^{-6} * 2.10^{-6}}{100.10^{-3}} = 11.3.10^{-6} \text{ L}$$

i.e. 11.3 μL of non-radiolabeled UTP at a concentration 100 mM should be mixed with 2 μL of the $[\alpha\text{-}^{35}\text{S}]\text{-UTP}$ stock solution (250 μCi) and adjusted to a final volume of 22.6 μL (using a 40 mM Tris-HCl pH 8.1 solution). This working solution can be stored at -20°C .

Preparation of template DNA must be taken into account when performing this assay. In order to avoid premature termination of the T7 transcription due to the particular sequences in the IRE constructs, and hence have a standard protocol for the T7RNP activity assay, it was decided to use a plasmid containing a T7 promoter as template: *pBluescript IISK+* (46). Although not required to determine the T7RNP

activity, knowledge of controlling the maximum number of U incorporated in the full-length transcript is necessary to estimate the T7 transcription reaction yield. The T7 transcription yield is defined as the number of full-length RNA molecules made per template DNA molecule, which is the parameter used in finding optimal conditions for the reaction. To avoid “run-around” transcripts and produce transcripts of defined length, the plasmid is linearized by Afl III, a specific endonuclease. This restriction enzyme hydrolyzes the chosen plasmid once, after the cytosine in the following recognition sequence: (+) DNA 5' ...C/TTAAG ...3', 508 nucleotides downstream the G₊₁ of the T7 promoter, so that the number of U incorporated in a full-length RNA is 121.

Assay conditions are under which T7RNP is limiting (pseudo first order kinetics) and having adjusted the [α -³⁵S]-UTP specific activity as required. The following protocol for T7 transcription and TCA precipitation/wash was designed and optimized by Philippe Bénas and myself (45):

1. Use one reaction per time point, in order to avoid concentration uncertainties that could occur if aliquots were taken (in particular if precipitation of the transcripts occurs as more RNA is made)
2. Use a minimum reaction volume of 17 μ L to minimize evaporation effect on concentrations. This allows one to remove a constant and precise volume of 15 μ L for each sample
3. The 17 μ L reaction mixtures contain 50 nM linearized *pBluescript II*SK+, 40 mM Tris-HCl pH 8.1 at 37°C, 0.01% (v/v) Triton X-100, 50 mM DTT, 8% (v/v)

PEG 8000, 50 $\mu\text{g/ml}$ BSA, 400 U/mL RNasin, 2.5 mM NTPs including [α - ^{35}S]-UTP at a specific activity of 17.7 Ci/mol (prepared as described above), 1 U/mL PPIase, 16 mM MgCl_2 , 2 mM spermidine and the T7RNP. The addition order is as listed and the T7RNP addition corresponds to $t = 0$. All solution but the T7RNP are brought to room temperature before addition to the reaction, spermidine and MgCl_2 being known to precipitate DNA at colder temperature (e.g. 4°C).

4. Reactions are carried out at 37°C , and 3 time points of 15 μL each are taken that varied depending on the assay from: 2.5, 5, 10, 15, and 20 min.
5. The T7RNP concentrations were the following: 233 nM for a commercially available T7RNP (*TaKaRa*), 207 nM T7RNP-2 (10 months old, stored in 50% glycerol), 5.8 μM T7RNP-3, and for 4.6 μM T7RNP-5.
6. One positive control is to deposit 15 μL out of 17 μL rxn. (contains all components) on 3 MM paper, that is not followed by a TCA precipitation or any wash. Ideally, there should be one positive control per time point, but in practice, a single positive control is sufficient. This simulates the maximum number of counts that could be incorporated into the full-length RNA transcripts.
7. A measurement of background, a 15 μL blank, contains all the components of the T7 transcription and 5% TCA, prior to T7RNP addition; several blanks are required for a good estimation of the background (e.g. one per time point); no correlation with incubation time was ever observed.

To count the amount of radioactivity, 1 cm x 1 cm cellulose 3MM paper (*Whatman*) pieces are previously pinned on a piece of *Styrofoam*. 15 μL out of the 17 μL

reaction mixture are pipetted onto a 3 MM paper, air-dried for 5 seconds and then dumped into 5% TCA, chilled by melting-ice (0°C). The TCA stops the reaction by denaturing the RNA polymerase and precipitates the oligonucleotides longer than 15 nt. At the end of the experiment, two additional washes of 10 min. each in chilled 5% TCA are carried out, followed by 3 washes of 10 min. each in 95% ethanol. The pieces of 3MM paper are put back on the *Styrofoam* for drying (either under a heating lamp for at least 1 hour or overnight at room temperature). Finally, each piece of paper is placed in a scintillation vial containing 1mL of scintillation liquid (*Ecoscint O*), and counted for 10 min. on a *Packar 2200 CA Tri-Carb* counter.

Results

The measurements obtained from the counter and initial speeds are summarized in Table 3, whereas Figures 25, 26, and 27 give a graphical representation the number of nmoles of [α -³⁵S]-UMP incorporated in transcripts as a function of reaction time.

Sample UTP [³⁵ S]	time (min)	CPMA	DPMI	SIS	Counting time (min)
	5	537500.00	565952.00	53.99	0.03
Blank					
	5	578.30	608.66	61.80	10.00
	10	91.20	95.98	71.66	10.00
	15	286.80	301.99	65.28	10.00
	20	272.50	286.78	64.64	10.00
T7 Com.					
	5	3539.31	3726.14	60.36	5.75
	10	7677.55	8086.77	57.81	2.65
	15	10285.70	10826.30	56.82	1.42
	20	3633.55	3831.90	58.09	1.28

Table 3: CPM and DPM counts for blanks and T7RNP preps, and initial rates of T7RNP preps

T7RNP-2					
	5	2151.64	2268.31	62.60	5.17
	10	2415.52	2540.70	59.18	4.64
	15	2823.93	2974.88	58.77	3.97
	20	4552.85	4794.03	59.09	2.46
T7RNP-3					
	5	23358.30	24619.40	58.13	0.48
	10	45632.00	48062.60	64.56	0.25
	15	74506.30	78439.30	60.15	0.16
	20	27685.40	29181.10	57.92	0.41
UTP [³⁵ S]-5					
	5	1065100.00	1208239.00	22.41	0.02
	10	1100400.00	1253542.00	22.14	0.02
Blank					
	2.5	9320.78	10617.30	24.69	1.54
	5	10469.90	11911.30	26.03	1.36
	10	18731.20	21310.80	24.38	0.77
	20	202600.00	231002.00	24.28	0.70
T7RNP-5					
	2.5	14007.80	15870.00	24.54	1.02
	5	14788.70	16818.20	24.62	0.97
	10	46546.90	52872.40	23.12	0.32
	20	287067.00	326580.00	23.97	0.60
T7 Com		T7-2		T7-3	
Time	Speed (nmol/min)	Speed (nmol/min)	Speed (nmol/min)	Speed (nmol/min)	Speed (nmol/min)
5	5.12	2.92	36.53	4.32	
10	11.67	3.33	71.78	29.43	
15	15.79	3.99	117.45	N/A	
20	5.28	6.72	43.39	220.09	
Speed (t=5)	1.02	0.58	7.31	1.46	
Speed (t=20)	1.17	0.33	7.18	0.86	
Speed 5-2.5	1.05	0.27	7.83	11.00	
Speed 20-10	1.31	0.08	7.05	0.26	
Average Dn/Dt	0.82	0.13	9.13	19.07	
Average n/t	1.07	0.11	8.09		
Average 20 min	1.08	0.39	7.44		
AS (nmol/hour)	1.08	0.28	7.70	15.03	
N units/ μ l	140.30	40.87	40.42	238.32	
N units/ml	2.50	0.73	0.72	4.25	

Table 3: continued

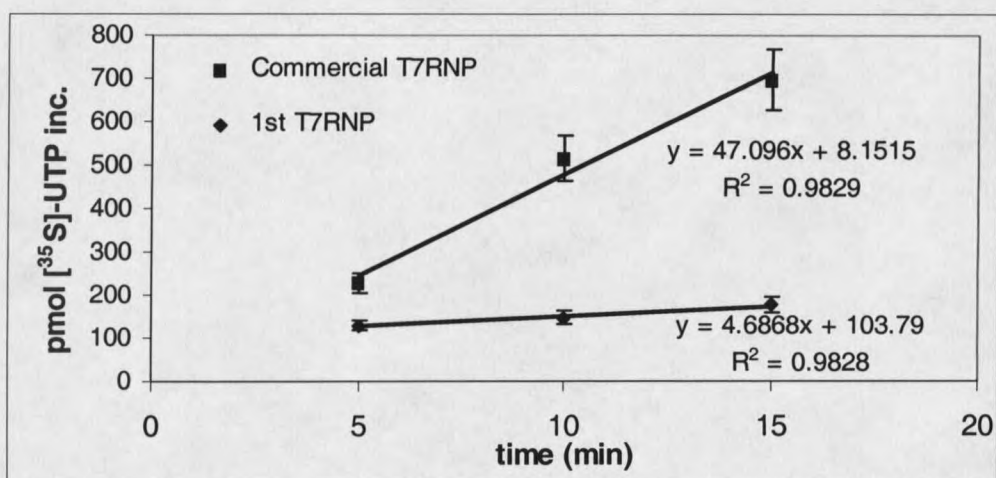


Fig. 25: $[\alpha\text{-}^{35}\text{S}]\text{-UTP}$ incorporation using the commercial or T7RNP-1

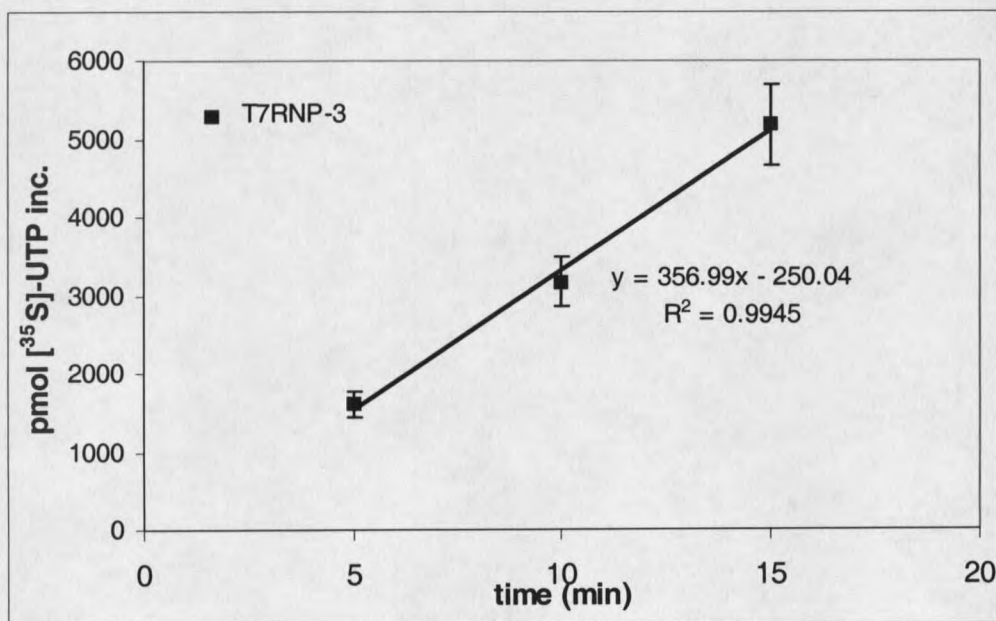


Fig. 26: $[\alpha\text{-}^{35}\text{S}]\text{-UTP}$ incorporation using T7RNP-3

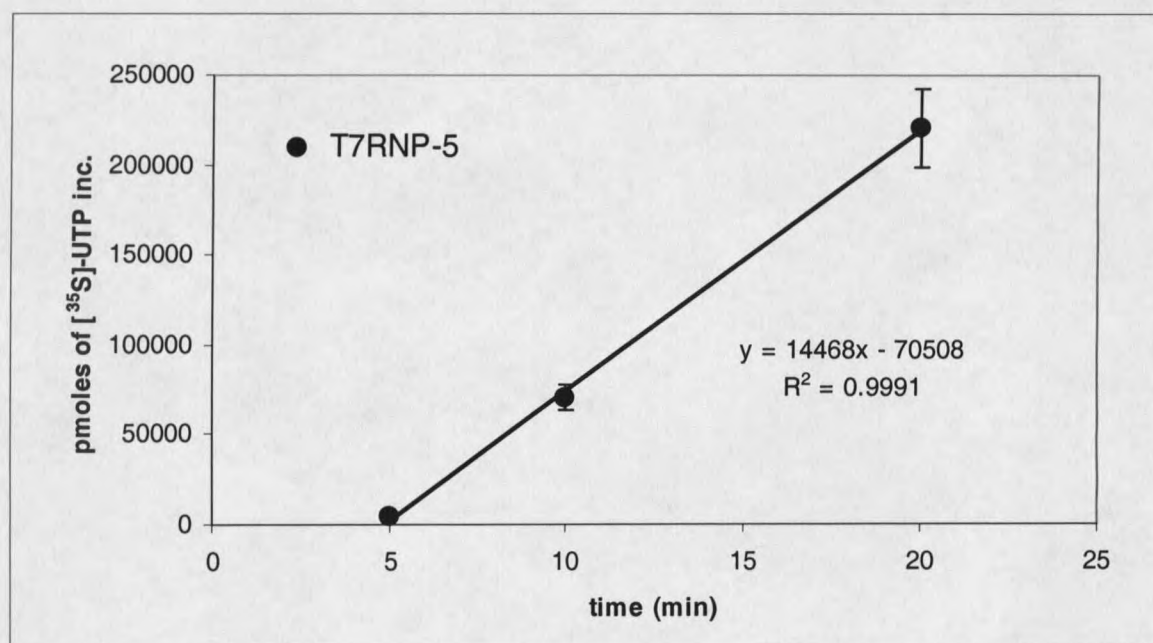


Fig 27: [α - ^{35}S]-UTP incorporation using T7RNP-5

Although there are only three time points per experiment they are linear, indicating that the initial reaction velocity is linear over time and 1st order in enzyme concentration. In addition, the enzymes concentrations used and the products formed are less than 10-fold and 5% of the initial NTPs concentration respectively, which corresponds to conditions required for kinetic analysis of order 1. Table 4 gives the relative specific activities for the three T7 RNA-polymerases used in the present experiments.

Table 4: Relative specific activities of the T7RNP

	Commercial T7RNP	T7RNP-2	T7RNP-3	T7RNP-5
Relative Specific Activity	100%	29.2%	28.8%	170%

Discussion and conclusions

The purpose of the experiment was to provide milligram amounts of active T7RNP at an affordable price comparable with the quality of the commercially available T7RNP. Purifications typically yielded 40 mg/L of T7RNP per liter of *E. coli*. The activity assay indicated that the T7RNP-2 and T7RNP-3 purifications had 3 fold lower specific enzymatic activity compared to the commercial one, while the latest prep (T7RNP-5) is about 2 fold more active than the commercial T7RNP. Hence, our purification scheme is rather well designed. In addition to determining the enzyme activity, the T7RNP was tested for RNase activity using the RNase Alert kit. Earlier enzyme purifications (T7RNP-2 and -3) contained a significant amount of RNase (~ 0.5 pg/ml), this possibly accounts for the lowered specific activities. The RNase contamination was first attributed to insufficient stringency during elution at the affinity chromatography step (Ni-NTA) and/or possible contamination during dialysis. Therefore, in the following purification (T7RNP-5), all solvent components were checked for possible RNase contamination before use by using the RNase Alert. The RNase assay still detected RNase activity from the eluted protein, but with extensive dialysis with RNase-Free storage buffer (8 washes of 500ml each), the T7 RNA polymerase showed no RNase activity. This suggested that it was not necessarily critical to have RNase-Free solutions during the column washes and elution. Of paramount importance is extensive dialysis against RNase free storage buffer, at least 8x500ml buffer exchange at 2 hour intervals. Obtaining milligram amounts of T7RNP that was 2-fold more active than that of the commercial polymerase and RNase free was critical for large scale synthesis of RNA.

PCR Amplification of Template DNA

In order to synthesize large amounts of RNA, there needed to be a ready supply of DNA template for the T7 transcription reactions. There are two ways to make DNA for T7 transcription reactions; one is to linearize a plasmid containing the T7 promoter followed by your sequence. The other is to use a PCR reaction to selectively amplify your sequence. The T7 promoter is either carried by the plasmid (with your sequence immediately following the T7 promoter, unless there is a ribozyme construct) or within the two primers needed for the PCR reaction. The PCR amplification method was decided upon because different constructs needed to be tested.

Three PCR templates were amplified; the HHR-IRE-HHR transcription (long transcript), where the template is a plasmidic DNA (pBPBE3) and one of the two primers contains the T7 promoter, the IRE-HHR transcription created the same way as the HHR-IRE-HHR transcription, and the T7 run off transcription, where the 2 primers are self-complementary and hence are also template, one of these primers containing the T7 promoter.

Methods

To be able to PCR amplify the plasmid DNA template the annealing temperature of the primers had to be optimized, this was done by using three different computer programs. By using *Anneal-Hybridize* (Olivier Friard, <http://annhyb.free.fr>), Nearest Neighbors Method, the melting temperature (T_m) of each primer was calculated. The

program takes into account the primer concentration and the sum of the monovalent cation salt concentrations (80 mM if just the minimal 10x buffer is used).

RNA-Structure 3.5 (David Mathews, <http://128.151.176.70/RNAstructure.html>) was used to find possible secondary structures within the primers. The intermolecular pairing option is used to get both the primer_i/primer_j and the primer₁/primer₂ possible base pairing.

The data from *RNA-Structure 3.5* was used in *RNA-Draw* (Ole Matzurato, <http://rnadraw.base8.se>) calculate the free energy at various temperatures, and the temperature that gave a null free energy corresponded to the T_m. The annealing temperature was obtained by taking the T_m – 7°C of the lowest temperature melting primer. MgCl₂ and dNTPs concentrations needed be adjusted between runs.

When performing PCR with primers used as template (self-complementary primers), the reaction is the same except that the template is made by the hybridization of the two primers. The primer concentration can be as high as 650 nM, or even more, provided the T_m of the strongest base paired DNA that could be formed stays reasonable (max 85°C). A typical PCR reaction procedure is shown in Table 5.

Depending on the type of RNA that was being made the PCR reaction would vary slightly. The PCR reactions that were optimized were for the wild type IRE, and the HHR-IRE-HHR constructs. The wild type IRE DNA was formed using BP-25-RIR primer (5' GTTCCGTTCAAgcactgttgaagcaggaa 3') and the BP-34-RIR primer (5' GAGCGCGCGTAATACGACTCACTATAGttcctgcttcaacagtgc 3'), the bases in lower case indicates the overlapping sequence.

Addition order	COMPONENTS OF THE REACTION	Comments
2	PCR "minimum" buffer, 10x TrisHCl pH 9.2 @ 25°C (500 mM) (NH ₄) ₂ SO ₄ (160 mM) Triton X-100 (1% v/v)	50 mM final concentration 16 mM in 1x solution 0.1 % final conc.
3	MgCl ₂ (10 mM)	0.5 - 3.5 mM final conc.; Free Mg ²⁺ ~ 1.5 mM [MgCl ₂] = Σ([dNTPs]) + (0.5 to 2.5) mM Error rate increases for conc. < 1.5 mM of free Mg ²⁺
3	BSA (1500 µg/ml)	10-100 µg/ml final; binds certain PCR inhibitors
3	DMSO (100% v/v)	1-10% final conc. improve the denaturation of GC-rich DNA
3	Glycerol (80% v/v)	5-20% final; improve the yield of amplification
3	Formamide (100% v/v)	1.25-10% final; lowers T _m of melt-resistant DNAs
3	Ammonium sulfate (500 mM)	15-30 mM final; alters T _m & enzyme activity
4	Template DNA (nM)	0.75 nM final conc. 0.38-1.515 nM final; average = 0.95 nM
5	Primer 5' (100 µM)	Final conc. 156 nM; 100-500 nM final
6	Primer 3' (100 µM)	Final conc. 156 nM; 100-500 nM final
7	dNTPs Mix (10mM)	final conc. 200 µM highest specificity & fidelity at the lowest conc.
8	Taq pol. (5 U/µl)	final conc. 0.125 U/µl Pfu pol. @ a final conc. of 0.0125-0.025 U/µL/kb
9	Vent pol. (0.01 U/µl)	0.5 µL/20µl
1	H ₂ O	

Table 5: PCR reaction using plasmid as template

The PCR reaction mixture was assembled using the following reagent conditions: 50mM Tris pH 9.2, 16mM $(\text{NH}_4)_2\text{SO}_4$, 2.3mM MgCl_2 , 625nM BP-34-RIR, 625nM BP-25-RIR, 0.1% Triton X-100, 200 μM dNTPS, Taq polymerase 0.05 Units/ μl , and 0.00025 Units/ μl Vent. The PCR reaction was cycled under the following conditions: 94°C for 90 seconds, 68°C for 45 seconds, 72°C for 45 seconds, and repeat 10 times; then 94°C for 90 seconds, 80°C for 45 seconds, 72°C for 45 seconds, and repeat 15 times. The DNA would be checked for purity and the correct base pair length by using PAGE gels (Fig. 28 and Fig. 29).

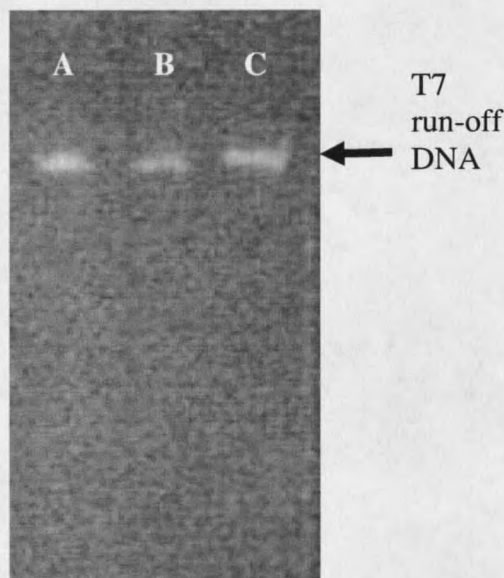


Fig. 28: Purified wild type IRE DNA generated by BP-25-RIR and BP-34-RIR run out on a 15% denaturing PAGE gel run at 300V to check its size and purity. Lanes A, B, and C indicate fractions from a desalting column to remove unincorporated dNTPs. PCR reactions were purified by performing a phenol/chloroform extraction, isopropanol precipitation, and run over a desalting column.

The HHR-IRE-HHR DNA was formed using the BP-37-RIR primer (5' GAGCGCGCGTAATACGACTCACTATAGGGCATGACGTCCTGATGAGTCCG 3'), the BP-22-RIR primer (5' GGGCCGACGGGTTTCGTCC 3'), and the template sequence (5'CGGCCCCGAGCTCGGGCCGACGGGTTTCGTCTCACGGACTCATCAGGACGGAGTCTAGACTCCGTCGACCCGTTCAAGCACTGTTGAAGCAGGGTCGACGGTCAGAAGCTTCTGACCGTTTCGTCTCACGGACTCATCAGGACGTCATGCGGGGGGCCCCGGCTCGAGCGGCCG 3') located in the pBPBE-3 vector.

The PCR reaction mixture was assembled using the following reagent conditions: 50mM Tris pH 9.2, 16mM (NH₄)₂SO₄, 2mM MgCl₂, 156nM BP-37-RIR, 156nM BP-24-RIR, 950nM pBPBE-3, 0.1% Triton X-100, 125μM dNTPS, Taq polymerase 0.125 Units/μl, and 0.00025 Units/μl Vent. The PCR reaction was cycled under the following conditions: 94°C for 90 seconds, 63°C for 60 seconds, 72°C for 60 seconds, and repeat 17 times; then 94°C for 90 seconds, 69°C for 60 seconds, 72°C for 60 seconds, and repeat 17 times; then 94°C for 90 seconds, 66°C for 90 seconds, 72°C for 60 seconds, and repeat 10 times. The DNA was checked for purity and the correct base pair length by using native PAGE gels (Fig. 29).

Other PCR reactions were performed. By using the primer BP-23-RIR (5' GACCCGTTCAAGCACTGTTGAAGCAGGGT 3') instead of BP-37-RIR only the 3'HHR would form. By using primers 1224 (5' CGCCAGGGTTTTCCAGTCACGAC 3') and 1233 (5' AGCGGATAACAATTTACACAGGA 3') instead of BP-37-RIR and BP-22-RIR, extra plasmidic DNA would be added to the ends of the HHRs.

These PCR reaction mixtures were assembled using the following reagent conditions:

50mM Tris pH 9.2, 16mM $(\text{NH}_4)_2\text{SO}_4$, 2mM MgCl_2 , 156nM BP-37-RIR, 156nM BP-24-RIR, 950nM pBPBE-3, 0.1% Triton X-100, 125 μM dNTPS, Taq polymerase 0.125

Units/ μl , and 0.00025 Units/ μl Vent. The PCR reaction was cycled under various annealing temperature conditions. The DNA was checked for purity and the correct base pair length by using native PAGE gels (Fig. 29).

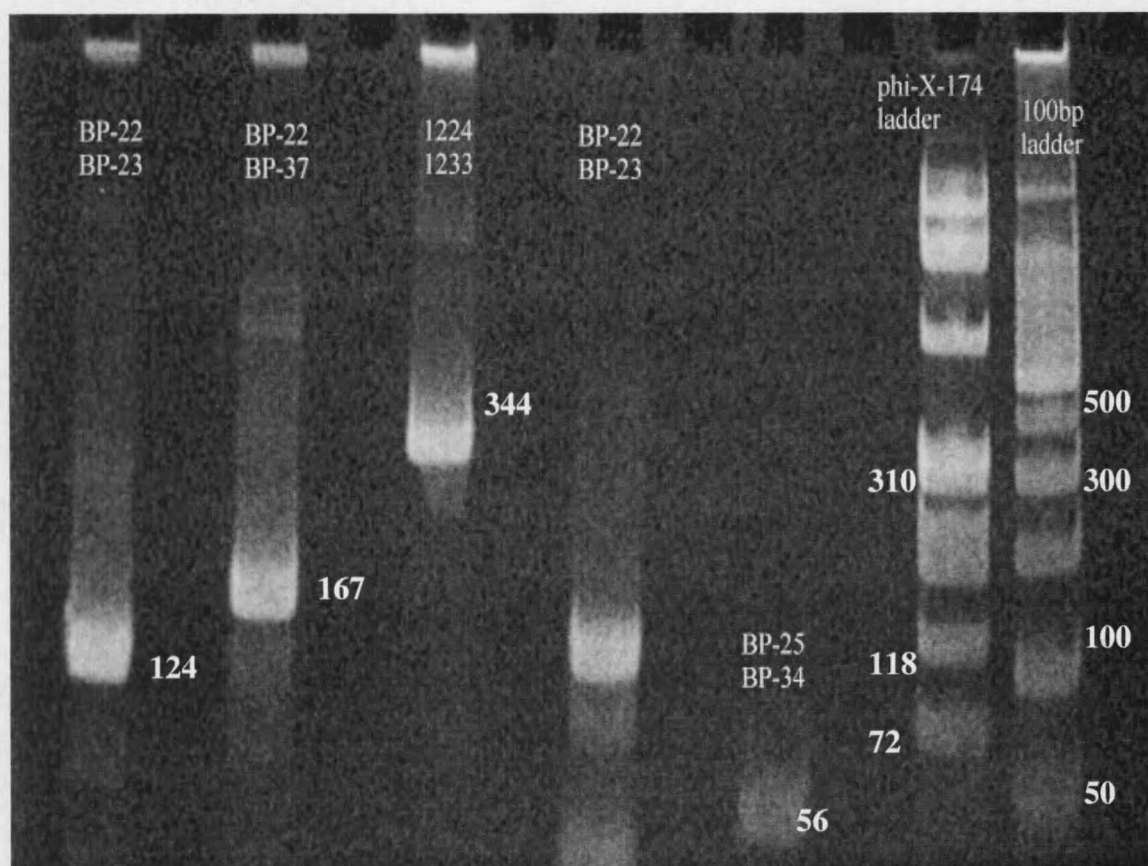


Fig 29: Raw PCR reactions. DNA fragments amplified by primers BP-22-RIR/BP-23-RIR (124mer), BP-22-RIR/BP-37-RIR (167mer), 1224/1233 (344mer), BP-25-RIR/BP-34-RIR (56mer) are shown on a 15% Native PAGE gel run at 300V.

After the PCR was completed the DNA fragments had to be purified to ensure that it would be RNase-Free and suitable for T7 transcription reaction. Typically the PCR reactions would undergo a phenol/chloroform (pH 7-9) extraction in order to denature all enzymes (Taq polymerase, Vent polymerase, and any RNases) present. A phenol/chloroform extraction procedure will be explained further in the IRE purification section. After the extraction the DNA fragments would be precipitated using 100% isopropanol, then run over a NAP-25 Column (*Pharmacia*) that removes excess dNTPs and salts, fractions would be checked by O.D. 260/280, and then the fractions would be isopropanol precipitated and resuspended into water. The DNA was checked by O.D. 260/280 to determine the concentration, the DNA was now ready to be used in T7 transcription reactions, and it is stable for months at -20°C.

Results

The DNA obtained through PCR was checked for purity and correct size on PAGE gels (see figures 28 and 29). For large scale amounts of DNA only primers BP-37-RIR/BP-22-RIR and BP-25-RIR/BP-34-RIR were used for PCR. The DNA from the large scale PCR reactions was phenol/chloroform extracted (see section in IRE purification for procedure), isopropanol precipitated, and run over a desalting column (NAP column, see section in IRE purification for procedure). It may be necessary to go back and perform large scale PCR amplification of different HHR constructs in order to obtain a homogenous IRE.

In vitro T7 transcriptions

Enzymatic synthesis of RNA is commonly accomplished using the T7 *in vitro* transcription system developed by Uhlenbeck et al. (39). In an *in vitro* T7 transcription, double stranded DNA is converted into RNA by a T7 RNA polymerase. The T7 RNA polymerase binds a specific sequence on the double stranded DNA called a T7 promoter. The polymerase begins synthesis immediately following the promoter and continues until it reaches the end of the DNA strand. There are many components and conditions that can affect the T7 reaction. The components and conditions in a T7 reaction usually requires titrating individual components to identify optimal reaction conditions. Optimal conditions should yield a high ratio of RNA to the amount of DNA template that is used. A general T7 reaction is shown in Table 6, the current T7 reaction conditions are listed in Table 7. Some general tips to consider when preparing for a T7 transcription reaction are as follows:

1. Prepare reactions at room temperature, since the presence of spermidine above 4 mM (and MgCl_2) can precipitate DNA at colder temperatures ($0-4^\circ\text{C}$) (47).
2. T7RNP: Remove from the -20°C freezer immediately prior to use (only component to be cold); Fresh DTT should be added to the T7RNP stocks every 6 months.
3. Normally, the reaction is allowed to proceed for 1 hour. However, addition of another aliquot of T7RNP after this period followed by a second hour of incubation will approximately double the yield (48).

Table 6: General T7 transcription reaction conditions

Addition order	COMPONENTS OF THE REACTION	Comments
3	T7 "minimum" buffer, 10x TrisHCl pH 8.1 @ 37C (400 mM) MgCl ₂ (40 M) Spermidine (20 mM) Triton X-100 (0.1 % v/v) DTT (50 mM) PEG 8k (27.5 % v/v)	40mM final conc.; pH 8.4 @ 25C 4 mM in 1x solution 2 mM in 1x solution 0.01 % in 1x solution 5 mM in 1x solution 2.75 % in 1x solution
2	Template DNA (nM)	5 - 500 nM final conc.
4	Additional NaCl (2 M)	15 mM final conc.; 100 mM for short transcripts (~ 50 nt)
5	Additional KCl (1.5 M)	15 mM final conc.; 100 mM for short transcripts (~ 50 nt)
6	Additional PEG 8k (30 %)	5 - 8 % final conc.
7	Additional DMSO (100%)	5 - 10 % final conc.
8	PPiase (200 U/ml)	0.15 - 5 U/ml final conc.; <i>Roche 108 987</i>
9	Additional BSA (1500 µg/ml)	50 µg/ml final conc.; not for large scale
10	RNasin (40000 U/ml)	100 - 1000 U/ml final conc.; <i>Fischer Sci.</i> OR <i>SUPERase-In (Ambion)</i>
11	Additional DTT (1.5 M)	5 - 50 mM final conc; 50 mM for short transcripts (~ 50 nt)
12	ATP (100 mM)	1 - 5 mM final conc.
12	UTP (100 mM)	1 - 5 mM final conc.
12	GTP (100 mM)	1 - 5 mM final conc.
12	CTP (100 mM)	1 - 5 mM final conc.
13	Additional Triton X-100 (1%)	0.01% final conc.
14	Additional MgCl ₂ (1.5 M)	Final conc. = Σ [NTPs] +6
15	Additional Spermidine (10 mM)	1 - 4 mM final conc.
16	T7 RNA pol (T7RNP)	4000 - 80 000 U/ml final conc. optimum: 0.01 – 0.2 mg/ml
1	H ₂ O	

Table 7: Current T7 transcription reaction conditions for run-off T7 transcript using a DNA template made from primers BP-25-RIR and BP-34-RIR

Addition order	COMPONENTS OF THE REACTION	Comments
3	T7 "minimum" buffer, 10x TrisHCl pH 8.1 @ 37C (400 mM) MgCl ₂ (40 M) Spermidine (20 mM) Triton X-100 (0.1 % v/v) DTT (50 mM) PEG 8k (27.5 % v/v)	40mM final conc.; pH 8.4 @ 25C 4 mM in 1x solution 2 mM in 1x solution 0.01 % in 1x solution 5 mM in 1x solution 2.75 % in 1x solution
2	Template DNA (nM)	50 nM final conc. of run-off T7 template
4	Additional NaCl (2 M)	0 mM final conc.
5	Additional KCl (1.5 M)	15 mM final conc.
6	Additional PEG 8k (30 %)	8 % final conc.
7	Additional DMSO (100%)	7.5 % final conc.
8	PPiase (200 U/ml)	0.15 U/ml final conc.; <i>Roche</i>
9	Additional BSA (1500 µg/ml)	0 µg/ml final conc.; not for large scale
10	RNasin (40000 U/ml)	100 U/ml final conc. <i>SUPERase-In (Ambion)</i>
11	Additional DTT (1.5 M)	50 mM for short transcripts (~ 50 nt)
12	ATP (100 mM)	3 mM final conc.
12	UTP (100 mM)	3 mM final conc.
12	GTP (100 mM)	3 mM final conc.
12	CTP (100 mM)	3 mM final conc.
13	Additional Triton X-100 (1%)	0.01% final conc.
14	Additional MgCl ₂ (1.5 M)	18 mM final conc.
15	Additional Spermidine (10 mM)	2 mM final conc.
16	T7 RNA pol (T7RNP)	2500 U/ml final conc. T7 RNA pol Prep #5
1	H ₂ O	

4. Large scale transcriptions often generate a considerable amount of a white precipitate (presumably $\text{Mg}^{2+}/\text{PPi}$ complexes) that can be reduced or even avoided by the use of PPIase. The pyrophosphatase will also prevent the backward reaction (49), i.e. RNA unpolymerization, thus increasing yields. Otherwise, remove this precipitate either by centrifugation or by addition of EDTA (RNase-free).
5. The T7 transcription will generate a 5' triphosphate extremity: Should this extremity be removed with phosphatase?
6. Although 40°C is the optimal temperature, incubation at 37°C generally yields fewer incomplete transcripts without a negligible drop in yield (46). The reaction works almost as well at 37°C as at 40°C, often with a significant drop premature termination of transcription. In fact, reducing the temperature to 30°C is often even more efficient at producing full-length transcripts (>300 nt), but the yield of RNA drops to about 50% (46).
7. Under optimal conditions, the template and T7RNP concentrations are limiting (45). Ensuring that none of the NTPs are not limiting, by increasing the concentration of these NTPs can yield significantly more full-length RNAs (long RNA, like mRNAs) (46).
8. Concentrations that should be optimized are: template, T7RNP, NTPs, MgCl_2 , NaCl, KCL, DTT, PEG, BSA, DMSO, and spermidine.

Results

T7 reactions were run under the conditions listed in Table 6 and Table 7, and run out on various denaturing PAGE gels. In figure 30 two different reactions were performed and compared to the synthetic RNA. This figure indicates that the run-off IRE transcript contains a major band at the same size as synthetic 30 nt IRE. The run-off IRE contains incomplete transcription products (products less than 30 nt) and additional transcription products (greater than 30 nt). While this transcription byproducts are expected some of the additional transcription products were too large be from an additional one or three nt (as expected with run-off transcriptions) added the IRE. The HHR-IRE-HHR in the figure 30 is too large in size to effectively separate the bands on a 20% denaturing PAGE gel. The HHR-IRE-HHR transcript shows no significant band that can be compared to the synthetic IRE, suggesting that the HHR-IRE-HHR can yet to undergo significant cleavage. When a similar HHR-IRE-HHR transcription reaction is run out on an 8% denaturing PAGE gel you can see multiple bands present. These multiple bands could be from incomplete transcription products or some intial cleavage of the HHRs. The reactions were also run out on a sequencing gel to determine exactly what bands are present in a T7 reaction. Figure 32 shows that the major band from the run-off reaction is IRE, while the HHR-IRE-HHR reactions show small amounts of IRE. Incomplete and additional transcription products are present as well. These gels indicate that the HHR cleavage needs to be optimized in order to get significant amounts of homogenous IRE.

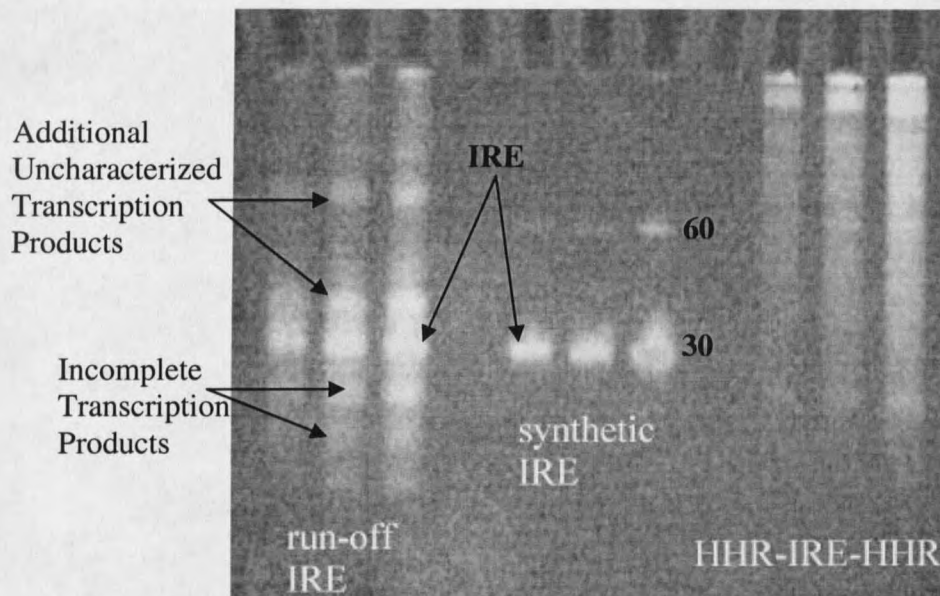


Fig. 30: Comparison of enzymatic synthesis versus chemical synthesis of IRE. T7 RNA polymerase transcription reactions of run-off IRE (DNA from primers BP25/BP34), and HHR-IRE-HHR (DNA from primers 1224/1233) run out on a 20% denaturing PAGE (7M UREA) gel run at 300V.

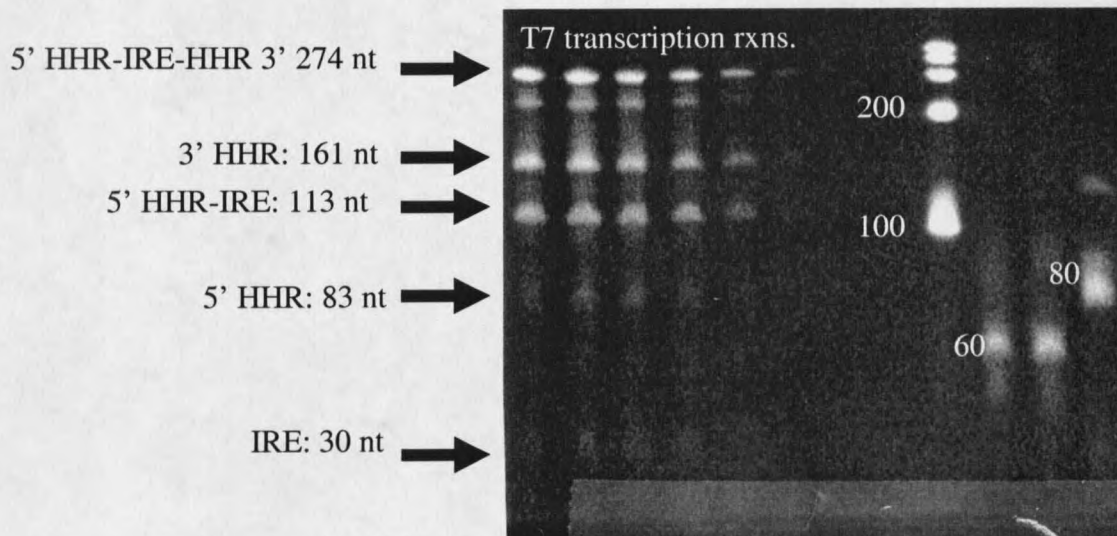


Fig. 31: Various T7 RNA polymerase transcription reactions of HHR-IRE-HHR (made from primers 1224/1233) run out on an 8% denaturing PAGE (7M UREA) gel run at 300V.

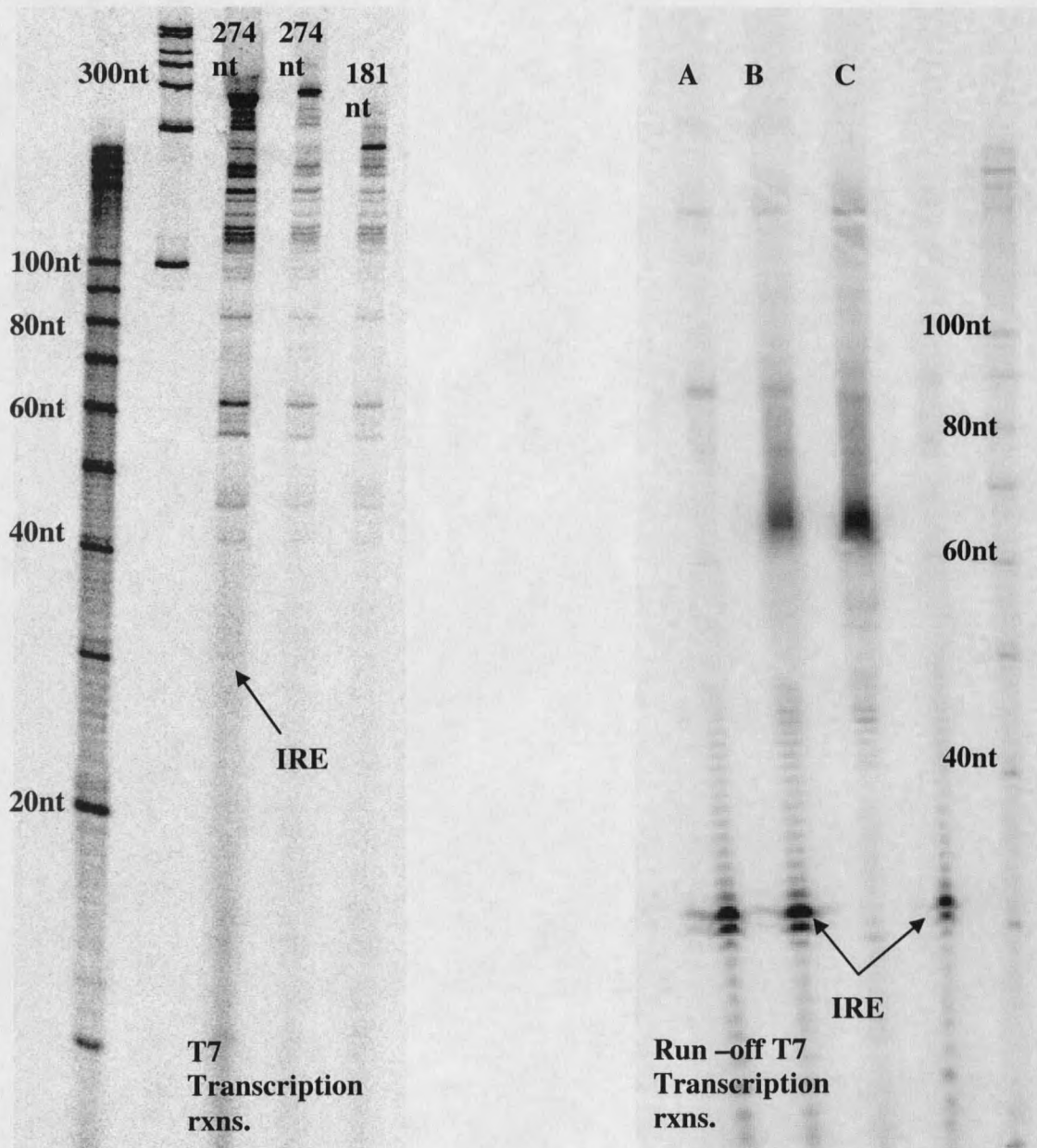


Fig. 32: Two different T7 transcription reactions that were labeled with α - ^{35}S UTP, single nucleotide resolution can be obtained using a denaturing PAGE sequencing gel. The gel on the left shows two different HHR-IRE-HHR, one is 274 nt (from primers 1224/1233) the other is 181 nt (from primers BP22/BP37). The gel on the right shows run-off IRE 30 nt (from primers BP25/BP34). Lanes A: Run-off template/improved conditions for large scale rxn, B: Run-off template/initial conditions for large scale rxn, C: Run-off template/conditions for small scale rxn. Reactions were run out on a 10% denaturing PAGE (7M UREA) sequencing gels run at 50 Watts for 20 cm.

IRE Purification

Following the synthesis of the IRE, subsequent purification is necessary to remove any potential RNases that might degrade the IRE, to remove salts that may inhibit IRP binding, and to separate the 30 nucleotide IRE from other RNA fragments.

Phenol/Chloroform Extractions

A Phenol/Chloroform extraction is used for initial purification of the RNA. Both phenol and chloroform denature proteins and the denatured protein partitions in the lower phenolic phase and/or at the interface, while nucleic acids remain in the aqueous phase. Depending on the pH of the phenol, DNA will partition into either the organic phase or the aqueous phase (50, 51). At $\text{pH} \geq 7.0$, both DNA and RNA partition into the aqueous phase. On the contrary, at an acidic pH, DNA will be denatured and precipitate into the organic phase and the interface, while the RNA remains in the aqueous phase.

Chloroform is mixed with phenol to reduce losses of nucleic acids at the interface. The chloroform's ability to denature proteins increases the efficiency of the extraction. Probably more, or at least as important, the phase separation is also enhanced by the chloroform, hence allowing the removal of the aqueous phase with minimal contamination from the organic phase and minimal loss of RNA.

Sometimes, for mRNA or rather long transcripts (> 300 nt), isoamyl alcohol (IAA) is added to phenol:chloroform (25:24:1) to reduce foaming, although there should be no reason not to use it whatever the length of the transcript. After centrifugation, the aqueous phase containing the nucleic acids is often re-extracted with an equal volume of

chloroform:isoamyl alcohol (50). This combination of extractions is thought to reduce the loss of RNA due to the formation of insoluble protein:RNA complexes at the interface. More important, oxidation products contained in the phenol or resulting from phenol oxidation should be neutralized by adding 8-hydroxyquinoline (0.1 g/100 ml phenol) to the phenol mixture prior to use.

Standard procedure for phenol extractions:

1. Determine the kind of phenol mixture to use (Table 8): e.g.
phenol:chloroform:IAA (25:24:1) pH 4.5 for mg amounts of RNA
2. First extraction: Mix 1 volume of the phenol mixture with 1 volume of the solution containing the RNA, vortex for 1 min, put on ice for 10 min, centrifuge at 12,000 times g for 3 min, take the top (aqueous) phase.
3. Second extraction: Mix 1 volume of the aqueous phase with 1 volume of chloroform:IAA (24:1), vortex for 1 min, put on ice for 10 min, centrifuge at 12,000g for 3 min, take the top aqueous phase.

After the IRE has undergone the phenol/chloroform extraction, it is precipitated using an equal volume of isopropanol (large scale) or 3 volumes ethanol:0.1 volumes of 3M NaOAc pH 4.6 (small scale) and placed at -20°C overnight or at -80°C for one hour. RNA is recovered by centrifugation at > 4000 g for one hour.

Table 8: Applications of pH specific phenol mixtures

* 8-hydroxyquinoline (0.1 g/100 ml phenol) should be added to the phenol mixtures prior to use.

Phenol mixture	pH	Application	Comments
Saturated phenol	7.9 to 9.0	DNA/RNA extraction	
Saturated phenol: CHCl ₃ (1:1)	7.9 to 9.0	DNA/RNA extraction	Often better for small scale (up to 1 ml) T7 transcriptions (more NA in the aqueous phase)
Sat. phenol:CHCl ₃ :IAA (25:24:1)	7.9	DNA/RNA extraction	Solution of choice for DNA extraction
Saturated acid phenol	4.5	DNA/RNA extraction	Acidic pH moves DNA into the organic phase
Sat. acid phenol:CHCl ₃ (5:1) or Sat. acid phenol:CHCl ₃ :IAA (25:24:1)	4.5	RNA extraction Removal of DNA	Acidic pH moves DNA (from in vitro transcriptions) into the organic phase CHCl ₃ and IAA increase phenol extraction efficiency (reducing the loss, increasing the phase separation and the protein denaturation) IAA prevents foaming when mixing

Nucleotide Removal

After the phenol/ chloroform extraction and precipitation, the free NTPS, and salts need to be removed. We have tried a variety of buffer conditions, column types and volumes, and flow rates. Gel filtration columns such as Superdex G25 (*Amersham Pharmacia*), NAP columns (*Amersham Pharmacia*), and P-4 (*Bio-Rad*) are particularly well suited for nucleotide removal. All of the gel filtration columns have pores that are too small for the IRE, so the IRE elutes in the void volume of the column, while the NTPs enter the matrix and are eluted after the void volume. The NAP column can handle

samples sizes of 0.5 ml to 1 ml, with a gel bed volume of 8.6 ml. A typical NAP column protocol is as follows; wash column with 3x5 ml of H₂O, apply 0.5 ml of sample to the column, elute with 3x5 ml of H₂O, and collect 0.5 ml fractions. The fractions are then checked by O.D.. By looking at the spectral profile we are able to determine which fractions contain IRE and which fractions contain NTPs (Fig. 33 and Fig. 34). The NAP columns work better for larger RNAs than for shorter RNA, primarily due to the fact that the column does not have large enough gel bed volume to effectively separate 30mer RNA from NTPs (Fig. 33).

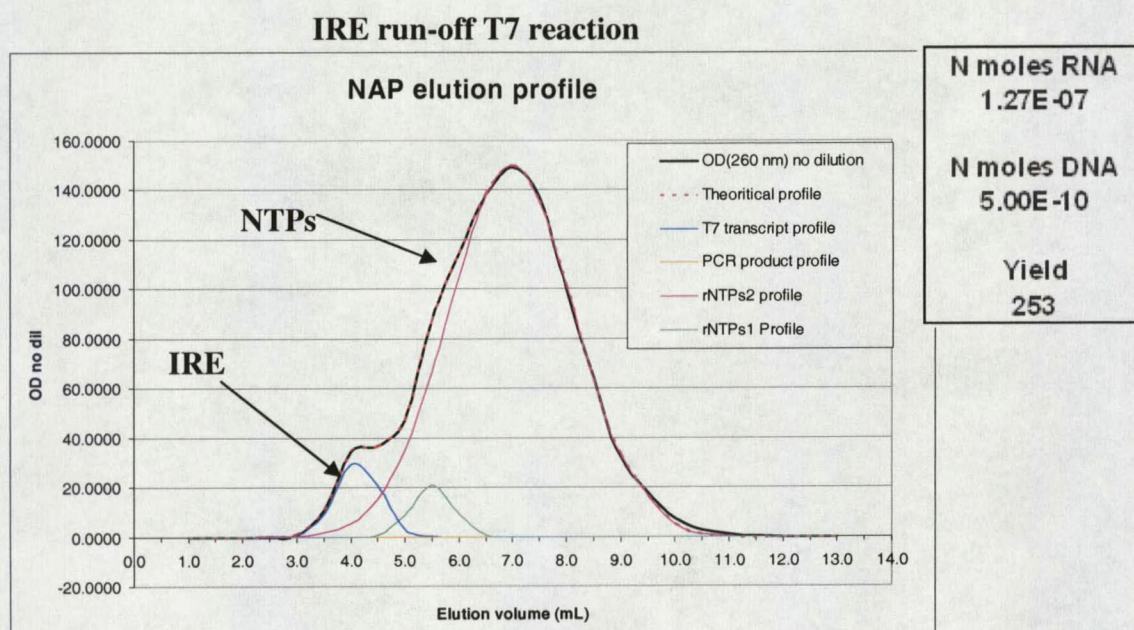


Fig. 33: Theoretical profiles of a 10ml run-off T7 transcription reaction run over a NAP column using O.D. 260 to indicate which peak contains the RNA and which peak contains the NTPs. The theoretical profiling can be used to estimate the number of moles of RNA produced.

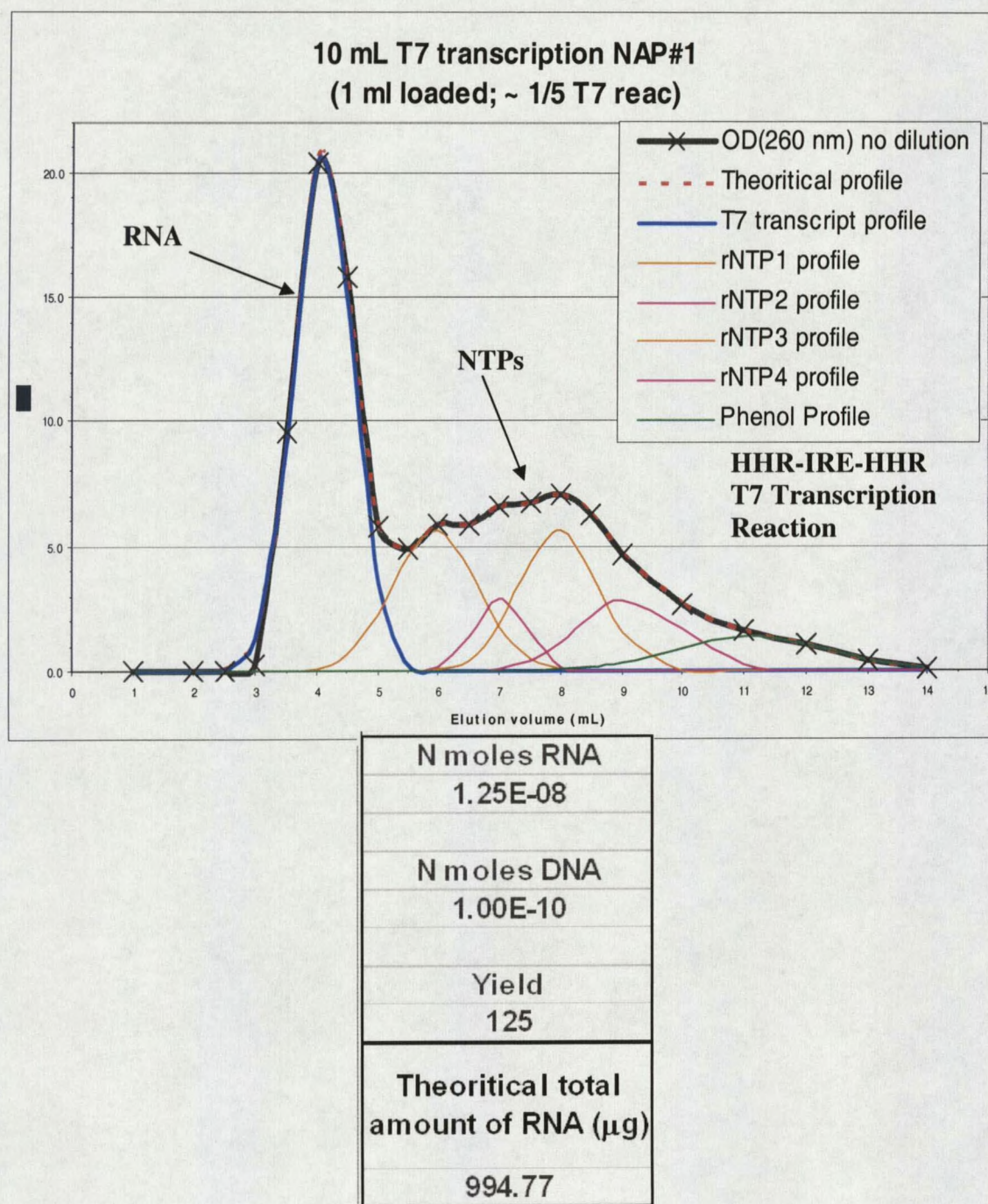


Fig. 34: Theoretical profiles of a 10ml HHR-IRE-HHR T7 transcription reaction run over a NAP column using O.D. 260 to indicate which peak contains the RNA and which peak contains the NTPs. The theoretical profiling can be used to estimate the number of moles of RNA produced.

The Bio-Rad P4 column works the same way as the NAP column, but since it is not a pre-packed column we are not limited by the gel bed volume. A P4 column with a bed volume of 130 ml can handle sample volumes of up to 10 ml, and effectively separate 30mer IRE from NTPs (Fig. 35). The RNA elutes in the void volume while the NTPs enter the matrix and thus elute later.

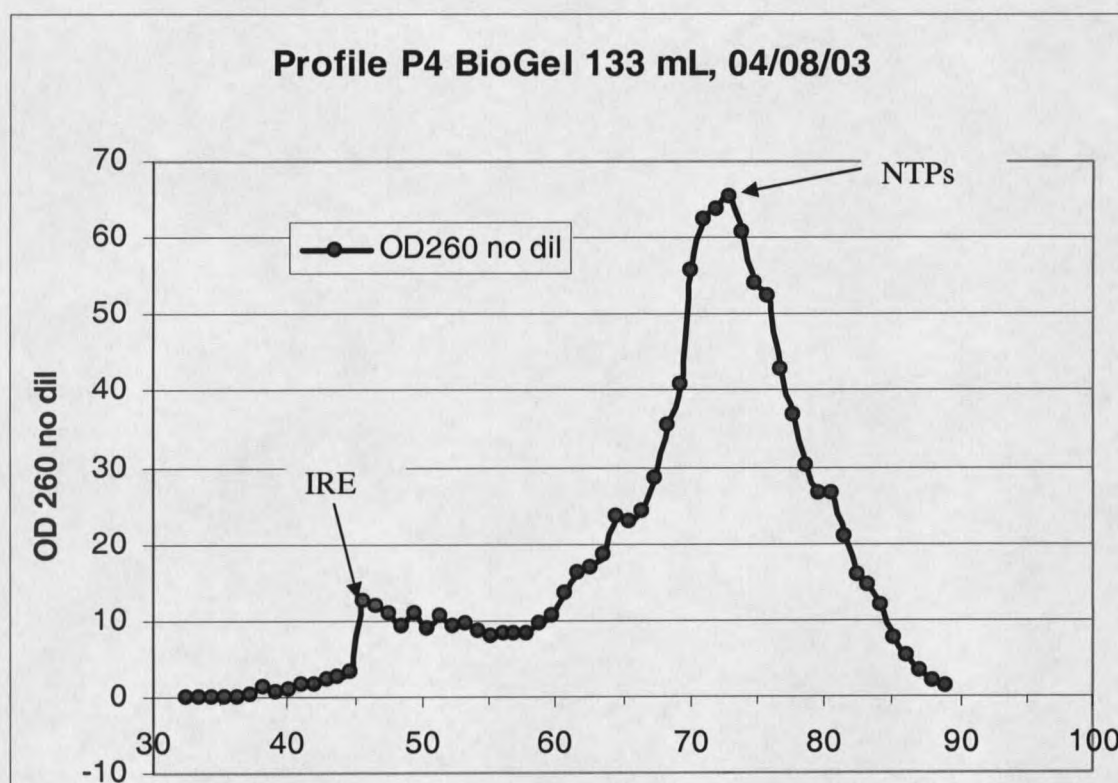


Fig 35: P4 BioGel column loaded with 10ml of 30mer IRE. First small peak is IRE, while the second larger peak is NTPS.

Due to the polyanionic character of nucleic acids it is possible to bind the DNA and RNA to an anionic exchange resin. Therefore, it should be possible to find loading conditions on the Mono Q column (*Amersham Pharmacia*), or CHT column (*Bio-Rad*) such that the IRE will bind and the unincorporated NTPs would be washed away, hence

bypassing the gel filtration step. After initial experiments performed by Philippe Bénas (Montana State University) using an anion exchange column, MonoQ, further developments were made to purify the IRE away from the HHRs and NTPs. The first MonoQ run was done using HHR-IRE-HHR as the sample made from DNA using primers BP-37/BP-22. The sample was loaded onto the column (0.5ml of a 10 ml reaction). Buffer A (50mM Tris-HCl pH 6.8 (at 4°C)) was used to equilibrate the column and the run starts at 100% buffer A, while buffer B: (50mM Tris-HCL pH 6.8 (at 4°C), 1M NaCl) was used to start the gradient (0% to 100% buffer B). The absorbance reading (1.0 Abs) was measured and fractions were collected at 0.5 ml intervals (Fig 36). Analysis of column fractions using denaturing (7M UREA) PAGE gels indicated that some NTPs copurified with the IRE, because the absorbance peak and the gel for the IRE did not correlate. Thus, a second MonoQ column run was developed with a stepwise elution holding the gradient constant at 10%, 20%, 30%, 40%, 50% buffer B for 5 ml each. The result was a second peak of NTPs that eluted before the major NTP peak that was indicated in the first MonoQ run (Fig. 37). Analysis of the column fractions using denaturing (7M UREA) PAGE gels indicated that some NTPs were still associated with the IRE, because the absorbance peak and the gel for the IRE did not correlate (figures 38, 39, and 40). The O.D. 260/280 ratio (1.2) and the spectral data indicated that the excess signal strength for the IRE peak probably came from the NTPs and not another buffer component.

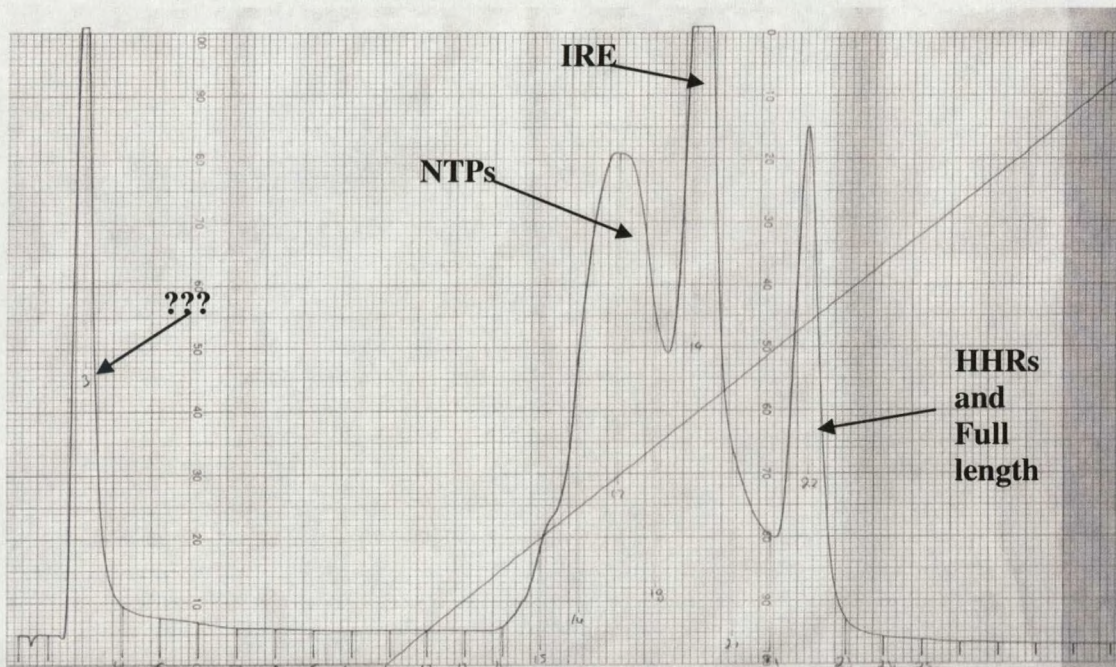


Fig. 36: Mono Q run at 4°C of HHR-IRE-HHR (BP37/BP22). Loaded 0.5 ml of a 10 ml T7 transcription reaction. Buffer A: 50mM Tris-HCl pH 6.8 (at 4°C) Buffer B: 50mM Tris-HCL pH 6.8 (at 4°C), 1M NaCl. Absorbance reading of 1.0

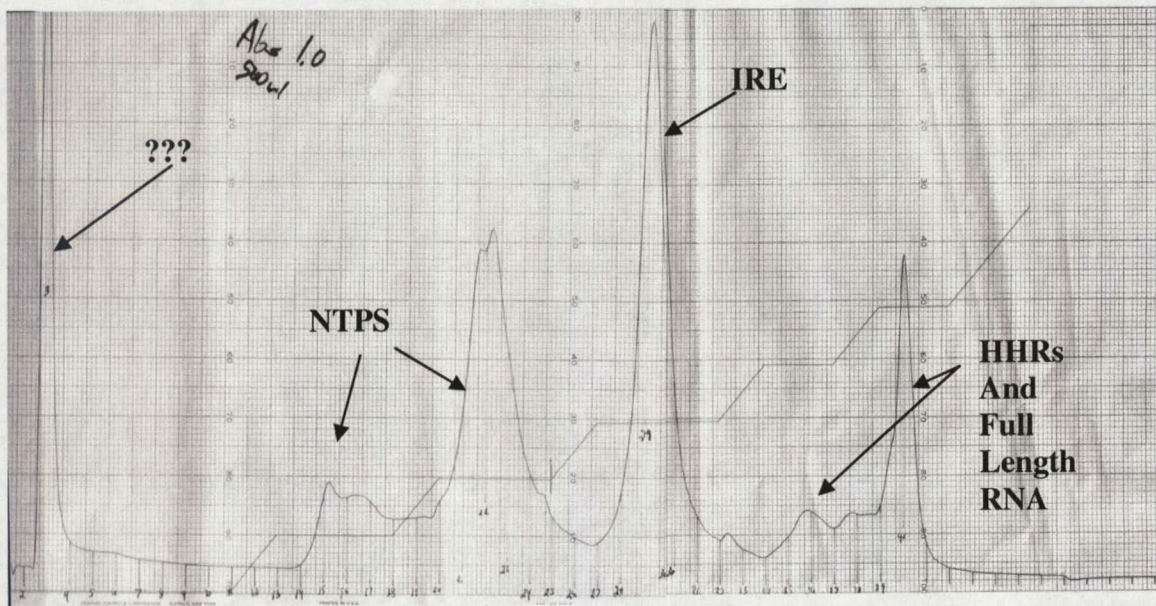


Fig. 37: Mono Q run at 4°C of HHR-IRE-HHR (BP37/BP22) with step gradient. Loaded 0.5 ml of the same 10 ml T7 transcription reaction, and used same buffers as in Fig. 32. Abs. 1.0

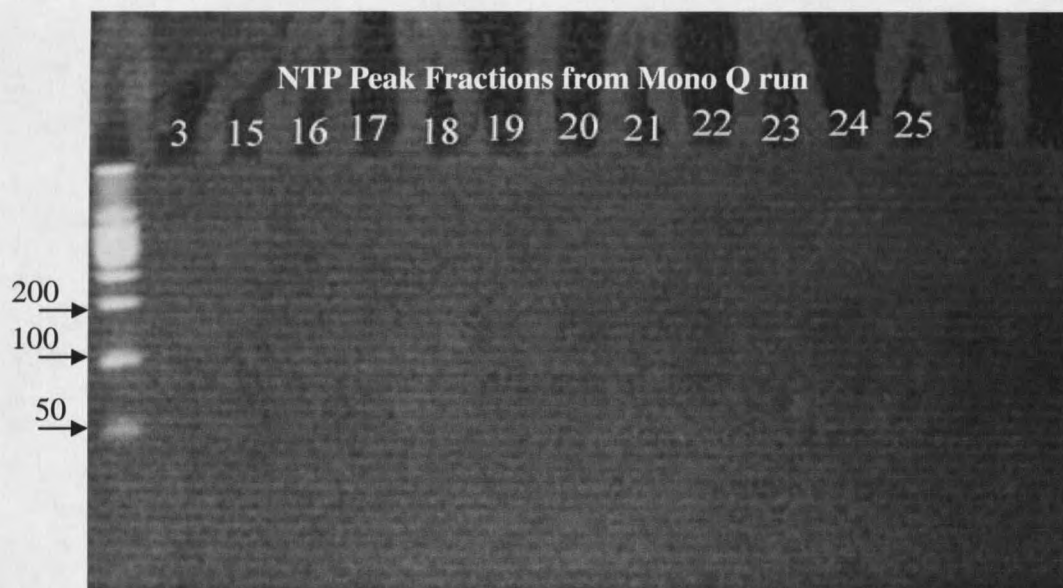


Fig. 38: 15% Denaturing (7M UREA) PAGE gel of fractions (3, 15-25) corresponding to the peak containing NTPs from the MonoQ run in Fig. 33.

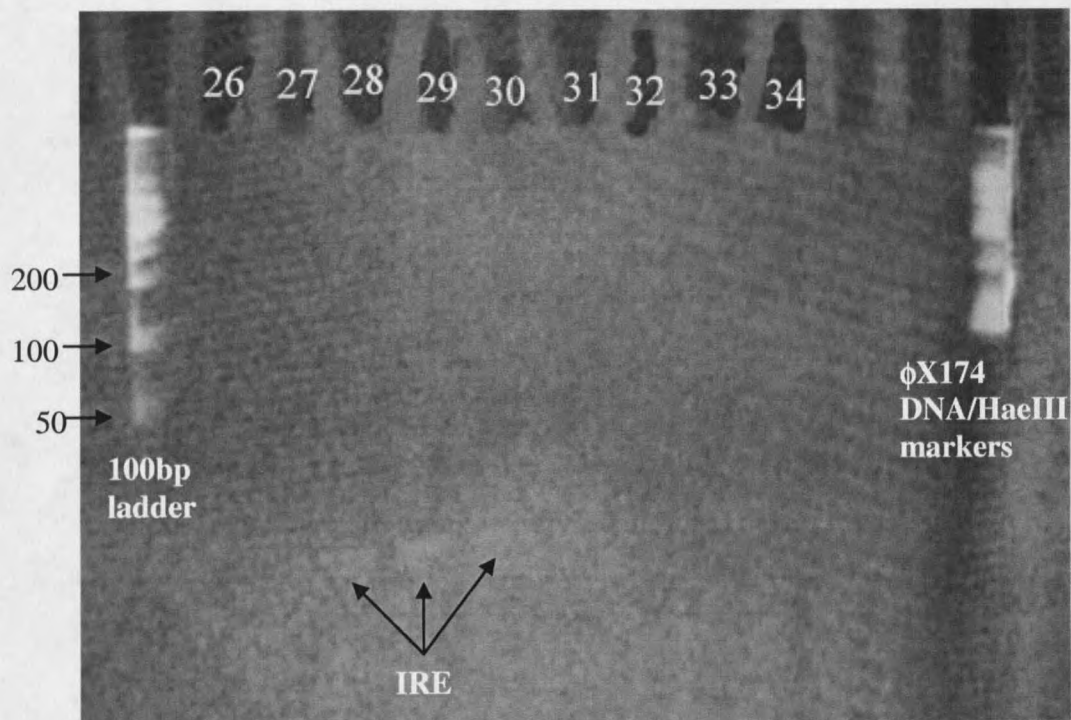


Fig. 39: 15% Denaturing (7M UREA) PAGE gel of fractions (26-34) corresponding to the peak containing IRE from the MonoQ run in Fig. 33.

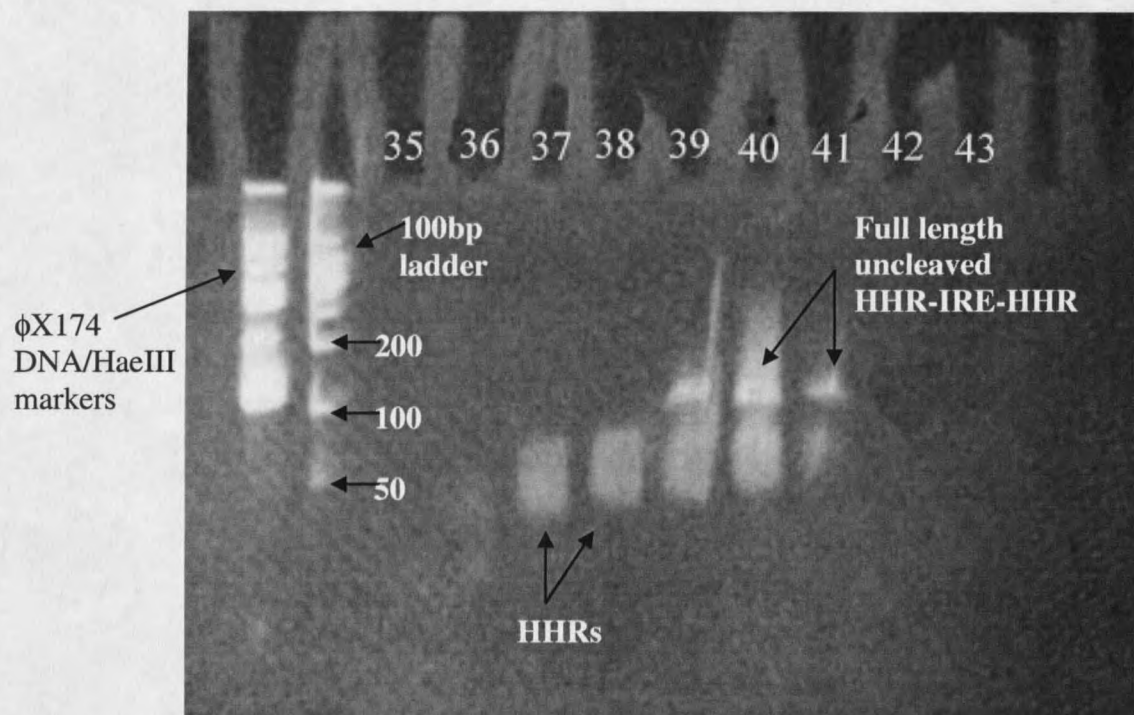


Fig. 40: 15% Denaturing (7M UREA) PAGE gel of fractions (35-43) corresponding to the peak containing HHRs from the MonoQ run in Fig. 33.

Results

Removal of the NTPs from the transcription reactions was achieved by using NAP columns and P-4 columns. The MonoQ column is able to bind the RNA and separate most of the NTPs away from the RNA. It is evident from the gels that some NTPs co-purify with the IRE peak seen in Figure 36 and 37 (the lanes on the gel containing the IRE are very faint Fig. 39). This suggests that either the IRE is degraded rapidly or the peak containing the IRE is receiving some extra absorbance from co-purified NTPs. The step gradient was able to remove NTPs (secondary peak of NTPs) suggesting that the conditions can be optimized further to remove all the NTPs.

IRE Quality Control

During and after IRE purification it is necessary to check the quality of the IRE. There are a variety of ways to accomplish this goal, but it usually involves UV absorbance or denaturing PAGE gels. UV absorbance is often selected because it offers a quick and accurate method for determining RNA concentration and protein contamination. Small denaturing PAGE gels are intermediate in time consumption and allow one to estimate the relative amounts of different signal transcripts. At times, single nucleotide resolution is necessary in which case sequencing gels loaded with radiolabeled RNA are used. Sequencing gels require several days to complete, but may be worth the time and effort in order to resolve the products at the single nucleotide level.

UV-absorbance

By checking the UV absorbance at 260 nm and 280 nm the quantity and quality of IRE can be determined. Absorbance at 260 nm translates into 40 μ g/ml per 1 OD, while the OD 260/280 ratio can tell you if there are any contaminants in the sample, typically 1.9 is a good ratio. Also, by following the absorbance at 260 nm during a run on the sizing columns or anion exchange columns, it can tell you where the RNA and NTPs elute. UV absorbance is a quick and effective method for determining the quantity of IRE present in a sample. It is useful to determine an accurate concentration of IRE before trying to form the complex with the IRPs.

Mini PAGE

A quick way to check for IRE presence and quality is to use a mini denaturing PAGE gel. The gel usually contains 7M urea, 1X TBE, 20% acrylamide and is run for one hour at 250-300 volts. This gel doesn't require any radiolabeled sample to be read, ethidium staining followed by UV illumination works fine. Radiolabeled samples can be run on the mini gels, if the acrylamide concentration is too high the gels will crack during the drying process. If the acrylamide concentration is too low the resolution will be poor. These gels typically work best for non-radio-labeled samples that need to be checked for the presence of IRE.

Sequencing Gels

To determine RNA quality and quantity one of the first things to try is to run radiolabeled RNA in a denaturing PAGE sequencing gel. Typically the IRE is either spiked during a T7 transcription reaction with radioactive α -³⁵S-UTP (use standard T7 reaction procedure but add the desired amount of radiolabel, typically 10 μ Ci/ μ l for a 50 μ l reaction volume) or is kinased with γ -³³P-ATP. The radiolabeled RNA is then loaded with 95% formamide gel loading dye onto a 7M urea, 20% formamide, 1X TBE, 10 % acrylamide sequencing gel and run for 20-25 cm at 50 watts. The gels were dried for 2 hrs (1 1/2 hours of 80°C heat and 2 hours under vacuum), and allowed to expose in a *Kodak* phosphoimager plate (Bill Dyer, Montana State University) overnight (at least 4 hours). The plate is then placed in a *Molecular Dynamics* phosphoimager (Al Jesaitis, Montana State University), and analyzed using *ImageQuant* software. A sequencing gel

is used in order to obtain single nucleotide resolution. From the gel, the quality of the IRE can be determined by comparing the different bands, and the quantity can be determined (during a T7 transcription labeling) by measuring the density of the bands. The disadvantages of running a sequencing gel is that it is time consuming taking anywhere from 2-3 days to complete, the gels are too thin to ethidium stain and check by UV, and radiolabeling may cause gel artifacts.

The sequencing gels also allow one to follow the HHR cleavage reaction, and to assay for optimal cleavage conditions (Fig. 41) (52, 53). Two 50 μ l α -[³⁵S]-UTP radiolabeled T7 transcription reactions were made for HHR cutting experiments. Cutting experiments were conducted on a 274 nt HHR-IRE-HHR (DNA template from primers 1224/1233), and 181 nt HHR-IRE-HHR (DNA template from primers 1224/BP22). The T7 reactions mixtures were passed through a G-25 microcentrifuge spin column (Amersham) to remove any salts and excess NTPs. One third of each reaction had varying concentrations of MgCl₂ added. The reactions contained 0mM, 30mM, and 60 mM MgCl₂ respectively, and were heated at 70°C for 30 min (52, 53). The reactions were then run out on a 10% denaturing (7 M urea) sequencing gel at 50 watts for 20 cm to separate the IRE. The sequencing gel showed the disappearance of the full length 274 nt and 181 nt long RNA, and the appearance of a 30 nt long IRE. The sequencing gel also showed that the 5'HHR ribozyme had cut and was visible at 83 nt in every lane, but no 3'HHR could be seen. The 274 nt long RNA should have generated a 161 nt long 3'HHR and the 181mer should have generated a 68 nt long 3'HHR once the cut was complete, but neither could be seen with certainty on the gel (Fig. 41).

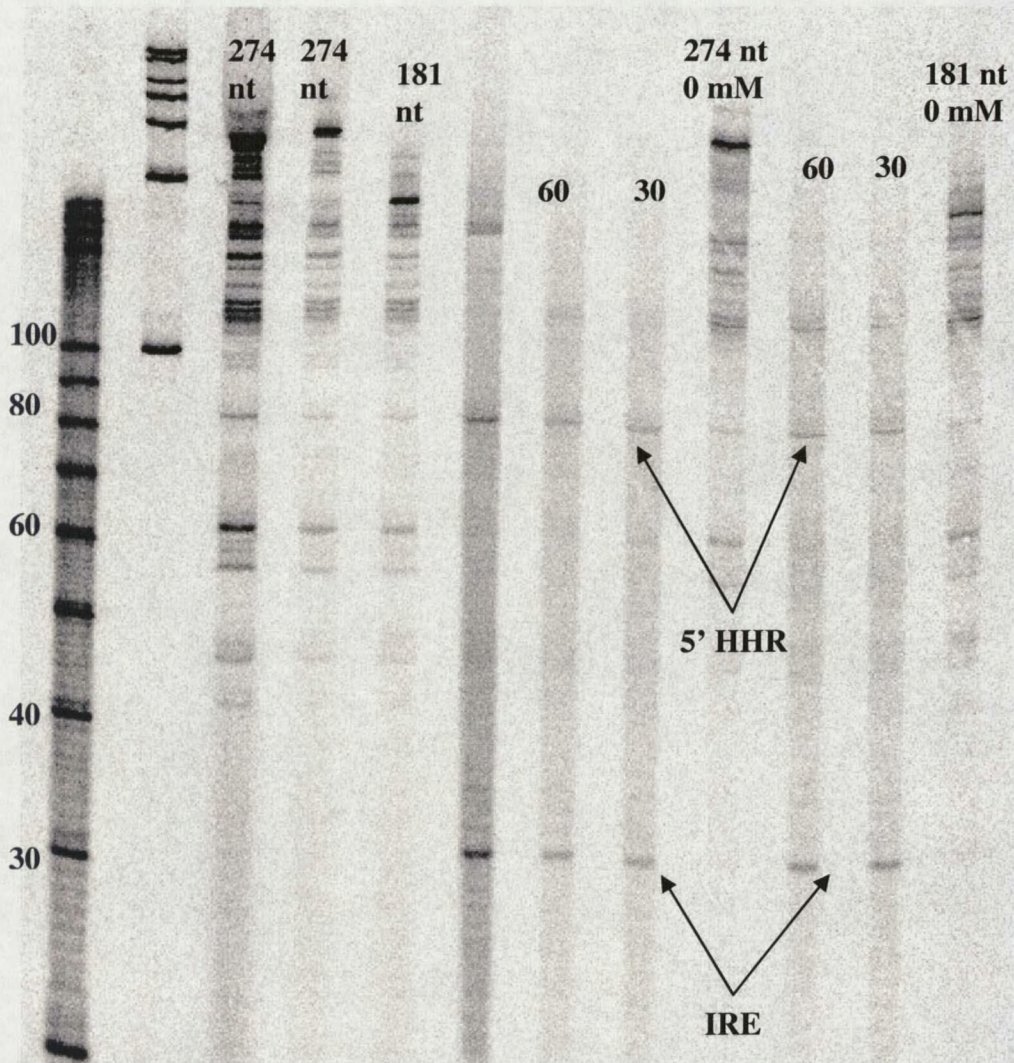


Fig. 41: Cutting experiment on HHR-IRE-HHR 274 mer (primers 1224/1233), and HHR-IRE-HHR 181mer (primers 1224/BP22). 0mM, 30mM, and 60 mM MgCl_2 were each added to a 20 μl T7 reaction that was heated at 70°C for 30 min. and run out on a 10% denaturing (7 M urea) sequencing gel at 50 watts for 20 cm.

Since, the reactions were heated in the presence of MgCl_2 , the RNA could have undergone chemical cleavage, which would cause the lack of expedited bands. Once the reactions volumes were increased to greater than 1ml, the cutting experiments never repeated the success that was seen in the small scale reactions. This could be due to a

number of reasons; including reaction volume, RNA concentration, MgCl_2 concentration, temperature effects, etc.

Band Shift Assays

One way to check for IRE/IRP binding activity is to run a band shift assay. In a band shift assay the IRE is radio labeled with either α - ^{35}S -UTP or γ - ^{32}P -ATP. Following Elizabeth Leibold's (University of Utah) band shift protocol, radio labeled RNA and IRP (1 or 2) are bound together at 0°C for 30 minutes in a solution containing: 10mM Hepes pH 7.6, 3mM MgCl_2 , 40mM KCL, 5% glycerol, and 1mM dithiothreitol (DTT) (24). The IRE/IRP complex is run on a pre-electrophoresed 4% non-denaturing polyacrylamide gel (19:1 acryl/bis acryl ratio), 0.25X TBE and run at 4°C for 60 min at 100 volts. The gel is dried, exposed to a phosphoimager plate, and developed. The band shift assay does not determine how much IRP or IRE is active; it only shows that the IRP can bind IRE (Fig 42). From the literature on IRP bandshift assays IRE/IRP-1 is always shifted higher than IRE/IRP-2. The bandshift assay did show that our "homemade" run-off T7 IRE could bind to IRP-1 and some of the IRP-2 (disappearance of the free IRE band and faint appearance of the IRE/IRP-2 band) indicating that the proteins and the IRE were active. The synthetic IRE was able to shift with IRP-1 and IRP-2, once again the IRE/IRP-2 shift was not as well defined as the IRE/IRP-1. IRP-2 not binding the IREs in this bandshift assay is probably due to the IRP-2 protein being a week old, we have seen the IRP-2 bind IRE stronger in previous bandshift assays. The band shift assay is a useful tool in determining if you have any active IRE and IRP.

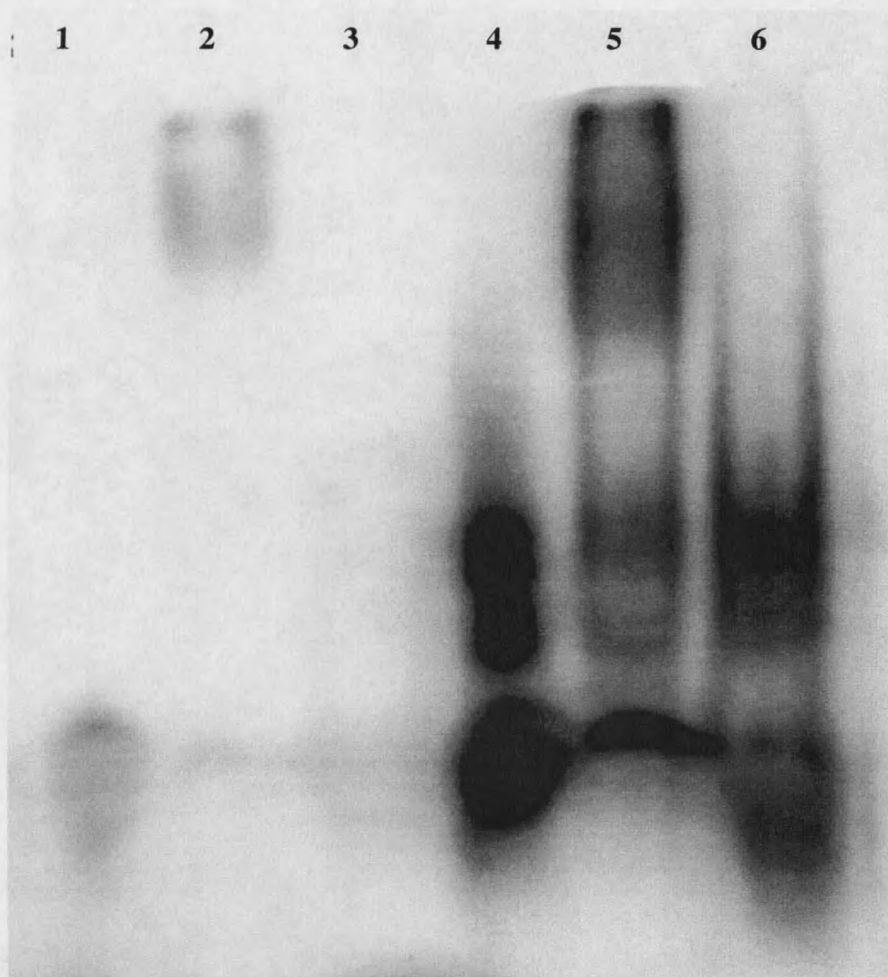


Fig. 42: Bandshift Assay (4% native PAGE gel, 0.25xTBE, run at 100 V for 1hr.) of radiolabeled synthetic IRE versus radiolabeled T7 run-off IRE being bound by IRP-1 and IRP-2. Lane 1: run-off IRE, Lane 2: run-off IRE/IRP-1, Lane 3: run-off IRE/IRP-2, Lane 4: commercial IRE, Lane5: commercial IRE/IRP-1, Lane 6: commercial IRE/IRP-2.

Conclusions

Sequencing gels and UV absorbance are good ways to check for RNA quality and quantity, while mini PAGE gels are quick and easy to use they just don't provide the resolution necessary to analyze small RNA fragments such as the IRE. Band shift assays indicated that IRP-1, and IRP-2 could bind homemade IRE to some extent.

CHAPTER 4

Conclusions

The IRE/IRP story is a challenging one that hopefully this research will help to unravel. Since, the Hentze and Kuhn 1996 review, few major advances in the story have been made. Hopefully when the structure of the IRP/IRE is solved some of the major questions will be answered. There were a few small advances made by this research.

IRP-2 minus degradation domain

While the expression of IRP-2 minus the degradation domain, has not yielded any substantial results the idea is sound. If the IRP-2 mutants express as well or better than the wild-type IRP-2 in yeast, the mutant IRPs should be easier to purify and crystallize. Currently IRP-2- Δ DD is expressing at 0.5mg/L in yeast cell culture. The method for obtaining the mutants is a powerful technique that could be used to generate more mutants of IRP-2 that might express and behave better.

IRE

The current protocol for T7 transcription reactions is using the run-off T7 transcript, and it is yielding approximately 1-2 mg of IRE per 60 ml of reaction. This yield can still be improved upon, but for right now it is generating enough IRE to be able to move forward. The HHR IRE constructs are still coming along, initial cleavage results looked promising at the small scale T7 transcription reaction stage, but when the T7

transcription reaction volumes were increased the HHR on longer cleaved under the same conditions as the small scale T7 transcription reactions. The yield of the T7 reaction is supposed to increase with the increased size of the transcript, but if HHR cleaves away the IRE only 50% of the time, usable IRE is approximately the same as the run-off transcript. The only advantage of the HHR IRE method is that the IRE is more suitable for crystallization, by itself and for making IRE/IPR complex.

The area of greater concern is purifying away the NTPs after the reaction is completed. The column that removes the nucleotides the best is the P-4 Column by *Bio-Rad*. Although, it still appears that some NTPs are still co-purifying with the IRE, because the absorbance readings and the gels don't agree with each other. The absorbance indicates that there should be 2-3 times the amount to IRE that is indicated by denaturing PAGE. There might be a couple of reasons for this discrepancy besides NTPs co-purifying with the IRE: one is that the IRE may be too small to be effectively stained with ethidium bromide so there should be more present than can be seen; another is that the absorbance reading is off due to instrument or user error; and the IRE may be degraded by RNases or some sort of chemical cleavage. It may turn out that some NTPs will always migrate with the IRE, leading to an overestimate of IRE. Still the majority of NTPs are separated from the IRE through size exclusion chromatography.

The good news is that the IRE that is being made is at the correct size (approximately 32 bases for run-off transcript), and can actively bind IRE, as is evident by the sequencing gels and band shift assays. Most recently, Philippe Bénas performed some complex formation/ purification experiments for IRE/IRP-1 that resulted in IRE binding IRP in a ratio of 1:1. It would be nice if the mini-PAGE gels would be able to

resolve the IRE better, because it would be nice to have a quick way to look for the IRE verses using a sequencing gel. The main problems in getting the gel to have good resolution are IRE size, gel size, and salt concentration in the IRE sample.

Future Experiments

While the research performed in the lab has been a step forward, there are many experiments left to try. Something that should be explored further is the HHR-IRE cleavage experiments, currently the lab is obtaining enough usable IRE by run-off transcription that the cleavage experiments are not a necessity. Another area of focus is to continue to work on IRP-2- Δ DD expression in yeast. Further purification of the IRE is necessary as well; while all of the columns currently used separate most of the NTPs and salts away from the IRE it would be nice to see distinct separate peaks for both of them. The main area of focus that has to be explored further is to form the IRE/IRP-1 complex and the IRE/IRP-2 complex so that crystallization trials can be completed. Once crystals of the complex are obtained, structure determination can begin.

REFERENCES CITED

1. Hentze, M. W., & Kuhn, L. C. (1996) *Proc. Natl. Acad. Sci. U. S. A.* 93, 8175–8182
2. Hentze, M. W., Caughman, S. W., Rouault, T. A., Barriocanal, J. G., Dancis, A., Harford, J. B. & Klausner, R. D. (1987) *Science* 238, 1570–1572.
3. Brown, P. H., Daniels-McQueen, S., Walden, W. E., Patino, M. M., Gaffield, L., Bielser, D. & Thach, R. E. (1989) *J. Biol. Chem.* 264, 13383–13386.
4. Zähringer, J., Baliga, B. S. & Munro, H. N. (1976) *Proc. Natl. Acad. Sci. USA* 73, 857–861.
5. Brown, P. H., Daniels-McQueen, S., Walden, W. E., Patino, M. M., Gaffield, L., Bielser, D. & Thach, R. E. (1989) *J. Biol. Chem.* 264, 13383–13386.
6. Goossen, B. & Hentze, M. W. (1992) *Mol. Cell. Biol.* 12, 1959–1966.
7. Gray, N. K. & Hentze, M. W. (1994) *EMBO J.* 13, 3882–3891.
8. Casey, J. L., Hentze, M. W., Koeller, D. M., Caughman, S. W., Rouault, T. A., Klausner, R. D. & Harford, J. B. (1988) *Science* 240, 924–928.
9. Casey, J. L., Di Jeso, B., Rao, K., Klausner, R. D. & Harford, J. B. (1988) *Proc. Natl. Acad. Sci. USA* 85, 1787–1791.
10. Koeller, D. M., Casey, J. L., Hentze, M. W., Gerhardt, E. M., Chan, L.-N., Klausner, R. D. & Harford, J. B. (1989) *Proc. Natl. Acad. Sci. USA* 86, 3574–3578.
11. Ward, J. H., Kushner, J. P. & Kaplan, J. (1982) *J. Biol. Chem.* 257, 10317–10323.
12. Rao, K. K., Shapiro, D., Mattia, E., Bridges, K. & Klausner, R. (1985) *Mol. Cell. Biol.* 5, 595–600.
13. Rao, K. K., Harford, J. B., Rouault, T., McClelland, A., Ruddle, F. H. & Klausner, R. D. (1986) *Mol. Cell. Biol.* 6, 236–240.
14. Müllner, E. W., Neupert, B. & Kühn, L. C. (1989) *Cell* 58, 373–382.
15. Casey, J. L., Koeller, D. M., Ramin, V. C., Klausner, R. D. & Harford, J. B. (1989) *EMBO J.* 8, 3693–3699.

REFERENCES CITED - CONTINUED

16. Seiser, C., Posch, M., Thompson, N. & Kühn, L. C. (1995) *J. Biol. Chem.* 270, 29400–29406.
17. May, B. K., Bhasker, C. R., Bawden, M. J. & Cox, T. C. (1990) *Mol. Biol. Med.* 7, 405–421.
18. Dunkov, B. C., Zhang, D., Choumarov, K., Winzerling, J. J. & Law, J. (1995) *Arch. Insect Biochem. Physiol.* 29, 293–307.
19. Hentze, M. W., Caughman, S. W., Casey, J. L., Koeller, D. M., Rouault, T. A., Harford, J. B. & Klausner, R. D. (1988) *Gene* 72, 201–208.
20. McCallum, S. A., & Pardi, A. (2003) *J. Mol. Biol.* 326, 1037–105
21. Barton, H. A., Eisenstein, R. S., Bomford, A. & Munro, H. N. (1990) *J. Biol. Chem.* 265, 7000–7008.
22. Beaumont, C., Leneuve, P., Devaux, I., Scoazec, J.-Y., Berthier, M., Loiseau, M.-N., Grandchamp, B. & Bonneau, D. (1995) *Nat. Genet.* 11, 444–446.
23. Henderson, B. R., Menotti, E., Bonnard, C. & Kühn, L. C. (1994) *J. Chem.* 269, 17481–17489.
24. Leibold, E. A. & Munro, H. N. (1988) *Proc. Natl. Acad. Sci. USA* 85, 2171–2175.
25. Henderson, B. R., Seiser, C. & Kühn, L. C. (1993) *J. Biol. Chem.* 268, 27327–27334.
26. Hentze, M. W., Rouault, T. A., Harford, J. B. & Klausner, R. D. (1989) *Science* 244, 357–359.
27. Kennedy, M. C., Mende-Mueller, L., Blondin, G. A. & Beinert, H. (1992) *Proc. Natl. Acad. Sci. USA* 89, 11730–11734.
28. Hirling, H., Henderson, B. R. & Kühn, L. C. (1994) *EMBO J.* 13, 453–461.
29. Neupert, B., Menotti, E. & Kühn, L. C. (1995) *Nucleic Acids Res.* 2579–2583.
30. Rouault, T. A. (2001) *Pediatr. Neurol.* 25(2), 130–137
31. Iwai K., Klausner R.D., & Rouault T.A. (1995) *EMBO J.* 14(21), 5350–5357

REFERENCES CITED - CONTINUED

32. Guo, B., Yu, Y. & Leibold, E. A. (1994) *J. Biol. Chem.* 269, 24252-24260.
33. Kang D.K., Jeong J., Drake S.K., Wehr N.B., Rouault T.A., & Levine R.L. (2003) *J. Biol. Chem.* 278(17), 14857-14864
34. Goessling L.S., Mascotti D.P., & Thach R.E. (1998) *J. Biol. Chem.* 273(20), 12555-12557
35. Grabill C., Silva A.C., Smith S.S., Koretsky A.P., & Rouault T.A. (2003) *Brain Res.* 971(1), 95-106.
36. Ho S.N., Hunt H.D., Horton R.M., Pullen J.K., & Pease L.R. (1989) *Gene* 77(1), 51-59
37. Horton R.M., Hunt H.D., Ho S.N., Pullen J.K., & Pease L.R. (1989) *Gene* 77(1), 61-68
38. Ke Y., Wu J, Leibold E.A., Walden W.E., & Theil E.C. (1998) *J Biol Chem.* 273(37), 23637-23640
39. Milligan J.F., Groebe D.R., Witherell G.W., & Uhlenbeck O.C. (1987) *Nucleic Acids Res.* 15(21), 8783-8798
40. McConnell T.S., Herschlag D., & Cech T.R. (1997) *Biochemistry* 36(27), 8293-8303
41. Doudna JA, & Cech TR. (2002), *Nature* 418(6894), 222-228
42. Ambion (2002) *Tech. Notes* 9(3)
<http://www.ambion.com/techlib/tn/93/9316.html>
43. Condon, C. (2003), *Microbiol Mol Biol Rev.* 67(2):157-174.
44. Chamberlin, M., & Ring, J. (1973), *J. Biol. Chem.* 248, 820-825
45. Arnold S., Siemann M., Scharnweber K., Werner M., Baumann S., & Reuss M. (2001), *Biotechnol. Bioeng.* 72, 548-561
46. Yisraeli, J.K., & Melton, D.A. (1989), *Methods Enzymol.* 180, 42-50
47. Hoopes, B.C., & McLure, W.R. (1981), *Nucleic Acids Res.* 9, 5493

REFERENCES CITED - CONTINUED

48. Cunningham, P.R., & Ofengand J. (1990), *Biotechniques* 9, 713-714
49. Melton D.A., Krieg P.A., Rebagliati M.R., Maniatis T., Zinn K., & Green M.R. (1984), *Nucleic Acids Res.* 12, 7035-7056
50. Wallace, D.M. (1987), *Methods Enzymol.* 152, 33-40
51. Wallace, D.M. (1987), *Methods Enzymol.* 152, 41-48
52. Herschlag D, & Khosla M. (1994,) *Biochemistry* 33(17), 5291-5297
53. Leibold E.A., & Munro H.N. (1988) *Proc Natl Acad Sci U. S. A.* 85(7), 2171-2175.

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