TESTS OF THE ASSOCIATION OF HEAT SHOCK PROTEIN 90 WITH A CAULIFLOWER MOSAIC VIRUS REVERSE TRANSCRIPTASE

By

BRESHANA QUIENE JOHNSON

Bachelor of Science

Xavier University of Louisiana

New Orleans, Louisiana

May 1997

Submitted to the Faculty of the
Graduate College of the
Oklahoma State University
in partial fulfillment of
the requirements for
the Degree of
MASTER OF SCIENCE
July, 2000

TESTS OF THE ASSOCIATION OF HEAT SHOCK PROTEIN 90 WITH A CAULIFLOWER MOSAIC VIRUS REVERSE TRANSCRIPTASE

Thesis Approved:

Ulrich Melcher
Thesis Advisor

Richard C. Esseles

Robert F Matts

Alfred Landgraf
Dean of the Graduate College

ACKNOWLEDGEMENTS

I sincerely thank my thesis advisor, Dr. Ulrich K. Melcher, for his guidance, supervision, expertise, and friendship. It was a pleasure to be a part of his lab and I will always appreciate his dedication to his students and encouragement. I thank my committee members Dr. Richard C. Essenberg and Dr. Robert L. Matts for their support and expertise. I also thank Dr. James B. Blair and the Department of Biochemistry and Molecular Biology for providing me with the opportunity to participate in the NIH Biomedical Graduate Students program and their financial support. I express my sincere gratitude to Dr. Steven D. Hartson for his guidance, suggestions, and inspiration throughout this project.

I would like to thank staff and students of the Department of Biochemistry and Molecular Biology, who provided me with assistance: Ann Williams, Dr. Bassam Fraij, Dr. Jerry Merz, Wenhao Wang, Qi Jiang, Kenji Onodera, Wenjun Huang, Jieya Shao, Abdel Bior, and Thomas Prince

I truly thank my parents for encouraging and supporting me throughout my academic pursuits. I am very grateful to them and blessed to have their love and concern.

Finally, I thank the Department of Biochemistry and Molecular Biology for being very helpful and supportive during my graduate studies.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

NOMENCLATURE

CaMV	Cauliflower mosaic virus
RT	Reverse transcriptase
HBV	Hepatitis B virus
Hsp90	Heat shock protein 90
DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid
dsDNA	Double-stranded DNA
ocDNA	Open circle DNA
cccDNA	covalently closed circular DNA
$tRNA^{i}_{met}$	transfer ribonucleic acid methionine initiator
pgRNA	Pregenomic RNA
ORF	Open reading frame
sORF	Small open reading frame
uORF	upstream open reading frame
nt	nucleotide
S	Svedberg
TCA	Trichloroacetic acid
IgM	Immunoglobulin M
IgG	Immunoglobulin G
cpm	counts per minute

mAb	monoclonal antibody
pAb	polyclonal antibody
P#	10 mM Pipes pH 7.0 and # mM NaCL
P#T	Pipes pH 7.0, # mM NaCl, and 0.05% Tween20
P#M	10 mM Pipes pH 7.0, # mM NaCl, and 20 mM Na_2MoO_4
R	Resin
IP	Immunoprecipitation
NBT	nitroblue tetrazolium
BCIP	5-bromo, 4-chloro, 3-indolyl phosphate
PNCA	p-nitrophenyl chloroformate-activated
PVDF	polyvinlylidene difluoride
TEMED	N, N, N', N'-Tetramethylethylenediamine
AHA	6-Amino-n-hexanoic acid
APS	Ammonium persulfate
DMF	N, N-Dimethylformamide
EDTA	Ethylenediaminetetraacetic acid
GA	Geldanamycin

LIST OF SYMBOLS

α	anti
ε	*cis*-acting packaging signal near 5'end of pgRNA

CHAPTER I

INTRODUCTION

Cauliflower Mosaic Virus

Cauliflower mosaic virus (CaMV) is a dsDNA containing plant virus in the caulimovirus genus of the Caulimoviridae family whose other members include the badnaviruses, PCVC-like viruses (Petunia vein-clearing virus), SbCMV-like viruses (Soybean chlorotic mottle virus), CsVMV-like viruses (Cassava vein mosaic virus), and RTVB-like viruses (Rice tungro bacilliform virus). *Brassica rapa,* the turnip plant, is the most extensively used host due to its high accumulations of virions[1]. Members of the Caulimoviridae and Hepadnaviridae constitute the group of dsDNA reverse transcribing viruses known as pararetroviruses. Rather than integrating viral DNA into the host genome and using this proviral DNA as a template for transcription, which is characteristic of retroviruses, pararetroviruses use a minichromosome that is cccDNA (covalently closed circular DNA) as a template for transcription[2].

The genome of CaMV, which is reminiscent of that of the hepadnaviruses, is organized into six major open reading frames (ORFs), two minor ORFs, and a large 0.6kb intergenic region[3]. ORFs 1-7 code for an intercellular transport protein, an aphid acquisition factor, a DNA binding protein, a capsid protein precursor, an RNA-dependent DNA polymerase known as reverse transcriptase (RT), inclusion body protein, and a

polypeptide of unknown function[4]. The intergenic region of 35S RNA contains nine short open reading frames (sORFs) and regulates important steps in the virus life cycle. It contains the primer binding site for reverse transcription at the 3' end, the terminally redundant region for template switching within the first 190 nt[5], an inefficient splice donor toward the end of the leader[6], and the polyadenylation signal[7]. The intergenic region in the 35S RNA was reported to fold into a stem-loop structure[8,9] known to inhibit downstream translation. This structure has also been observed in the hepatitis B virus (HBV) RNA and is conserved among all caulimoviruses[8] and in RTBV[10]. Examination of the stem-loop structure in HBV RNA showed that specific nt sequences in the loop and upper stem regions are critical for RNA packaging[11] which may suggest a similar role for such structures in the CaMV 35S RNA. Small upstream ORFs (uORFs) seen in retroviruses also affect downstream translation just as in pararetrovirses, but translation of these uORFs, specifically uORF 3 in Rous sarcoma virus, is a prerequisite for RNA packaging[12]. While it has not been determined if this structure is involved in RNA packaging for CaMV, computer analysis and infectivity studies *in planta*[13] suggested that the position of the stop codon in sORF A and the landing sequence near the 3' end of the leader flanking the base of the stem-loop are critical for efficient ribosome shunting during translation[14].

During the replication cycle, CaMV employs RT[15] to replicate through an RNA intermediate and the host $tRNA^{i}_{met}$ as a primer for (-) DNA strand synthesis[16]. The RT is synthesized from the *pol* gene (ORF 5) as part of a polyprotein that includes RNase H and an aspartic protease[17]. Two facts concerning CaMV replication are the presence of supercoiled DNA in the nucleus as a transcriptionally active minichromosome which is not present in mature virus particles[1] and the presence of two CaMV specific polyA

mRNAs, 19S and 35S that were isolated from infected turnip leaves[18]. In the virus particle, CaMV DNA exists as open-circular DNA (ocDNA) with three single-stranded discontinuities resulting from short sequence overlaps during replication[18, 3]. Upon infection, the virus particle is uncoated in the cytoplasm and the DNA transported to the nucleus. In the nucleus, the DNA exists as circularly closed minichromosomes that produce the 19S and 35S RNA. The 19S RNA is translated into inclusion body protein in the cytoplasm of infected cells. The inclusion bodies are the sites of reverse transcription and translation of the 35S RNA for the production of dsDNA and viral proteins for packaging into mature infectious virions, respectively. The 35S RNA is bifunctional such that it acts as a template for reverse transcription and a full-length transcript for ORFs 1-5.

Hepatitis B Virus

HBV is a partially dsDNA[19] virus in the hepadnavirus genus of the Hepadnaviridae family whose members are the orthohepadnaviruses and avihepadnaviruses. HBV infects the hepatocytes of humans and mammals and the duck hepatitis B virus (DHBV) infects the hepatocytes of birds. HBV is also one of the six viruses that contribute to the development of hepatocellular carcinoma (HCC). The genome is organized into four ORFs that encode the polymerase (P), core (C), surface (S), and X proteins[20-24]. These proteins are expressed from different promoters based on the presence of different 5' ends, (i.e. initiation sites) in all four transcripts[25,26]. HBV also employs a RT to replicate through an RNA intermediate known as the pregenomic RNA (pgRNA)[27] and has a replication cycle that is parallel to that of CaMV. The RT is also expressed as part of a polyprotein that includes a terminal protein (TP), spacer, and RNase H domain. The

HBV RT plays a bifunctional role in the replication cycle being responsible for producing dsDNA[28] and being involved in RNA packaging[29].

After production and transportation of pgRNA into the cytoplasm, it is encapsidated within subviral nucleocapsids composed of core proteins and reverse transcribed into dsDNA. RNA packaging is known to be a highly selective process that discriminates between pgRNA and other genomic and subgenomic transcripts that are produced such that only the pgRNA is encapsidated[30,31]. RNA packaging depends on a *cis*-acting packaging signal, epsilon (ε), that is located within the stem-loop structure near the 5' end of the pgRNA[32]. It has been shown to direct RNA packaging and is conserved among mammalian and avian hepadnaviruses[33].

Encapsidation of pgRNA has been tightly coupled to encapsidation of the RT such that one is not encapsidated without the other[34]. The process is triggered by the recognition and binding of ε by the polymerase such that both are packaged together[28,29]. The formation of this ribonucleoprotein (RNP) complex between ε and the polymerase is transient[35,36] and has been shown to require a molecular chaperone, heat shock protein (hsp90) for the polymerase activity in DHBV and HBV[37]. This complex also requires ATP hydrolysis and the hsp90 co-chaperone p23[38]. Immunoprecipitation experiments with deletion mutants of each domain in the human HBV polymerase protein showed hsp90 to interact with the C-terminal region of both the TP and RT domains[39].

Heat Shock Protein 90

Hsp90 is a ubiquitous high molecular weight protein that is found in the cytoplasm of eubacteria, yeast, and multicellular organisms. It is a dimer consisting of conserved N and C-terminal regions connected by a variable charged linker. It functions to facilitate

the correct folding pathway of non-native proteins and prevents inter- and intramolecular aggregation of proteins during protein folding and heat stress. It is part of a multi-chaperone complex that includes the hsc70, hsp40, Hip, Hop, p23, and one of the large immunophilins (FKBP51, FKBP52, or cyclophilin 40). Hsp90 is well known for regulating protein conformations of signal transducing molecules such as steroid hormone receptors and nonreceptor tyrosine kinases[40]. Studies have also shown it to possess refolding activity on heat denatured firefly luciferase[41]. Hsp90 also interacts with the tumor suppressor protein, p53[42], nitric-oxide synthase[43], the heme-regulated eIF-2α kinase[44], and RT. In the case of RT for the HBV, hsp90 does not dissociate after protein folding. It remains bound to the RNP complex between ε and the polymerase and is packaged simultaneously within the nucleocapsid[38]. This demonstrates the importance of hsp90 to the HBV assuring that the polymerase remains competent for protein priming and reverse transcription.

Purpose of the Study

Based on structural and replication similarities between CaMV and HBV with regard to the DNA genome and the use of a RT, it is possible that the requirement of hsp90 for HBV RT folding may be applied to that of a CaMV RT. The purpose of this project is to determine whether hsp90 is required for CaMV RT folding. If evidence shows that hsp90 is required for a CaMV RT, this may (*i*) indicate an evolutionary relationship between HBV and CaMV, (*ii*) suggest the use of CaMV as a potential model to study HBV, and (*iii*) lead to an alternative strategy to develop antiviral agents against HBV.

CHAPTER II

MATERIALS AND METHODS

Construction of pBJ1

Polymerase Chain Reaction of Reverse Transcriptase Gene

Plasmid pUM52, a partially dimeric plasmid that contained between one and two copies of the CaMV strain CabbS[45] was used as the PCR template to amplify the RT gene. The sense primer, 5'gggggggatccgccaccatggatcatctacttctgaag3', contained the Kozakian sequence[46] gcc(a/g)ccatgg including the atg start codon, and a *Bam*HI site (g^gatcc). The antisense primer, 5'ggggctcgagttaggaattaaccttattgaattctcttgaaag3', contained a linker upstream from the tta antistop codon consisting of a *Xho*I site (c^tcgag) and four guanylates. To be consistent with the Kozakian sequence, a CaMV isolate that contained nts flanking the atg codon most identical to the Kozakian sequence was chosen. For that reason, the CabbS CaMV isolate was used as the PCR template in this strategy. A 50 µl PCR reaction was prepared with 1 u Taq polymerase (Promega), 2 mM $MgCl_2$, 10X Taq polymerase buffer (Promega), 0.2 mM dNTPs, and 49.5 ng of pUM52. PCR was carried out at 94°C for melting, 51°C for primer annealing, and 72°C for primer extension at one minute each. These steps were repeated 30 times followed by additional extension at 72°C for five minutes. The PCR reaction was purified by a PCR

quick clean up kit (Promega) and analyzed on a 1% agarose gel. The desired fragment was excised and recovered from the gel by the freeze-thaw method described below.

Freeze-Thaw Method

The desired fragment was excised from the gel with a sharp razor blade and placed in a 1.5 ml Eppendorf tube. With a pipet tip, the gel slice was broken into many pieces. The gel pieces were frozen at –70°C for 1 hour and thawed at 37°C. After thawing, the mixture was vortexed and centrifuged. The supernatant containing the DNA was collected.

Plasmid Size Quick Check Method

To a 1.5 ml Eppendorf tube, 50 µl of a 1:1 mixture of buffer saturated phenol/chloroform, 50 µl of TE (10 mM Tris 1 mM EDTA pH 8), and 15 µl of DNA stop solution (50% glycerol, 10 mM EDTA pH 8, and 0.025% Bromophenol blue) were added. A single white isolated bacterial colony from a LB Amp plate was added to the tube, vortexed for 10 seconds. The solution was centrifuged at 10,000 rpm for 3 minutes in a microfuge. The aqueous layer was collected and loaded onto an agarose gel.

PCR Cloning

Using the TNT Cloning Kit (Promega), the 2.1 kb PCR product was cloned at the single 3' terminal thymidine overhang site of the pGEM-T vector. Four ligation reactions were prepared containing 33 ng and 100 ng of the RT PCR insert with vector DNA at 1:1 and 1:3 vector/ insert ratios, respectively, vector DNA with control insert DNA provided with the kit, and vector DNA alone. Each ligation reaction was incubated for 1 hr at room temperature and overnight at 4°C. Two microliters of each reaction were

transformed by heat shock into 50 µl of JM109 cells supplied with the pGEM-T vector system and spread onto LB ampicillin (50 µg/ml) X-gal (2%) IPTG (100 mM) plates.

After transformation, 20 single white isolated colonies were picked and plasmids were analyzed by the plasmid size quick check method. Plasmids 14, 15, 16, and 17 (p14-p17) were re-transformed into JM109 cells and purified from the resulting transformants. Two colonies from each LB Amp plate were picked, designated as A and B for each plasmid (i.e. p14A and p14B), incubated separately in 4 ml LB with 0.10 mg of ampicillin at 37°C with shaking for 19 hours. The plasmids were purified with the Qiagen miniprep plasmid kit and analyzed on a 1% agarose gel. Plasmids 14-17 were digested with *Nae*I to determine the orientation of the insert. The 5' and 3' end of the inserts in p14 and p16 were sequenced with M13 forward and reverse primers. The sequencing result led to the choice of p14B, henceforth designated pGTRT-14B, for subsequent procedures.

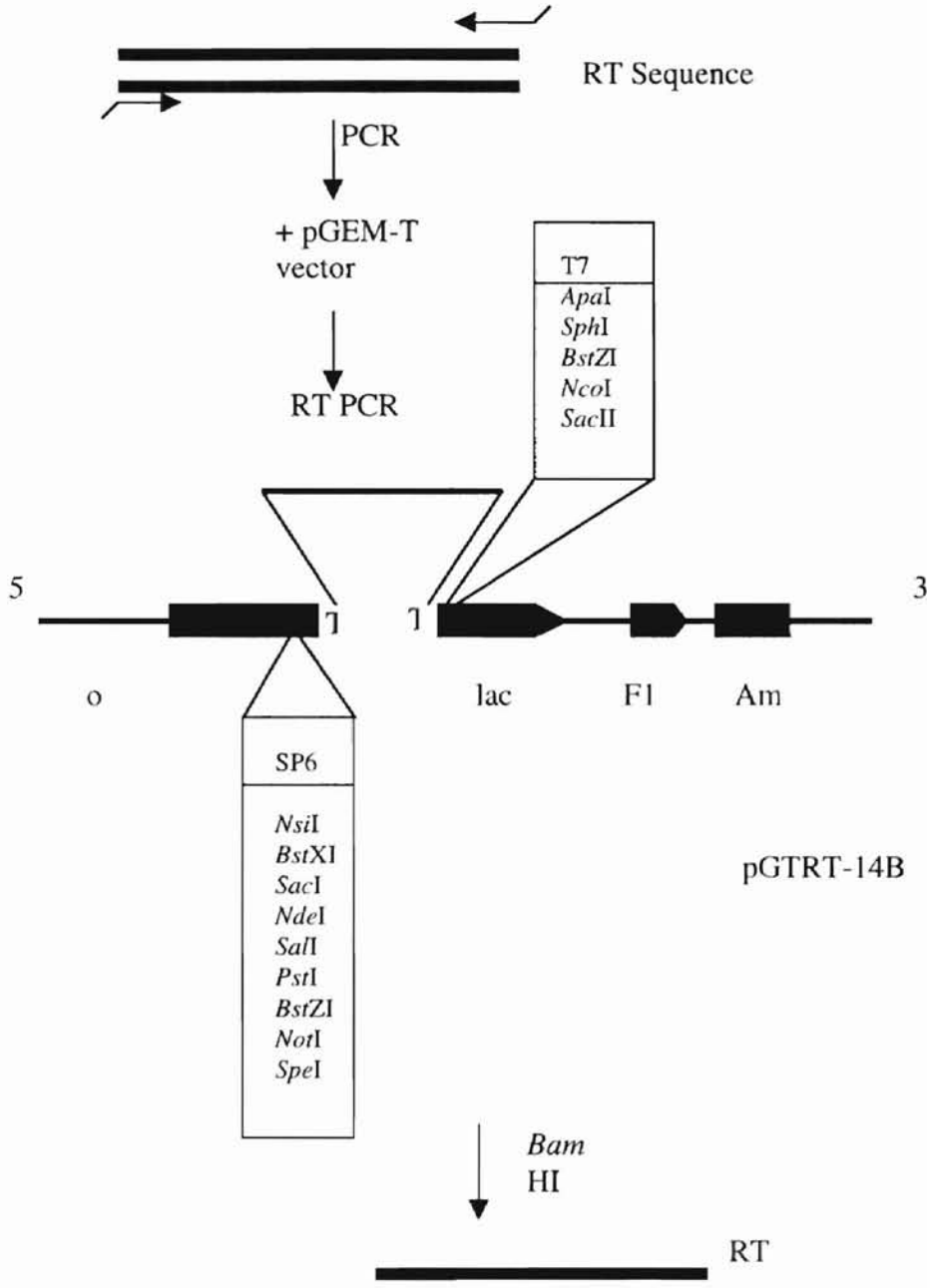
Sequencing

Sequencing was done by the Recombinant DNA/Protein Resource Facility (Oklahoma State University).

Indirect Cloning with pSP64T

Plasmid pGTRT-14B was digested with *Bam*HI and *Xho*I. Plasmid pSP64T was digested with *Bgl*II and *Sal*I. Products from both digestions were checked on a 1% agarose gel and recovered by the freeze-thaw method. The *Bam*HI/*Xho*I fragment was cloned at the *Bgl*II/*Sal*I site of pSP64T (figure 1) and transformed by heat shock into *E. coli* JM109 cells. Seven colonies were picked and analyed by the plasmid size quick

FIGURE 1
CONSTRUCTION OF pBJ1



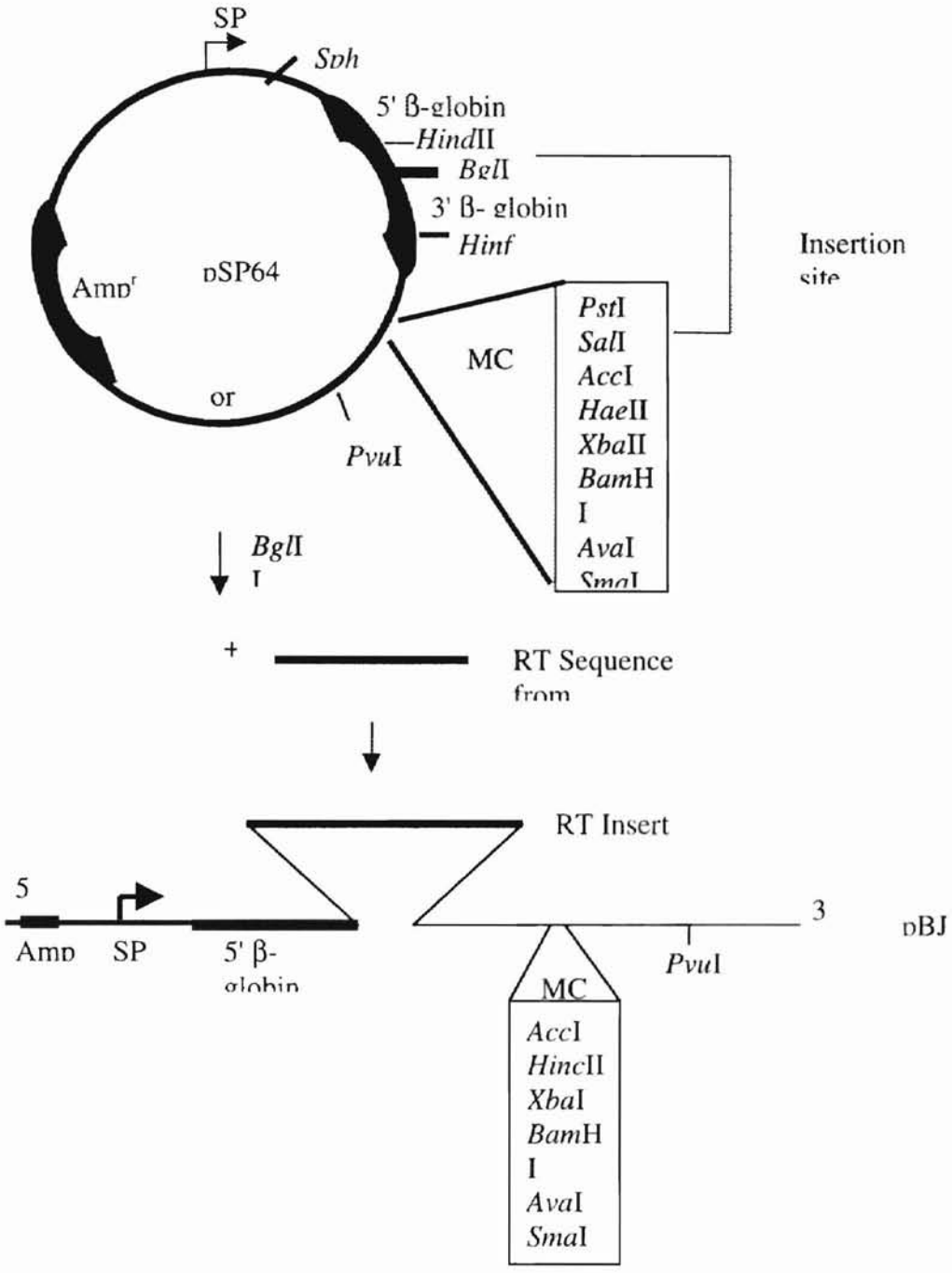

SP
Sph
5' ß-globin
—HindII
BglI
3' ß- globin
Hinf
Insertion
site
Amp
pSP64
MC
PstI
SalI
AccI
HaeII
XbaII
BamH
I
AvaI
SmaI
or
PvuI
BglI
I
+
RT Sequence
from
RT Insert
5
3
pBJ
Amp
SP
5' β-
globin
PvuI
MC
AccI
HincII
XbaI
BamH
I
AvaI
SmaI

FIGURE 2
INDIRECT CLONING OF RT INTO pSP64T

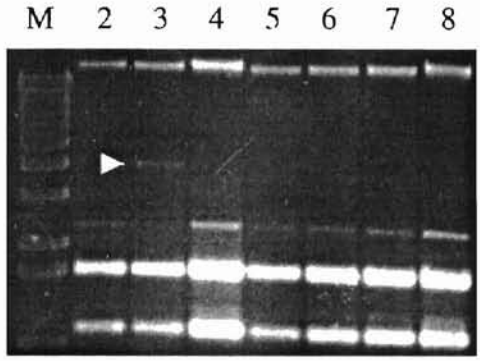


Figure 2. Ligation of RT sequence from pGTRT-14B and pSP64T. M, marker; lanes 2-8, plasmids 1-7. White arrow indicates indicates plasmid chosen as pBJ1.

PROTEIN SYNTHESIS

Coupled *In Vitro* Transcription-Translation in Rabbit Reticulocyte Lysate

The RT was expressed from pBJ1 and pGTRT-14B utilizing the SP6 polymerase promoter in RRL (Promega SP6 TNT Quick Coupled Transcription-Translaion System) to choose the most suitable plasmid for further experiments. Four reactions A, B, C, and D, were prepared with pBJ1, pGTRT-14B, control luciferase DNA, and no DNA, respectively. Each reaction contained master mix (rabbit reticulocyte lysate, SP6 RNA polymerase, TNT reaction buffer, RNasin, and amino acid mixture minus methionine) and 40 μCi [^{35}S]-met (250-1000 mCi/mmol, New England Nuclear) and was incubated at 30°C for 90 minutes. The incorporation of [^{35}S]-met at 30, 60, and 90 minutes was determined by a TCA assay and scintillation counting in EcoLume (Research Products International Corporation). The protein products from each reaction were separated on 8% SDS-PAGE as described below. The protein products were electroblotted onto a PVDF membrane, stained, and audoradiographed with Kodak X-ray film.

SDS-PAGE

An 8% separating gel was prepared with 14.19ml water, 8ml acrylamide: bisacrylamide (30%:0.8%), 7.5 ml 1.5 M Tris pH 8.8, 0.3 ml 10% SDS, 0.15 ml 0.1 g/ml APS (prepared fresh), and 12 μl TEMED. The stacking gel was prepared with 7 ml water, 1.5 ml acrylamide:bisacrylamide (30%:0.8%), 1.25 ml 1.0 M Tris pH 6.8, 0.1 ml 10% SDS, 0.1 ml 0.1 g/ml APS (prepared fresh), and 10 μl TEMED.

TCA Assay

The translation reaction was spotted onto Whatman #1 filter paper and placed in ice cold 10% TCA. Filter papers were then boiled in 5% TCA and stirred in 5% TCA at room temperature with excess methionine. Filter papers were washed in 95% ethanol, acetone, and dried. Dry filter papers were de-stained in 15% H_2O_2, then washed sequentially in 95% ethanol, acetone, and dried.

Electroblot of Proteins to PVDF Membrane

Four pieces of Whatman #1 filter paper and 1 piece of PVDF membrane were cut to the same size to match the size of the gel. One filter paper each was placed in the anode 1 solution (A1, 0.23 M Tris·HCl 20% methanol), anode 2 solution (A2, 0.19 M Tris·HCl and 20% methanol), and the other 2 filter papers were placed in the cathode solution (C, 0.19 M Tris·HCl, 0.4 M 6-AHA, and 20% methanol). The membrane was wetted with methanol, then rinsed with water. The membrane was placed on the gel followed by paper A2, then A1. The gel was trimmed to match the filter paper and membrane and inverted onto the anode of the blotting apparatus (PolyBlot Transfer System, American Bionetics). Two layers of filter paper C were positioned on the gel upon which the cathode was then placed. The transfer ran at 2.5 mA/cm^2 for 30 minutes.

Staining PVDF Membrane

The membrane was washed in TBS for 10 minutes and in water for 5 minutes. The membrane was stained in a Coomassie blue staining solution (1 part 0.2% Coomassie Brilliant Blue R-250 (Biorad)/50% methanol and 6 parts 10% isopropanol/25% acetic acid) for 1 minute with vigorous shaking. The membrane was then washed in a destaining solution (10% isopropanol/25% acetic acid) for 1 minute with vigorous

shaking. The destaining was repeated with fresh destain solution for 5 minutes with vigorous shaking and dried under a heat lamp.

IMMUNOPRECIPITATION OF CaMV RT WITH HSP90

Antibodies and Resins

8D3 is a mouse IgM mAb against hsp90[47]. D7α is a mouse IgG mAb against hsp90[48]. Anti-hsp90 is a mouse IgG pAb raised by the Hybridoma Center for the Agricultural and Biological Sciences (Oklahoma State University). Non-specific IgM antibodies were mouse antibodies from MOPC-104E hybridoma (Sigma). Non-specific IgG antibodies were mouse antibodies from MOPC-21 hybridoma (Sigma).

Resins were goat anti-mouse IgM or IgG antibodies covalently coupled to PNCA agarose (Sigma) as previously described[49]. All antibodies and resins were supplied by Dr. Steven Hartson.

Rabbit Reticulocyte Lysate System

8D3 or a non-specific antibody was bound to two IgM resins (R1 or R2, respectively) by 1-hour incubation at 4°C with rocking. Following the 1-hour incubation at 4°C, the resins were pre-washed twice with 1 ml P150 and 1 ml P20. The RT was expressed from pBJ1 in RRL at 30°C for 25 minutes. During the incubation, a TCA assay was done at 0, 10, 15, 20, and 25 minutes. At 20 minutes 0.4 M Na_2MoO_4 was added to the reaction which was then incubated for an additional 5 minutes. The translation reaction (I1) was clarified by centrifugation at 14,000 rpm for 10 minutes and then designated as (I2) after

clarification. Three microliters of I2 were added to 20 µl R1 and R2 and stirred on ice for 2 hours. The resins were washed twice with 1 ml P0 and the supernatants were collected as 1S and 2S. Resins 1 and 2 were then washed twice with P0, once with P50T, and twice with P0. Both resins were eluted twice with P500 and the pooled fractions were labeled 1P and 2P. I1, I2, 1S, 2S, 1P, 2P, R1, and R2 were separated by SDS-PAGE. The products were electroblotted onto PVDF membrane, audoradiographed, and western blotted for hsp90.

A non-specific Ab, D7α mAb, or α–hsp90 pAb was bound to three IgG resins (R3, R4, or R5 respectively). Immunoprecipitation was done as described above with the following changes. Resins were pre-washed twice with 1 ml P150 and 1 ml P100. After the 1-hour incubation, all resins were washed twice with 50 µl P150. The translation reaction was designated as A1 and after clarification as A2. Supernatants were collected as 3S, 4S, and 5S. Eluted pellets were collected as 3P, 4P, and 5P. A_1, A_2, 3S-5S, 3P-5P, and R3-R5 were separated by SDS-PAGE, electroblotted onto PVDF membrane, stained, audoradiographed, and western blotted for hsp90.

Western Blot for Hsp90

The PVDF membrane was washed in methanol with shaking until the blue stain was removed and then switched to water. The membrane was blocked in TBS/5% skim milk for 1 hour, placed in a heat sealed bag containing a mouse anti-hsp90 pAb, and incubated overnight at 4°C with shaking. The membrane was washed twice in TBS/0.5% Tween20 for 5 minutes and reblocked with TBS/5% skim milk for 5 minutes at room temperature. The membrane was placed in a heat sealed bag containing 10 ml TBS, 10 ml TBS/5% skim milk, and 8 µl AffiniPure Goat anti-mouse AP-conjugated IgG, (Jackson

Immunoresearch) at room temperature for 2 hours. The membrane was then washed three times in TBS/0.5% Tween20 for 5 minutes at room temperature and then washed in TBS for 5 minutes at room temperature. The membrane was developed in 30ml alkaline phosphatase buffer (0.1 M Tris HCl pH 9.5, 0.1 M NaCl, and 0.1 M magnesium acetate) warmed to 32°C containing 100 µl BCIP (50 mg/ml in premixed 70% DMF/30% water) and 200 µl NBT (50 mg/ml in100% DMF). Once blue bands appeared, the membrane was rinsed in water and washed in methanol. The blot was then dried under a heat lamp.

IP in a Wheat Germ Lysate System

Immunoprecipitation was also done with the RT expressed in wheat germ lysate (WGL, Promega SP6 TNT Quick Translation-Translation System). Resins 6 and 7 were anti-mouse IgM Abs crosslinked to PCNA agarose and R8, R9, and R10 were anti-mouse IgG Abs crosslinked to PCNA agarose. The non-specific MOPC-104E and 8D3 antibodies were adsorbed onto IgM resins 6 and 7, respectively, and the non-specific MOPC-21, D7α, and α–hsp90 antibodies were absorbed onto IgG resin 7, 8, and 9, respectively, by a 1-hour incubation at 4°C. Following the 1-hour incubation, all resins were pre-washed twice with 1 ml of P150T. Resins 6 and 7 were then pre-washed twice with 1 ml P20M and R8-10 were pre-washed twice with 1 ml P100M. The RT was expressed from pBJ1 in WGL at 30°C for 25 minutes. At 20 minutes, 0.4 M Na_2MoO_4 was added to the translation reaction, which was then incubated for an additional 5 minutes. At 25 minutes, the translation reaction was clarified as described earlier and designated as T2. Three microliters of T2 was added to each pre-washed resin and the mixture stirred on ice for 2 hours. After stirring, resins 6 and 7 were washed twice with 50 µl P0 and R8-10 were washed twice with 50 µl P150. The supernatants of resins 6-10 were collected as 6S, 7S, 8S, 9S, and 10S, respectively. After collecting the supernatants,

resins 6 and 7 were washed 5 times with 1 ml P75 and R8-10 with 1 ml P150. The supernatant from each wash was discarded. Resins 6 and 7 were eluted twice with 50 µl P500 and designated as 6P and 7P. Resins 8-10 were eluted by boiling in hot SDS sample buffer and designated as 8P-10P. T2, 6S-10S, and 6P-10P were analyzed by SDS-PAGE, transferred to a PVDF membrane, audoradiographed, and western blotted for hsp90.

Protease Assay of RT

A protease assay buffer (20 mM PIPES pH 7.0, 0.1 M NaCl, 4 mM $CaCl_2$, and 0.1 mM EDTA) was prepared. The RT was expressed from pBJ1 in RRL with 40 µCi [^{35}S]-met and 0 or 1 µg/80 µl translation reaction of geldanamycin for 30 minutes. Each reaction was diluted 1:1 with protease assay buffer. To 30 µl of protease assay buffer, 0, 58 ng, or 8.6 µg of trypsin (288 µg/ml) was added. Thirty microliters of each diluted translation reaction was added to the protease assay buffer containing either 0, 58 ng, or 8.6 µg of trypsin and digested for 6 minutes on ice. The reaction was stopped by transferring immediately to boiling SDS sample buffer. The products were separated by 8% SDS-PAGE, electrobloted to PVDF membrane, stained, and audoradiographed.

CHAPTER III

RESULTS

Construction of pBJ1

PCR and Cloning

To take adavantage of the activity of Taq polymerase that adds an adenine onto the ends of PCR products independent of the template, the RT sequence was cloned into pGEM-T that contained terminal thymidine overhangs. Amplification of the RT sequence resulted in a 2.1kb PCR product (figure 3 lane 1). The 2.1kb RT PCR product was cloned into the pGEM-T vector (3 kb) at the thymidine overhang site (figure 4). To determine the ligation background that may result from the vector self-annealing, a reaction containing the pGEM-T vector only was done (lane 5). After the ligation incubation, the ligation background showed the 3kb vector and no band caused by the vector self-annealing. To determine ligation efficiency, a 458 bp control insert (provided with Promega TNT Cloning kit) was ligated into pGEM-T that should have resulted in a 3.5kb band (lane 4). However, a distinct 3.5kb band was not detected. In the 1:1 (insert:vector) reaction (lane 2), the 2.1 kb RT insert was detected, but not the 3 kb vector or a product of the vector and insert ligation. Therefore, the PCR insert must have ligated into the vector at the 1:1 vector to insert ratio. A 2, 3, and 6 kb band were observed in the 3:1 (insert:vector) reaction (lane 3) that corresponded to the RT insert, undigested

FIGURE 3
PCR OF RT GENE FROM pUM52

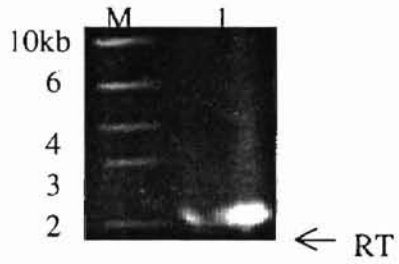


Figure 3. M, marker; lane 1, RT PCR product from pUM52.

FIGURE 4
PCR CLONING OF RT INTO pGEM-T

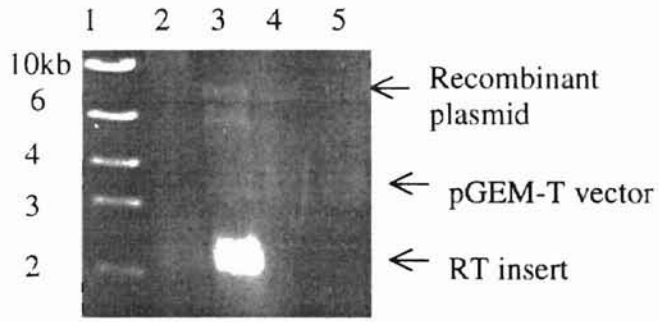


Figure 4. Ligation of RT insert and pGEM-T vector. Lane 1, marker; lane 2, 1:1 insert/vector ligation; lane 3, 3:1 insert/vector ligation; lane 4, vector with control insert DNA; lane 5, vector only.

pGEM-T vector, inter- and intramolecular recombination of the non-T tailed vector, respectively. This indicated some of the RT insert and vector did not ligate. A band migrating close to the 7 kb marker band in lane 3 was also observed which suggests that the RT insert did ligate into the vector and migrated consistent with a relaxed circle containing vector and RT insert.

After transforming the 7kb plasmid from the 3:1 ligation reaction (figure 4 lane 3) and the vector control (figure 4 lane 5), 20 bacterial colonies from the 3:1 ligation reaction were analyzed by the plasmid size quick check method (figure 5). The vector control (lane 1) showed the vector to migrate to the 3kb marker as expected. This would help distinguish ligation products from the vector DNA. Four single bands were observed (lanes 15-18, plasmids 14-17) that migrated between the 3 and 4kb DNA markers. A plasmid containing the vector and insert was calculated to be 5066bp. Therefore, it was suspected that plasmids 14-17 (p14-p17) were plasmids containing the vector and insert DNA and migrated as relaxed circle DNA. Therefore, p14-p17 were re-transformed into JM109 cells and purified.

Transformations of bacteria with the p14-p17 plasmids all resulted in transformants with the exception of p15. Plasmid DNA from three bacterial colonies produced from the transformation of p14, 16, and 17 (colonies picked were each labeled as A, B, and C for p14-17, i.e. 14A, 14B, and 14C) were purified and digested with *Nae*I to determine the presence and orientation of the insert. If the insert was present in the correct orientation, a 2.1 and 2.9kb band were expected. If the insert was present in the wrong orientation,

FIGURE 5
PLASMID SIZE OF pGTRT DNAs

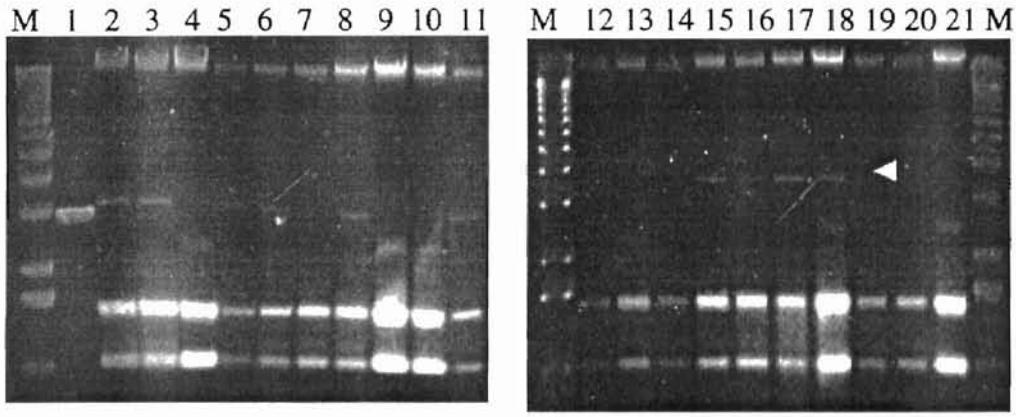


Figure 5. M, marker; lane 1, pGEM-T vector DNA only. Lanes 2-21 are pGTRT plasmids produced from PCR cloning of pGEM-T vector and RT PCR insert. White arrow points to the plasmids chosen for analysis.

then a 4.4kb and 650bp were expected (figure 6). If the insert was not present, then a single 4.7kb band was expected. Undigested plasmids were used as controls and showed 3 bands migrating near the 10, 5, and 4kb marker bands (figure 7). Digestion of p14C with *Nae*I (lane 6) produced a single 4.7kb band. Digestion of p16A (lane 8) and p17 (not shown) produced approximately 4.7 and 2.9kb fragments. Therefore, p14C was not digested at all and p16A and p17 were only partially digested, respectively. Digestion of p14A, B, p16B, and C produced approximately 4.7, 2.9, and 2.1kb fragments (lanes 2, 4, 10, and 11). All plasmids were only partially digested because of the presence of the 4.7kb band, but shown to contain the insert in the correct orientation.

Sequence analysis of the insert in p14A and B showed both plasmids contained an error near the 5' end of the insert that resulted in the amino acid change I97T. Plasmid p14A also had two missing nts at position 4132 and 4133. The I97T change and the two missing nts were not observed to be sequence artifacts. Plasmids 16B and C also had errors near the 5' end of the insert that were not observed as sequence artifacts. These resulted in the amino acid changes of T19A (nt A3251 to G) and A150V (nt C4081 to T). Since it was possible that the I97T change in p14B would not affect the activity of the polymerase, p14B was chosen as pGTRT-14B for further experiments.

To construct pBJ1, the RT sequence from pGTRT-14B was digested with *Bam*HI and *Xho*I and cloned at the *Bgl*II/*Sal* site of pSP64T. Ligation resulted in a band migrating near the 4kb marker band (lane 3). This band had a migration pattern similar to the bands produced from PCR cloning of the RT and pGEM-T (figure 4 lanes 15-18). Therefore, this plasmid was designated as pBJ1.

FIGURE 6
ILLUSTRATION OF RT ORIENTATION IN pGTRT

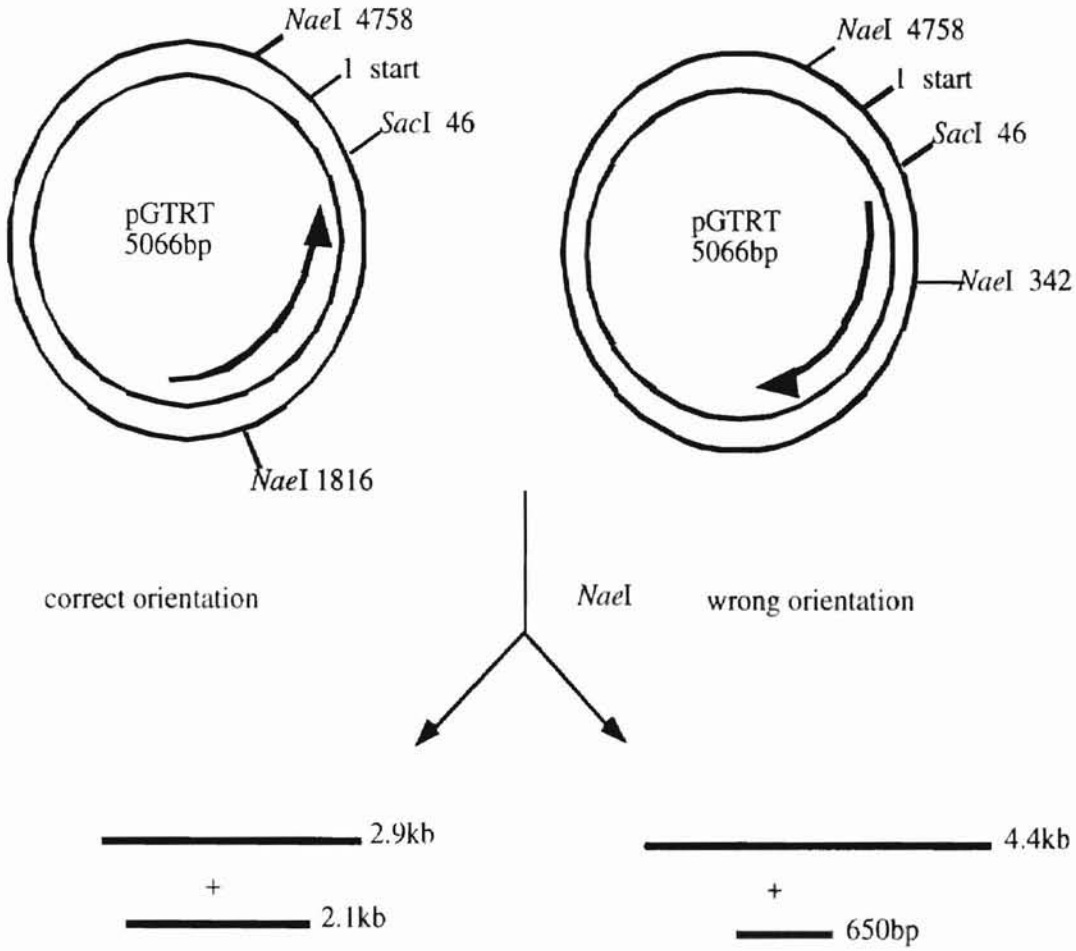


Figure 6. Illustration of *Nae*I digest of pGTRT-14B.

FIGURE 7
*Nae*I DIGEST OF pGTRT DNAs

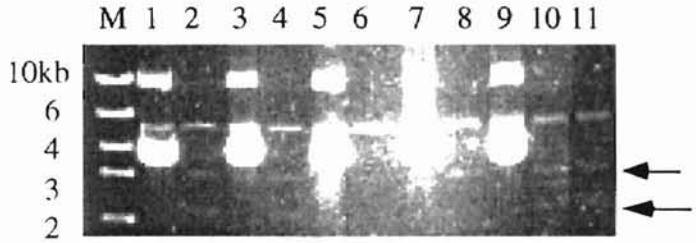


Figure 7. *Nae*I digests of pGTRT-14A, B, C, 16A, B, and C. M, marker; lanes 1, 3, 5, 7, and 9 are undigested DNAs pGTRT-14A, B, C, 16A, and B, respectively. Lanes 2, 4, 6, 8, 10, and 11 are *Nae*I digested plasmids pGTRT-14A, B, C, 16A, B, and C, respectively. Top and Bottom arrow correspond to the 2.1 and 2.9kb *Nae*I fragments, respectively.

Protein Expression in Rabbit Reticulocyte Lysate

RT Expression from pBJ1 and pGTRT-14B

Expression from the control luciferase DNA (C) produced the 61kDa monomeric luciferase protein (figure 8). Smaller protein products were also produced from the control luciferase DNA. No protein products were observed in the no DNA control (D) indicating the RRL did not contain any endogenous mRNA and all protein bands observed were translation products from the DNA template. *In vitro* transcription-translation of pBJ1 (A) and pGTRT-14B (B) produced major 79 and 52kDa protein bands. Minor protein products were also produced from pGTRT-14B and pBJ1. The molecular weight of the polymerase was calculated to 78.8kDa suggesting the 79kDa band observed was the polymerase. The major 52kDa band and the small minor protein products may have resulted from internal translation initiation, premature translation termination, or post-translational cleavage. The amount of incorporation at 30, 60, and 90 minutes for pBJ1 was higher than for pGTRT-14B and not significantly less than for the control luciferase plasmid (figure 9). The percent of incorporation of [^{35}S]-met and fold stimulation over background at 90 minutes for pBJ1 were 9.9% and 77-fold, respectively (table 1). The percent incorporation and fold stimulation over background for pGTRT-14B was only 0.89% and 25-fold, respectively (table 1). The fold stimulation by pBJ1 was not much less than that by the control luciferase plasmid suggesting that translation did proceed efficiently. These results showed that translation of pBJ1 stimulated the incorporation of [^{35}S]-met 3X more and incorporated 10X more [^{35}S]-met than pGTRT-14B. These results demonstrated that pBJ1 was a better template for *in vitro* transcription-translation and thus more suitable to determine the association of hsp90 with the RT.

FIGURE 8
REVERSE TRANSCRIPTASE EXPRESSION FROM
pGTRT-14B AND pBJ1

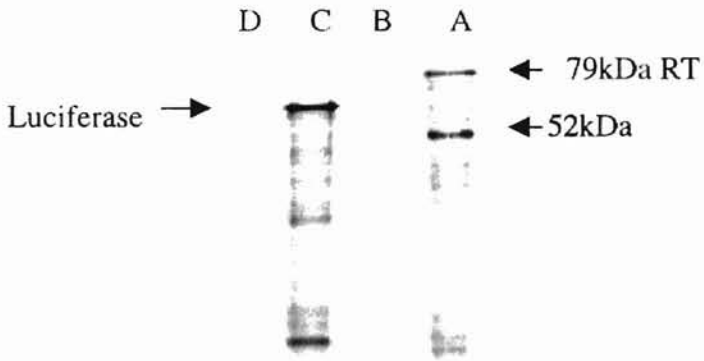


Figure 8. Audoradiograph of 8% SDS/PAGE of *in vitro* transcription-translation of pGTRT-14B and pBJ1. Lane D, no DNA; C, luciferase control DNA; B, pGTRT-14B; A, pBJ1.

FIGURE 9
[^{35}S]-MET INCORPORATION FROM *IN VITRO*
TRANSCRIPTION-TRANSLATION TEMPLATES

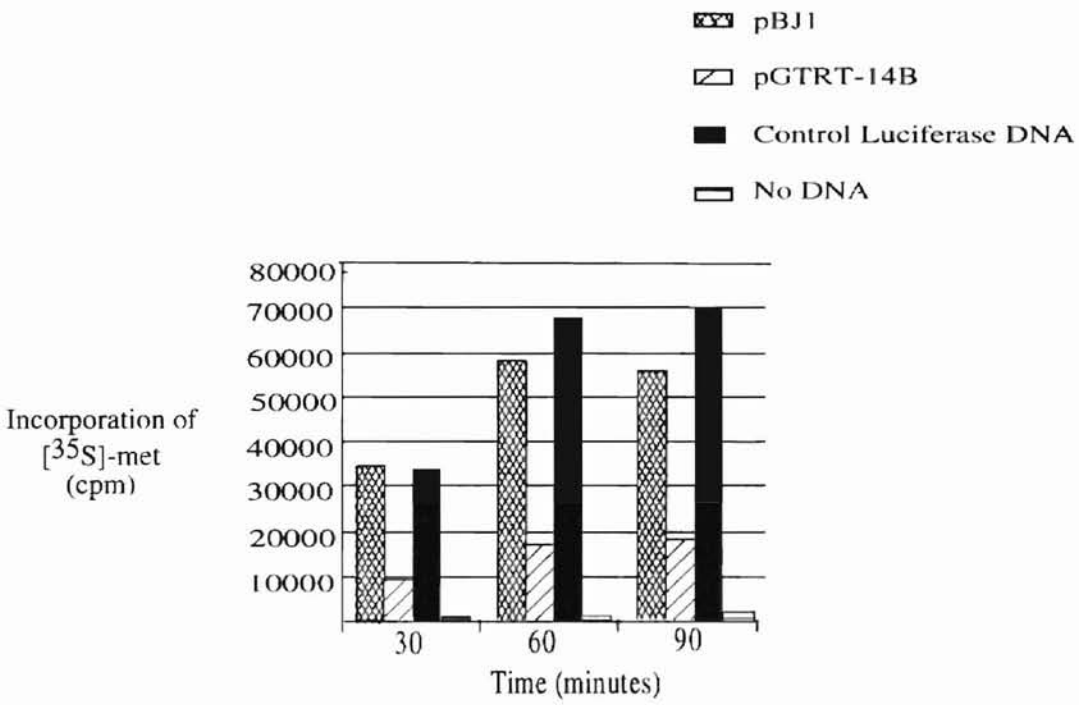


Figure 9. The amount of [^{35}S]-met incorporated from *in vitro* transcription-translation of pBJ1, pGTRT-14B, control luciferase DNA, and no DNA.

TABLE 1
COMPARISON OF [^{35}S]-MET INCORPORATION
AND FOLD STIMULATION FROM *IN VITRO*
TRANSCRIPTION-TRANSLATION TEMPLATES

	% incorporation at 90 min	Fold stimulation over background
pBJ1	9.9	77
pGTRT-14B	0.89	25
Control luciferase DNA	3.7	96
No DNA	0.08	1

Table 1. Comparison of percentage of incorporation and fold stimulation over background at 90 minutes of *in vitro* transcription-translation.

Immunoprecipitation of CaMV RT with Hsp90

Immunoprecipitation with IgM Resins and Protein Expression in RRL

To determine if hsp90 was associated with the RT in RRL, the translation reaction was immunoprecipitated using IgM resins attached to a non-specific control antibody (1) or 8D3 (2), a monoclonal antibody against hsp90. It was observed that some of the RT did not bind to the non-specific control antibody (1S, figure 10 lane 3) and a similar amount of the RT did bind to the control antibody (1P, lane 4). This showed that there was some non-specific binding of the RT to the IgM resin. The RT band that was observed in lane 5 showed that some of the RT did not bind to the 8D3 IgM resin (2S). The interaction between the hsp90 and the 8D3 antibody is sensitive to high salt concentrations. Therefore, a high salt elution was done to remove the bound protein from the 8D3 resin. The RT band observed in lane 6 after the high salt elution showed that some of the protein did bind to the 8D3 resin (2P). The RT bands observed in lanes 7 and 8 (R1 and R2, respectively) indicated that a portion of the polymerase remained on the resin after the high salt elution of the non-specific and 8D3 resin. The amount of protein that was observed to bind to the 8D3 resin (2P) was slightly more than the amount that did not bind to the 8D3 resin (2S). So it seemed as if the RT showed specific binding to the 8D3 resin. However, the amount of protein that bound to the 8D3 resin (2P) compared to the amount of non-specific binding (1P) may suggest that the amount of protein that bound to the 8D3 resin may have bound non-specifically.

FIGURE 10
IMMUNOPRECIPITATION OF RT AND HSP90
USING IgM RESIN

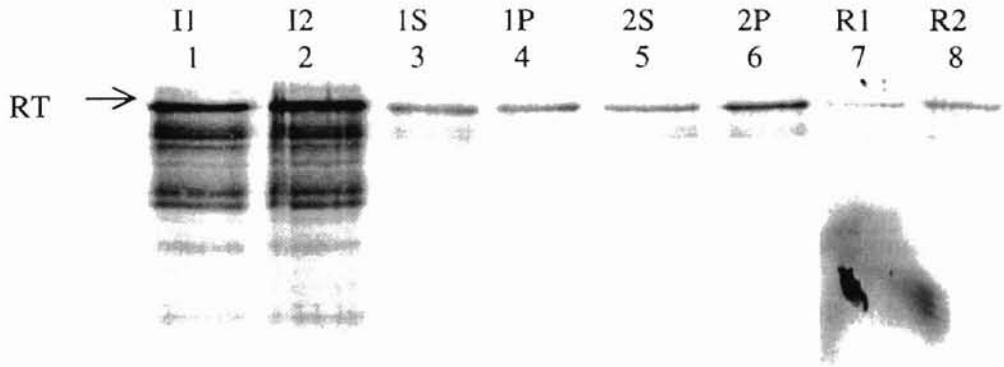


Figure 10. Audoradiograph of 8% SDS/PAGE of immunoprecipitation of RT and hsp90 expressed in RRL. I1, translation reaction; I2, clarification of translation reaction; 1S non-specific IgM supernatant; 1P, non-specific IgM eluate; 2S, 8D3 IgM supernatant; 2P, 8D3 IgM eluate; R1, non-specific resin; R2, 8D3 resin.

The western blot for hsp90 showed the presence of hsp90 in RRL (figure 11) and that most of it was recovered after removing protein aggregates (lane 2). A comparison of lanes 3 and 4 showed that all of the hsp90 was recovered in the supernatant (1S) and therefore not bound to the resin. These results were expected because the hsp90 should not bind to the non-specific resin. A faint hsp90 band was detected in 2S (lane 5) and a very strong hsp90 band in 2P (lane 6). A comparison of lanes 5 and 6 showed that most of the hsp90 bound specifically to the 8D3 resin. That no hsp90 band was detected in R1 (lane 7) showed that none of the hsp90 remained on the resin after the high salt elution. A very faint hsp90 band was detected in R2 (lane 8), but the amount that was observed was insignificant. Therefore, none of the hsp90 remained on the 8D3 resin after the high salt elution. The hsp90 bands observed in 2S and 2P, (lanes 5 and 6) were comparative to the RT bands observed in 2S and 2P (figure 8 lanes 5-8). The hsp90 bands observed in 1S, 1P, R1 and R2 (figure 11 lanes 3, 4, 7, and 8) were not parallel with the RT bands observed in 1S, 1P, R1 and R2 (figure 10 lanes 3, 4, 7, and 8). Therefore, this experiment failed to test the association between hsp90 and the RT because of the non-specific binding of the RT to both resins.

Immunoprecipitation with IgG resins and Protein Expression in RRL

Immunoprecipitation of hsp90 with the RT was attempted using IgG resins bound to a non-specific control antibody (3), D7α (4), a mAb against hsp90, and α–hsp90 (5), a pAb against hsp90. The RT was observed in the control, D7α, and α-hsp90 supernatants (3S-5S, figure 12 lanes 3, 5, and 7). The RT was also not precipitated in high salt eluate of the control, D7α, and α-hsp90 resins (3P-5P lanes 4, 6, and 8). This showed that the RT did not bind non-specifically to the IgG resins. The RT was observed in R3-5 (lanes 9-11) after the high salt elution. However, the amount of RT that absorbed onto the D7α

FIGURE 11
WESTERN BLOT OF HSP90 FROM IMMUNOPRECIPITATION
IN RRL

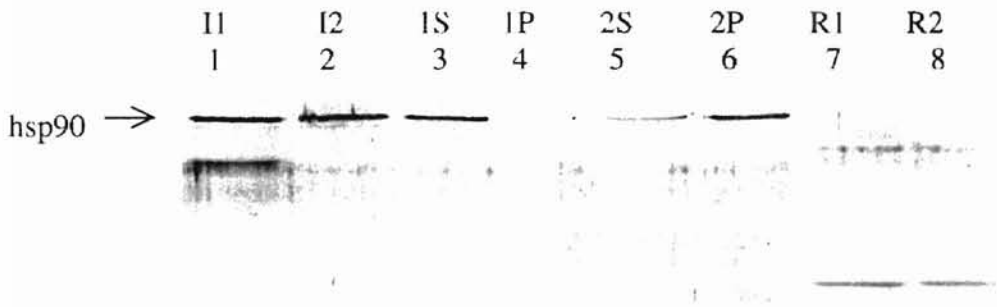


Figure 11. Western blot of hsp90 from immunoprecipitation in RRL. I1 and I2 are translation reactions before and after clarification, respectively. 1S and 1P are non-specific IgM supernatant and eluate, respectively. 2S and 2P are 8D3 specific IgM resin supernatant and eluate, respectively. R1 and R2 are non-specific and specific 8D3 IgM resin, respectively.

FIGURE 12
IMMUNOPRECIPITATION OF RT AND HSP90
USING IgG RESIN

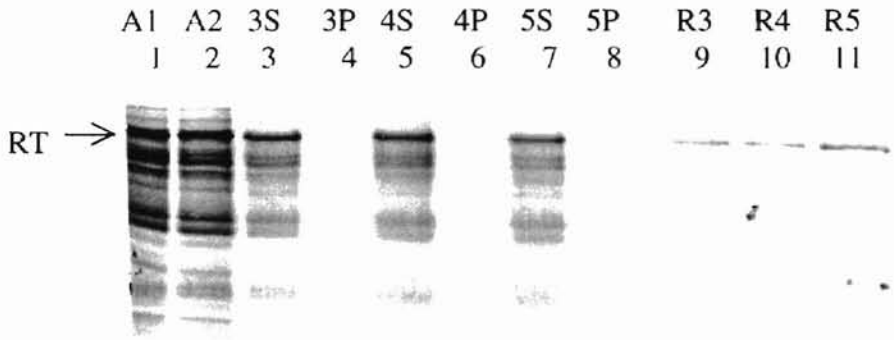


Figure 12. Audoradiogragh of 8% SDS/PAGE of immunoprecipitation of RT and hsp90 expressed in RRL using IgG resins. A1 and A2 are translation reactions before and after clarification, respectively. 3S and 3P are non-specific resin; 4S and 4P are D7α resin; 5S and 5P are α-hsp90 resin.

and α-hsp90 resins was similar to the amount absorbed on the control antibody resin. Therefore, the RT that remained absorbed to the D7α and α-hsp90 resins was due to non-specific binding.

The western blot (figure 13) showed all of hsp90 in the non-specific supernatant (3S, lane 3), therefore, it was not absorbed onto the non-specific resin. The presence of an hsp90 band in 4S (lane 5) indicated that the D7α resin did not bind all of hsp 90. An hsp90 band was observed in 5S (lane 7), but was insignificant relative to the amount of hsp90 present in the translation reaction before immunoprecipitation. Immunoadsorbing salt-resistant hsp90 complexes do not allow precipitation of the hsp90 complexes with high salt concentrations. Therefore, that no hsp90 bands were precipitated in the high salt eluate of the control, D7α, and α-hsp90 resins (4P and 5P lanes 6 and 8) indicated that hsp90 was not absorbed non-specifically to the control, D7α, and α-hsp90 antibodies. Hsp90 bands were observed in R4 and R5 (lanes 10 and 11) after the high salt elution. This indicated that hsp90 absorbed specifically to the D7α and α-hsp90 antibody resins. These results taken together with the autoradiogram (figure 12) showed that the RT did not bind to D7α or α-hsp90 specifically and that hsp90 did bind specifically to D7α and α-hsp90. This suggested there was no physical association between hsp90 and the RT.

FIGURE 13
WESTERN BLOT OF HSP90 FROM
IMMUNOPRECIPITATION USING IgG RESIN

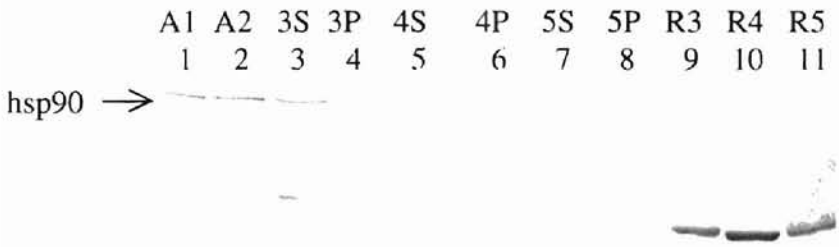


Figure 13. Western blot of hsp90 from immunoprecipitation of RT and hsp90 expressed in RRL using IgG resins. A1 and A2 are translation reactions before and after clarification, respectively. 3, non-specific; 4, D7α; 5, anti-hsp90 IgG resins; S, supernatant; P eluate; R, resin.

Immunoprecipitation with IgM and IgG and Protein Expression in Wheat Germ Lysate

Immunoprecipitation of hsp90 with the RT expressed from pBJ1 in WGL was also attempted because there may be some plant proteins present in WGL that are required to facilitate the interaction between the proteins. Immunoprecipitation was done with IgM resins bound to a non-specific control antibody (R6) and 8D3 (R7) and IgG resins were bound to a non-specific control antibody (R8), D7α (R9), and α-hsp90 (R10). The RT was detected in 6S and 6P (figure 14 lanes 2 and 3). This showed that only a portion of the RT bound non-specifically to the IgM resin. The RT was also observed in 7S and 7P (lanes 4 and 5) which showed the same band pattern observed in 6S and 6P. Some of the RT also remained on R6 and R7 after the high salt elution (lanes 12 and 13) which also indicated non-specific binding. The RT was also recovered in 8S, 9S, and 10S (lanes 6-8). Instead of a high salt elution, R8-10 were boiled in SDS sample buffer after collecting the low salt fractions. Resins 8-10 showed the same amount of radioactive protein (lanes 11-13). That the RT absorbed non-specifically to the control antibody indicated the RT also absorbed non-specifically to the 8D3, D7α, and α-hsp90 resin in WGL.

No western blot was provided for this experiment. If hsp90 showed the same band pattern as the autoradiogram, then this would mean that hsp90 was also absorbed non-specifically to the IgM and IgG resins. Therefore, the western blot is needed to verify that hsp90 was absorbed specifically to the resins. However, because the results of the autoradiogram were similar to the results from earlier experiments in RRL, it was concluded that hsp 90 and the RT do not associate in RRL or WGL.

FIGURE 14
IMMUNOPRECIPITATION OF RT AND HSP90
IN WGL

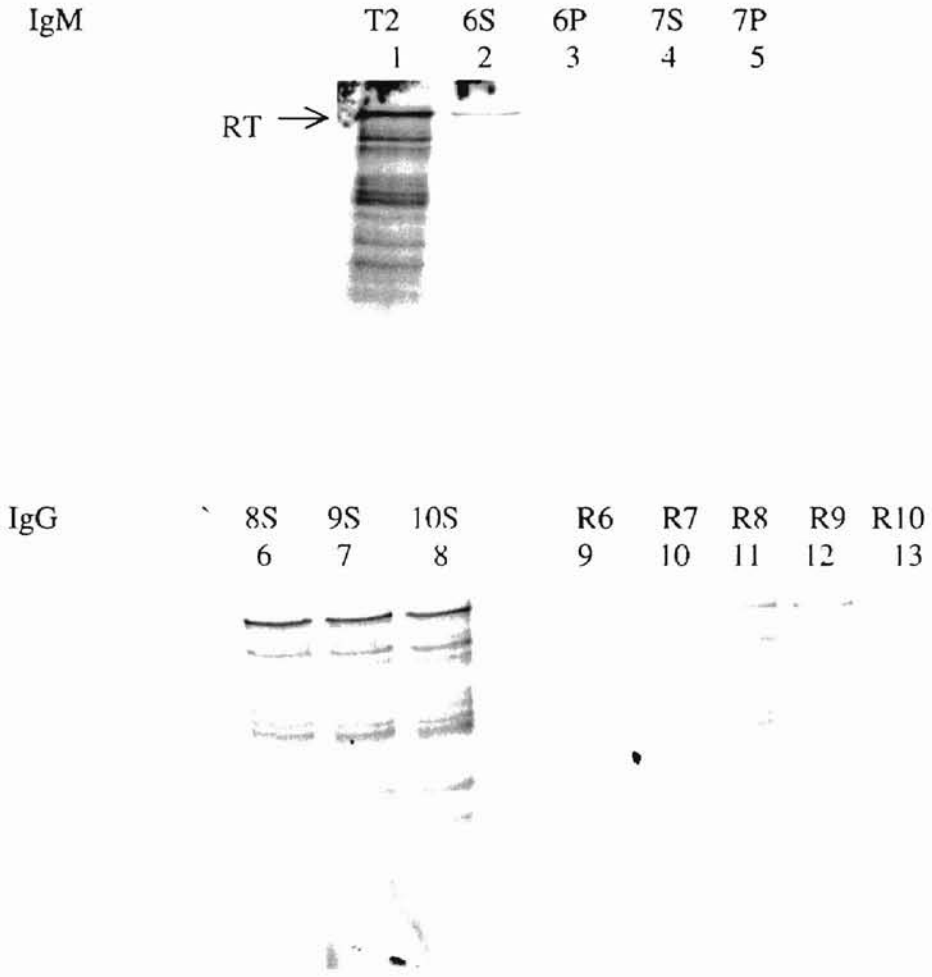


Figure 14 . Audoradiogragh of 8% SDS/PAGE of immunoprecipitation of RT and hsp90 expressed in WGL using IgM and IgG resins. S and P are supernatant and eluate, respectively. T2, translation reaction after clarification; 6, non-specific Ab; 7, 8D3 ; 8, non-specific; 9, D7α; 10, anti-hsp90 Abs; R, resin.

Proteolytic Assay of RT

To further test whether hsp90 was required for RT folding, a protease assay of the RT was done in the presence of GA. Geldanamycin inhibits hsp90 function as a molecular chaperone during protein folding. During the onset of folding, if the RT folding required hsp90, the RT would be sensistive to trypsin cleavage. If the RT did not require hsp90, then the RT would be resistant to trypsin cleavage. In the control experiment, the RT was expressed in the presence and absence of GA without trypsin (figure 15). The 79kDa RT protein band was observed with and without functional hsp90 and when no trypsin was added to the reaction (lane 1 and 4). With 58 ng of trypsin, the 79kDa RT and partial hydrolysis products were observed in the presence and absence of GA (lane 2 and 5). When the amount of trypsin was increased 8.6 μg, the 79kDa RT was still detected, but the amount of the radioactive 79kDa RT decreased significantly (lanes 3 and 6) and very small fragments were detected. The only difference observed was the change in the amount of radioactive protein with respect to the amount of trypsin. Therefore, in the presence of GA, RT folding was protease-resistant and independent of hsp90 function.

FIGURE 15
PROTEASE ASSAY OF RT

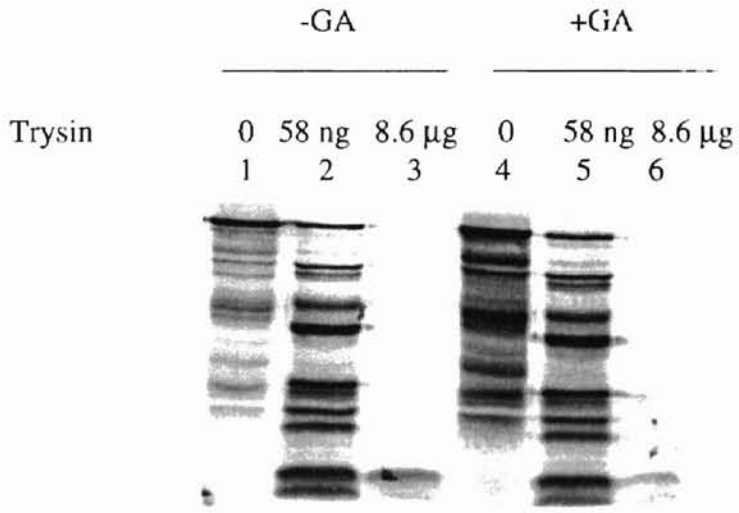


Figure 15. Trypsin cleavage of RT expressed in RRL in the absence (-GA) or presence (+GA) of geldanamycin.

CHAPTER IV

DISCUSSION

pGTRT DNAs Analysis

Construction of a plasmid competent for protein expression was achieved by PCR cloning with pGEM-T and direct cloning into pSP64T. PCR cloning with pGEM-T provided the advantage of having single thymidine overhangs flanking the insertion site of the vector and adenine overhangs on the RT PCR product produced from the Taq polymerase during PCR. Sequence analysis of the inserts in p14A, B, 16B, and C showed that pGTRT-14B to be a more suitable template. Although p14B had an I97T change near the 5' end of the insert, the activity of the polymerase may not have been affected. The pUM52 plasmid was a partial dimer plasmid that contained two copies of the CaMV CabbS DNA as direct repeats. The region of the pUM52 template that was amplified contained the modified region of the CabbS DNA that was cloned into the parent plasmid to create pUM52. When the infectivity of pUM52 was tested, it was still infectious when transfected onto plants[45]. So, it was suspected that the I97T change in pUM52 would not affect the activity of the polymerase.

Indirect Cloning with pSP64T

Indirect cloning of the RT insert into pSP64T could be more easily achieved than by PCR cloning. Cloning the RT into pSP64T was attempted previously by two-step PCR.

However, transformants were not produced after transformation of the ligation product into *E. coli* JM109 cells. It was possible that the two-step PCR cloning did not produce a recombinant plasmid. The recombinant plasmid could not be detected by gel analysis, but the insert and vector DNA could be detected. It was assumed that the recombinant plasmid was made since the amount of the vector and insert DNA observed was less than the amount observed before PCR cloning.

This problem was also encountered when the RT was cloned into pBluescript KS$^+$. One difference is a ligation product could be detected. However, transformants were not produced. After several attempts and very long incubations, transformants were produced. But recovery of the plasmid resulted in a much smaller plasmid than what was observed before the transformation. The difficulty in obtaining a recombinant plasmid with the RT insert has not been an uncommon phenomenon. A report by Fütterer[50] revealed the presence of a pseudo-bacterial promoter in the C-terminal half of ORF IV, coding for the coat protein, of which a portion overlaps ORF V, directed the expression of a nucleic acid binding protein. This protein was toxic to *E.coli* and caused insertions and/or deletions in clones rendering them unstable[50]. The portion of the RT gene used in this cloning procedure also contained a part of the C-terminal that overlapped with the RT gene. Recovering a plasmid that was 5kb smaller than what was transformed into *E. coli* was consistent with the presence of the pseudo-bacterial promoter in the region of the RT gene sequence being cloned that resulted in unstable clones. This suggested the clone between the RT and pBluescript KS$^+$ was toxic to the bacteria that took up the clone resulting in considerable deletions. It was suspected that this was also true for the clone produced by two-step PCR cloning of the RT and into pSP64T.

To ligate the RT insert into pSP64T, it was more efficient to obtain the RT insert by digestion of pGTRT-14B than from a PCR product with restriction sites near the 5' and 3' end. Therefore, a recombinant plasmid could be obtained by removing the RT sequence from pGTRT-14B and inserting it into pSP64T to produce pBJ1. But after recovering pBJ1, which contained the full-length RT gene, from *E. coli* cells, it was speculated that the problem in obtaining a recombinant plasmid made in earlier experiments may not have been the presence of a psuedo-bacterial promoter within the RT sequence alone. Similar problems would have been expected when PCR cloning the RT into pGEM-T and pSP64T. It was also speculated that cloning difficulties did not arise from the use of pBluescript since this vector has been used in *E. coli*. Therefore, it was suspected that an unexplainable event between pBluescript and the CaMV RT gene occurred in *E. coli*.

Comparison of Protein Expression from pBJ1 and pGTRT-14B

Protein expression from pGTRT-14B and pBJ1 was compared. *In vitro* transcription-translation of pGTRT-14B and pBJ1 first showed that a CaMV RT could be expressed in RRL. Both plasmids produced a 79kDa protein whose size corresponds to that expected from the entire reading frame. The CaMV RT gene has been expressed before in RRL, but as a fusion protein with N-terminal deletions[51]. This is the first demonstration of the complete CaMV RT ORF expressed as a single protein in a mammalian system. Both plasmids also produced a 52kDa protein. This protein may have been the result of downstream aug codons, premature translation termination, or cleavage of the 79kDa protein post-translation. The latter is more likely since part of the reading frame codes for a protease. Differences in the time of the translation reactions affected the expression pattern when the reactions were incubated for 30 and 90 minutes. The translation

reaction at 90 minutes (figure 6 lane) showed the 52kDa protein. At 30 minutes (figure 8), the 52kDa protein could not be distinguished. Because the polymerase is synthesized as part of a polyprotein, allowing translation to proceed for 90 minutes would permit enough time for the protease to cleave the polymerase and produce a 52kDa protein. But, translation for 25 minutes may not be enough time to produce the 52kDa protein. When a translation reaction was monitored at 5 minute intervals and stopped at 25 minutes, after 20 minutes, the reaction began to slow down (data not shown). Thus, the time of the translation reaction affects the expression pattern of the protein. Protein products smaller than the 52kDa protein may have been due to premature translation termination, downstream aug codons, or translational pausing.

Differences in the vector features proved to have a significant effect on the translation efficiency. The starting vector, pSP64T contained 5' and 3' regions of β-globin mRNA that enhance translation efficiency of any cloned DNA[52]. When pBJ1 was compared to pGTRT-14B, pBJ1 was a better template for *in vitro* transcription-translation. Although cloning the RT sequence into the *Bgl*II and *Sal*I site of pSP64T removed the 3' UTR, translation would still proceed efficiently due to the presence of the 5'UTR. It is possible that the amount of protein produced from pBJ1, which lacked the 3'UTR, may be less than had it contained the 3'UTR. However, because of restriction site constraints, this possibility was not explored.

Immunoprecipitation of RT with Hsp90

Immunoprecipitation of the CaMV RT with hsp90 was attempted with hsp90 specific antibodies. An interaction between hsp90 and the RT could not be determined because the RT seemed to have a non-specific affinity for the IgM resin in RRL and WGL. Using

a plant system to test the hsp90-RT interaction did not prove to have any affect on detecting an hsp90-RT interaction. The RT did not have non-specific affinity for the IgG resin in WGL. Most of the RT was recovered in the D7α and α-hsp90 supernatant, thus not showing any specific binding to the D7α or α-hsp90 resin. Because no western blot for hsp90 was provided for the immunoprecipitation test in WGL, definite interpretation of the results could not be made about the hsp90-RT interaction. However, a comparison of the autoradiogram results from WGL to the autoradiogram results from RRL led to the presumption that hsp90 and the RT did not associate. RT folding measured by protease-resistance was independent of the hsp90 function revealed by GA insensitivity. Therefore, this made the hypothesis that a CaMV RT associated with hsp90 unlikely.

The retroid action of the RT requires it to bind to an RNA template and synthesize DNA. Protein-protein interactions that are needed for specific functions require stable conformations of the participating proteins to exist. This is also true for protein-RNA and protein-DNA interactions. During protein folding, non-native proteins tend to aggregate and fold incorrectly. For this reason, molecular chaperones are employed in protein folding pathways. Tests showed that RT folding did not require hsp90, but that does not exclude its need for another heat shock protein. Because the sequence of the pUM52 insert in the region of change has not been determined, the nucleotide change near the 5' end of the polymerase sequence could have prevented recognition of the RT by hsp90. The region of the insert in pGTRT-14B that was not sequenced may also have nt changes that could affect the association of RT with hsp90.

Immunoprecipitation tests using specific antibodies against heat shock proteins other than hsp90 would determine which hsp does associate with the RT. Moreover, antibodies

against a CaMV RT or protein fused to the RT for immunoprecipitation tests would be an alternative method to test for a RT-hsp association. In addition to the need of a western blot for the WGL test, a control to show that GA was active for the protease assay and an assay of the RT activity with and without GA would prove definitely if a CaMV RT associated with hsp90.

CHAPTER V

REFERENCES

1. Hull, R., Shepherd, R. J., & Harvey, J.D. (1976) Cauliflower mosaic virus: an improved purification procedure and some properties of the virus particles. *J. Gen. Virol.* 31, 93-100
2. Hohn, T. & Fütterer J. (1991) Pararetroviruses and retroviruses: a comparison of expression strategies. *Semin. Virol.* 2, 55-69
3. Melcher, U. & Chenault, K. D. (1994) Patterns of nucleotide sequence variation among cauliflower mosaic virus isolates *Biochemie* 76, 3-8
4. Covey, S., N. (1991) Pathogenesis of plant pararetroviruses: cauliflower mosaic virus *Semin.Virol.* 2, 151-159
5. Bonneville, J.-M. & Hohn, T. (1993) A reverse transcriptase for cauliflower mosaic virus: state of the art, 1992, p. 357-390. *In* N. Skalka and S. Goff (ed.), Reverse transcriptase. Cold Harbor Laboratory Press, Cold Spring Harbor, N.Y.
6. Kiss-Laszlo, Z., Blanc, S. & Hohn, T. (1995) Splicing of cauliflower mosaic virus 35S RNA is essential for viral infectivity. *EMBO J.* 14, 3552-3562.
7. Sanfaçon, H. & Hohn, T. (1990) Proximity to the promoter inhibits recognition of cauliflower mosaic virus polyadenylation signal. *Nature* 346, 3131-3139

8. Fütterer, J., Gordan, K., Bonneville, J. M., Sanfaçon, H., Pisan, B., Penswick, J. & Hohn, T. (1988) The leading sequence of caulimovirus large RNA can be folded into a large stem-loop structure. *Nucl. Acids. Res.* 16, 8377-8390

9. Melcher, U. (1988) A readable and space efficient DNA sequence representation: application to caulimoviral DNAs. *Comput. Appl. Biosci.* 4, 93-96

10. Hay, J., M., Jones, M. C., Blakebrough, M.L., Dasgupta, I., Davies. J. W. & Hull, R. (1991) An analysis of the sequence of an infectious clone of rice tungro bacilliform virus, a plant pararetrovirus. *Nucl. Acids Res.* 19, 2615-2621

11. Pollack, R. R. & Ganem, D. (1993) An RNA stem-loop structure directs hepatitis B virus genomic RNA encapsidation. *J. Virol.* 67, 3254-3263

12. Donze, O., Damay, P. & Spahr, P-F. (1995) The first and third uORFs in RSV leader RNA are efficiently translated: implication for translational regulation and viral RNA packaging. *Nucl. Acids Res.* 23, 861-868

13. Pooggin, M.M., Hohn, T. & Fütterer, J. (1998) Forced evolution reveals the importance of short open reading frame A and secondary structure in the cauliflower mosaic virus 35S RNA leader. *J. Virol.* 72, 4157-4169

14. Poogin, M.M., Fütterer, J., Skryabin, K. & Hohn, T. (1999) A short open reading frame terminating in front of a stable hairpin is the conserved feature in pregenomic RNA leaders of plant pararetroviruses. *J. Virol.* 80, 2217-2228.

15. Menissier, J., Lacquel, P., Lebeurier, G. & Hirth, L. (1984) A DNA polymerase is associated with cauliflower mosaic virus. *Nucl. Acids Res.* 12, 8769-8778

16. Pietrzak, M. & Hohn. T. (1987) Translation products of cauliflower mosaic virus ORF V, the coding region corresponding to the retrovirus *pol* gene. *Virus Genes* 1, 83-86

17. Torruella, M., Gordon, K. & Hohn, T. (1989) Cauliflower mosaic virus produces an aspartic proteinase to cleave its polyproteins. *EMBO J.* 8, 2819-2825

18. Maule, A. J. (1985) Replication of caulimoviruses in plants and protoplasts. *Mol. Plant Virol.* 2, 161-190

19. Summers, J., O'Connel, A., & Millman, I. (1975) Genome of hepatits B virus: restriction enzyme cleavage and structure of DNA extracted from Dane particles. *Proc. Natl. Acad. Sci. USA.* 72, 4597-4601.

20. Ganem, D. & Varmus, H.E. (1987) The molecular biology of hepatitis B viruses. *Annu. Rev. Bioch.* 56, 651-693

21. Nassal, M. & Schaller, H. (1993) Hepatitis B virus replication. *Trends Microbiol.* 1, 221-228

22. Kaneko, S. & Miller, R.H. (1988) X-region-specific transcript in mammalian hepatitis B virus-infected liver. *J. Virol.* 62, 3979-3984

23. Siddiqui, A., Jameel, S. & Napoles, J. (1987) Expression of the hepatitis B virus X gene in mammalian cells. *Proc. Natl. Acad. Sci. USA.* 84, 2513-2517

24. Gough, N.M. (1983) Core and E antigen synthesis in rodent cells transformed with hepatitis B virus DNA is associated with greater than genome length viral messenger RNAs. *J. Mol. Biol.* 165, 683-699

25. Tiollais, P., Pourcel, C. & Dejean, A. (1985) The hepatitis B virus. *Nature* 317, 489-495

26. Huan, B. & Siddiqui, A. (1993) Regulation of hepatitis B virus gene expression. *J. Hepatol.* 11, Suppl. 3, S22-S23

27. Summers, J. & Mason, W. S. (1982) Replication of the genome of a hepatitis B-like virus by reverse transcription of an RNA intermediate. *Cell*, 29, 403-415

28. Wang, G. & Seeger, C. (1992) The reverse transcriptase of hepatitis B virus acts as a protein primer for viral DNA synthesis. *Cell*, 71, 663-670

29. Pollack, J. & Ganem, D. (1994) Site specific RNA binding by a hepatitis B virus reverse transcriptase initiates two distinct reactions: RNA packaging and DNA synthesis. *J. Virol.* 68, 5579-5587

30. Endens, G., H., Ganem, D. & Varmus, H. E. (1987) 5' Terminal sequences influence the segregation of ground squirrel hepatitis virus RNAs into polyribosomes and viral core particles. *J. Virol*, 61, 35-41

31. Hirsch, R., Lavine, J., Chang, L., Varmus, H. & Ganem, D. (1990) Polymerase gene products of hepatitis B viruses are required for genomic RNA packaging as well as for reverse transcription. *Nature* 344, 552-555

32. Hirsch, R., Loeb, D.D., Pollack, J.R. & Ganem, D. (1991) *cis*-acting sequences required for encapsidation of duck hepaitits B virus pregenomic RNA. *J. Virol.* 65, 3309-3316

33. Junker-Neipmann, M., Bartenschlager, R. & Schaller, H. (1990) A short *cis*-acting sequence is required for hepaitits B virus pregenome encapsidation and sufficient for packaging of foreign RNA. *EMBO J.* 9, 3389-3396

34. Bartenschlager, R. & Schaller, H. (1992) Hepadnaviral assembly is initiated by polymerase binding to the encapsidation signal in the viral RNA genome. *EMBO J.* 11, 34130-3420

35. Wang, G-H. & Seeger, C. (1993) Novel mechanism for reverse transcription of hepatitis B virus. *J. Virol.* 67, 6507-6512

36. Wang, G-H., Zoulim, F., Leber, E.H., Kitson, J. & Seeger. C. (1994) Role of RNA in enzymatic activity of the reverse transcriptase of hepatitis B viruses. *J Virol.* 68, 8437-8442

37. Hu, J. & Seeger, C. (1996) Hsp90 is required for the activity of a hepatitis B virus reverse transcriptase. *Proc. Natl. Acad. Sci. USA* 93, 1060-1064

38. Cho, G., Park, S. & Jung, G (2000) Localization of hsp 90 binding sites in the human HBV polymerase. *Bioc. Biophys. Res. Comm.* 269, 191-196

39. Ju., J., Toft, D. & Seeger, C. (1997) Hepadnavirus assembly and reverse transcription requires a multi-component chaperone complex which is incorporated into nucleocapsids. *EMBO J.* 16, 59-68

40. Pratt, W.B. & Toft, D. O. (1997) Steroid receptor interaction with heat shock proteins and immunophilin chaperone. *Endocrinol. Rev.* 18, 306-360

41. Schneider, C., Sepp-Lorenzion, L., Nimmesgem, E., Querfelli, O., Danishefsky, S., Rosen, N. & Hartl, F. U. (1996) Pharmacologic shifting of a balance between protein refolding and degradation mediated by hsp 90. *Proc. Natl. Acad. Sci. USA* 93, 14536-14541

42. Blagosklonny, M.V., Toretsky, J., Bohen, S. & Neckers, L. (1996) Mutant conformation of p53 translated *in vitro* or *in vivo* requires functional hsp 90. *Proc. Natl. Acad. Sci. USA* 93, 8379-8383

43. Garcia-Cardena, G., Fan, R., Shah, V., Sorrentino, R., Cirino, G., Papapetropoulos, A. & Sessa, W. (1998) Dynamic activation of endothelial nitric oxide synthase by hsp 90. *Nature* 392, 821-825

44. Uma, S., Hartson, S. D., Chen, J. J. & Matts, R. L. (1997) Hsp 90 is obligatory for the heme-regulated eIF-2alpha kinase to acquire and maintain conformation. *J. Biol. Chem.* 272, 11648-11656

45. Melcher, U., Choe, I., Lebeurier, G., Richards, K. & Essenberg, R. (1986) Selective allele loss and interference between cauliflower mosaic virus DNAs. *Mol. Gen. Genet.* 203, 230-236

46. Kozak, M. (1987) An analysis of 5' noncoding sequences from 699 vertebrate mRNAs. *Nucl. Acids Res.* 15, 8125-8130.

47. Perdew, G. & Whitelaw, M. L. (1991) Evidence that the 90-kDa heat shock protein exists in cytosol in heteromeric complexes containing hsp 70 and three other proteins with Mr 63,000, 56,000, and 50,000. *J. Biol. Chem.* 266 (11), 6708-6713

48. Brugge, J., Yonemoto, W. & Darrow, D. (1983) Interaction between the Rous sarcoma virus transforming protein and two cellular phosphoproteins: analysis of turnover and distribution of this complex. *Mol. Cell Biol.* 3, 9-19

49. Kaufman, B. T. (1974) Methotrexate-agarose in the purification of dihydrofolate reductase. *Methods Enzymol.* 34, 272-281

50. Futterer, J., Gordan, K., Pfeiffer, P. & Hohn, T. (1988) The instability of a recombinant plasmid, caused by a prokaryotic-like promoter within the eukaryotic insert, can be alleviated by expression of antisense RNA. *Gene* 67, 141-145

51. Ranu, R., Scholthof, H., Gowda, S. & Shepherd, R. (1999). Synthesis of figwort mosaic virus coat protein-reverse transcriptase fusion protein in reticulocyte lysates from a full-length transcript. *J. Plant Bioch. Biotech.* 8, 81-85

52. Krieg, P. & Melton, D. (1984) Functional messenger RNAs are produced by SP6 *in vitro* transcription of cloned cDNAs. *Nucl. Acids. Res.* 12, 7057-7070

VITA

Breshana Quiene Johnson

Candidate for the Degree of

Master of Science

Thesis: TESTS OF THE ASSOCIATION OF HEAT SHOCK PROTEIN 90 WITH A CAULIFLOWER MOSAIC VIRUS REVERSE TRANSCRIPTASE

Major Field: Biochemistry and Molecular Biology

Biographical:

Personal Data: Born in New Orleans, Louisiana on July 20, 1976 the daughter of Jacqueline and Rev. Wilfret Johnson.

Education: Graduated from McDonogh #35 Senior High School, New Orleans, Louisiana in May 1993; received Bachelor of Science degree in Chemistry from Xavier University of Louisiana, New Orleans, Louisiana in May 1997. Completed the requirements for the Master of Science degree with a major in Biochemistry and Molecular Biology in July 2000.

Experience: Employed by Xavier University of Louisiana, Department of Chemistry as an undergraduate research assistant and Oklahoma State University, Department of Biochemistry and Molecular Biology as a graduate research assistant.