Final Progress Report for Research Projects Funded by Health Research Grants

Instructions: Please complete all of the items as instructed. Do not delete instructions. Do not leave any items blank; responses must be provided for all items. If your response to an item is "None", please specify "None" as your response. "Not applicable" is not an acceptable response for any of the items. There is no limit to the length of your response to any question. Responses should be single-spaced, no smaller than 12-point type. The report **must be completed using MS Word**. Submitted reports must be Word documents; they should not be converted to pdf format. Questions? Contact Health Research Program staff at 717-783-2548.

1. **Grantee Institution:** Duquesne University

2. **Reporting Period (start and end date of grant award period):** 01/01/2009 – 06/30/2012

3. **Grant Contact Person (First Name, M.I., Last Name, Degrees):** Julie H. Christy, B.S.

4. **Grant Contact Person's Telephone Number:** 412-396-1886

5. **Grant SAP Number:** 4100047633

6. **Project Number and Title of Research Project:** 1 - Investigation of the Hepatitis C Virus 3'-Untranslated Region, as a Potential Target for New Antiviral Nucleic-acid Based Strategies

7. **Start and End Date of Research Project:** 01/01/2009- 06/30/2012

8. **Name of Principal Investigator for the Research Project**: Mihaela Rita Mihailescu, Ph.D.

9. **Research Project Expenses**.

   9(A) Please provide the amount of health research grant funds spent on this project for the entire duration of the grant, including any interest earned that was spent:

   $ 47,118.96

   9(B) Provide the last names (include first initial if multiple individuals with the same last name are listed) of **all** persons who worked on this research project and were supported with health research funds. Include position titles (Principal Investigator, Graduate Assistant, Post-doctoral Fellow, etc.), percent of effort on project and total health research funds expended for the position. For multiple year projects, if percent of effort varied from year to year, report in the % of Effort column the effort by year 1, 2, 3, etc. of the project (x% Yr 1; z% Yr 2-3).

Last Name	Position Title	% of Effort on Project	Cost
Mihailescu	P.I.	11%	$ 2,600
Shetty	Graduate Assistant	50%	$ 10,400

9(C) Provide the names of **all** persons who worked on this research project, but who *were not* supported with health research funds. Include position titles (Research Assistant, Administrative Assistant, etc.) and percent of effort on project. For multiple year projects, if percent of effort varied from year to year, report in the % of Effort column the effort by year 1, 2, 3, etc. of the project (x% Yr 1; z% Yr 2-3).

Last Name	Position Title	% of Effort on Project
Shetty	Graduate Assistant	50%
Henessey	Undergraduate Researcher	20%

9(D) Provide a list of **all** scientific equipment purchased as part of this research grant, a short description of the value (benefit) derived by the institution from this equipment, and the cost of the equipment.

Type of Scientific Equipment	Value Derived	Cost
None		

10. Co-funding of Research Project during Health Research Grant Award Period. Did this research project receive funding from any other source during the project period when it was supported by the health research grant?

Yes_____ No__x_____

If yes, please indicate the source and amount of other funds:

11. Leveraging of Additional Funds

11(A) As a result of the health research funds provided for this research project, were you able to apply for and/or obtain funding from other sources to continue or expand the research?

Yes___x_____ No_____

If yes, please list the applications submitted (column A), the funding agency (National Institutes of Health—NIH, or other source in column B), the month and year when the application was submitted (column C), and the amount of funds requested (column D). If you have received a notice that the grant will be funded, please indicate the amount of funds to be awarded (column E). If the grant was not funded, insert "not funded" in column E.

Do not include funding from your own institution or from CURE (tobacco settlement funds). Do not include grants submitted prior to the start date of the grant as shown in Question 2. If you list grants submitted within 1-6 months of the start date of this grant, add a statement below the table indicating how the data/results from this project were used to secure that grant.

A. Title of research project on grant application	B. Funding agency (check those that apply)	C. Month and Year Submitted	D. Amount of funds requested:	E. Amount of funds to be awarded:
Backbone Modified Peptide-nucleic Acids as Antiviral Agents Against Hepatitis C (RUTH L. KIRSCHSTEIN NATIONAL RESEARCH SERVICE AWARDS (NRSA) FOR INDIVIDUAL SENIOR FELLOWS)	☒ NIH ☐ Other federal (specify:_____) ☐ Nonfederal source (specify:_)	04/06/2011	$ 59,918	$ 62,030
Development of chemically modified peptide nucleic acids with antiviral activity	☒NIH ☐ Other federal (specify:____) ☐ Nonfederal source (specify:_)	07/13/2012	$ 1,738,375	Pending
CAREER: Investigation of the Hepatitis C Virus 3'-Untranslated Region: a Molecular Switch Mediated by Kissing Interactions?	☐NIH ☒ Other federal (specify:__NSF_) ☐ Nonfederal source (specify:_)	07/21/09	$ 596,018	Not funded

11(B) Are you planning to apply for additional funding in the future to continue or expand the research?

Yes_x_____ No_____

If yes, please describe your plans:

As indicated above I have applied for an NIH grant (1R21AI105940-01) on which I am the PI, in collaboration with Dr. Danith Ly at Carnegie Mellon University, and Drs. Yan Yang and Lishan Su at the University of North Carolina at Chapel Hill. The work on this grant will target specifically the Hepatitis B virus, however, the preliminary results used in this grant application as proof of concept were the results obtained during the study funded by the CURE grant. In the near future the same team of researchers will apply for an R01 grant at

the NIH with the focus of continuing the work with peptide nucleic acids with antiviral activity against the Hepatitis C virus.

12. Future of Research Project. What are the future plans for this research project?

This work will continue in collaboration with Dr. Danith Ly (peptide-nucleic acids synthesis) and Drs. Yan Yang, Lishan Su and Stanley Lemon at the University of North Carolina at Chapel Hill (in vivo cell culture and animal model testing), with the ultimate goal to develop the peptide-nucleic acids into viable therapeutics against hepatitis C virus.

13. New Investigator Training and Development. Did students participate in project supported internships or graduate or post-graduate training for at least one semester or one summer?

Yes____x_____ No_____

If yes, how many students? Please specify in the tables below:

	Undergraduate	Masters	Pre-doc	Post-doc
Male				
Female	1		1	
Unknown				
Total	**1**		**1**	

	Undergraduate	Masters	Pre-doc	Post-doc
Hispanic				
Non-Hispanic	1		1	
Unknown				
Total	**1**		**1**	

	Undergraduate	Masters	Pre-doc	Post-doc
White	1		1	
Black				
Asian				
Other				
Unknown				
Total	**1**		**1**	

14. Recruitment of Out-of–State Researchers. Did you bring researchers into Pennsylvania to carry out this research project?

Yes_____ No___x_____

If yes, please list the name and degree of each researcher and his/her previous affiliation:

15. Impact on Research Capacity and Quality. Did the health research project enhance the quality and/or capacity of research at your institution?

Yes_____ No____x_____

If yes, describe how improvements in infrastructure, the addition of new investigators, and other resources have led to more and better research.

16. Collaboration, business and community involvement.

16(A) Did the health research funds lead to collaboration with research partners outside of your institution (e.g., entire university, entire hospital system)?

Yes___x_____ No_____

If yes, please describe the collaborations:

As described above, I am currently collaborating on research projects stemming from the current grant with Dr. Danith Ly at Carnegie Mellon University and with Drs. Drs. Yan Yang, Lishan Su and Stanley Lemon at the University of North Carolina at Chapel Hill.

16(B) Did the research project result in commercial development of any research products?

Yes_____ No_____x_____

If yes, please describe commercial development activities that resulted from the research project:

16(C) Did the research lead to new involvement with the community?

Yes_____ No____x_____

If yes, please describe involvement with community groups that resulted from the research project:

17. Progress in Achieving Research Goals, Objectives and Aims.
List the project goals, objectives and specific aims (as contained in the grant application's strategic plan). Summarize the progress made in achieving these goals, objectives and aims <u>for the period that the project was funded (i.e., from project start date through end date).</u> Indicate whether or not each goal/objective/aim was achieved; if something was not

achieved, note the reasons why. Describe the methods used. If changes were made to the research goals/objectives/aims, methods, design or timeline since the original grant application was submitted, please describe the changes. Provide detailed results of the project. Include evidence of the data that was generated and analyzed, and provide tables, graphs, and figures of the data. List published abstracts, poster presentations and scientific meeting presentations at the end of the summary of progress; peer-reviewed publications should be listed under item 20.

This response should be a DETAILED report of the methods and findings. It is not sufficient to state that the work was completed. Insufficient information may result in an unfavorable performance review, which may jeopardize future funding. If research findings are pending publication you must still include enough detail for the expert peer reviewers to evaluate the progress during the course of the project.

Health research grants funded under the Tobacco Settlement Act will be evaluated via a performance review by an expert panel of researchers and clinicians who will assess project work using this Final Progress Report, all project Annual Reports and the project's strategic plan. After the final performance review of each project is complete, approximately 12-16 months after the end of the grant, this Final Progress Report, as well as the Final Performance Review Report containing the comments of the expert review panel, and the grantee's written response to the Final Performance Review Report, will be posted on the CURE Web site.

There is no limit to the length of your response. Responses must be single-spaced below, no smaller than 12-point type. If you cut and paste text from a publication, be sure symbols print properly, e.g., the Greek symbol for alpha (α) and beta (ß) should not print as boxes (□) and include the appropriate citation(s). DO NOT DELETE THESE INSTRUCTIONS.

Project Overview

Hepatitis C virus (HCV) infection poses a major health problem worldwide, with more than 170 million people being chronically infected. The HCV infection is the most common reason for liver transplantation in the United States; and HCV, together with the hepatitis B virus, is currently responsible for 75% of all cases of liver disease worldwide. No HCV protective vaccine is available to date, making it imperative for new therapeutic strategies to be developed. HCV is a small, enveloped virus, whose genome is encoded by a positive strand RNA molecule, which is involved in three essential processes: replication, translation into the viral proteins and packaging into new virions. The RNA genome has an open reading frame and highly conserved 5'- and 3'-untranslated regions (UTRs), which are essential for the virus replication and/or translation. Within the 3'-UTR there is a 98 nucleotide (nt) fragment named X RNA, which is absolutely required for HCV replication. The first 55 nucleotides of X RNA, which are 100% conserved among all HCV strains, contain a 7 nucleotide sequence that has been proposed to be involved in long-range kissing interactions with a novel cis-acting RNA element located within the viral RNA polymerase (NS5B) coding sequence. The biological significance of these interactions has been established by showing that mutations that prevent their formation lead to a complete block of the viral replication. This project has the following goals:

<u>Specific aim 1.</u> *Molecular characterization of the proposed long-range kissing interactions between the HCV 3'-UTR highly conserved X RNA region and the NS5B coding region.* Specifically, we will obtain quantitative information about the interactions of the first 55 nt of X RNA with the cis-acting RNA element located in the NS5B coding region by fluorescence spectroscopy. We will also employ NMR spectroscopy to obtain higher resolution structural information about these interactions.

<u>Specific aim 2.</u> *Screening for nucleic acid based aptamers that block the formation of the long range kissing complex between the HCV 3'-UTR highly conserved X RNA region and the NS5B coding region, preventing the HCV replication.* We will design peptide-nucleic acid (PNA) aptamers that will be tested using the fluorescence spectroscopy assays developed under specific aim 1 for the potential to inhibit HCV RNA replication, by inhibiting the interactions between X RNA and the NS5B coding region.

Final Report

Materials and Methods

RNA samples

A series of RNA oligonucleotides (wild type X55 RNA (55 nt), IIId RNA (37 nt), and Alt (31 nt), truncated 5BSL32TRUNC (38 nt) and 5BSL3.2_short (24 nt)) were transcribed from synthetic DNA templates using T7 RNA polymerase *in vitro* transcription reactions. The RNAs were purified by 10% polyacrylamide, 8 M urea gel electrophoresis, recovered by electrophoretic elution, and dialyzed against either 1 mM cacodylic acid, pH 6.5. The wild type 48 nt 5BSL3.2 RNA was chemically synthesized from Dharmacon, Inc since the *in vitro* transcription of 5BSL3.2 required insertion of extra G's on the 5'end of 5BSL3.2 RNA and this altered its

folding. Unless otherwise specified, all RNA samples were annealed prior to use by heating at 95°C for 5 minutes, followed by snap-cooling on ice.

HCV core peptide

The first two basic domains of the HCV core protein, comprising amino acid residues 2 – 23 and 38 -74, were combined to form a 58 amino acid sequence core peptide, which was named the "2BD core" peptide. The core 2BD peptide was chemically synthesized and purified by the Peptide Synthesis Unit at the University of Pittsburgh, Center for Biotechnology and Bioengineering. The peptide was reconstituted in 1 mM cacodylic acid, pH 6.5 at a final concentration of 618 μM.

Native gel electrophoresis

The RNA samples were diluted from stock solutions to 0.05-20 μM concentrations, and following boiling and snap-cooling, they were incubated in the presence of 0.2 -10 mM $MgCl_2$ either at 22°C (temperature at which a kissing complex conformation is maintained) or at 55°C (temperature which promotes the formation of the duplex conformation) for 2.5 hours. The samples were electrophoresed on 10-15% native gels in either TBE (Tris Borate EDTA) buffer or TBM (Tris Borate Magnesium) buffer at 4ºC.

To study the effect of the 2BD core peptide on the magnesium treated IIId-5BSL3.2 complex samples, the peptide was directly added to the RNA, followed by incubation for 30 minutes at room temperature. These samples were subsequently treated with proteinase K for peptide digestion (concentration of 190 μg/ml) for 1 h prior to being run on native gels. The native gels were run at 4ºC, 40-100 V for 1.5 - 10 hours and were visualized by UV shadowing at 254 nm using an AlphaImager HP (AlphaInnotech, Inc) or by using SYBr Gold stain for gels containing nanomolar concentrations of RNA.

Fluorescence spectroscopy

Experiments were performed on a J.Y. Horiba Fluoromax-3 fluorimeter equipped with variable temperature control. The excitation wavelength was set at 310 nm, and the emission spectra were recorded from 330-450 nm. All experiments were performed at 22°C.

X55-5BSL3.2 RNA heterodimerization. All RNA constructs used in the fluorescence assays were numbered serially from 5' to 3' beginning with 1; however the true position in reference to the full-length HCV genome is given in parentheses. For the fluorescence spectroscopy experiments, 2-aminopurine (2-AP), an adenine fluorophore analog, was used to monitor the interactions between X55 RNA and 5BSL3.2 RNA. 5BSL3.2 RNA was 2-AP labeled at position 21 (nucleotide 9283 in full length genome), forming 5BSL3.2_21AP that was chemically synthesized and purified by Dharmacon, Inc. The dissociation constant of the kissing complex formed by 5BSL3.2_21AP and wild type X55 RNA, was determined by titrating a fixed concentration of the fluorescent 5BSL3.2_21AP (76 nM) in 1 mM cacodylic acid pH 6.5 containing 5 mM $MgCl_2$ with increasing concentrations of wild type X55. The kissing complex dissociation constant was determined by fitting the binding curve to equation 1:

$$F = 1 + \left(\frac{I_B}{I_F} - 1\right) \cdot \frac{(K_d + [5BSL3.2_21AP]_t + [X55]_t) - \sqrt{(K_d + [5BSL3.2_21AP]_t + [X55]_t)^2 - 4 \cdot [5BSL3.2_21AP]_t \cdot [X55]_t}}{2 \cdot [5BSL3.2_21AP]_t} \quad (1)$$

in which I_B and I_F are the steady state fluorescence intensities of the free and bound 5BSL3.2_21AP, $[5BSL3.2_21AP]_t$ is the total concentration of 5BSL3.2_21AP RNA and $[X55]_t$ is the total concentration of X55 RNA.

5BSL3.2-IIId or 5BSL3.2-Alt heterodimerization: The interactions of 5BSL3.2 RNA with IIId or Alt were monitored by fluorescence spectroscopy by using a 5BSL3.2 RNA labeled at position 35 (nucleotide 9297 in full length genome) by 6-methyl isoxanthopterin (6MI), a guanine fluorophore analog. The labeled 5BSL3.2_35_6MI was chemically synthesized by Fidelity Systems, Inc.. The dissociation constants of the kissing complexes formed by 5BSL3.2_35_6MI with wild type IIId or with Alt RNA, were determined by titrating a fixed concentration of the fluorescent 5BSL3.2_35_6MI (100 nM) in 1 mM cacodylic acid pH 6.5 containing 5 mM $MgCl_2$ with increasing concentrations of either wild type IIId or Alt. The kissing complex dissociation constant was determined by fitting the binding curves to an equation similar to equation (1) in which, $[5BSL3.2_21AP]_t$ is replaced by $[5BSL3.2_35_6MI]_t$, which is the total concentration of 5BSL3.2_35_6MI RNA, and $[X55]_t$ is replaced by either $[IIId]_t$ *or* $[Alt]_t$ which are the total concentration of IIId or Alt RNA, respectively.

Results

<u>Specific aim 1.</u> *Molecular characterization of the proposed long-range kissing interactions between the HCV 3'-UTR highly conserved X RNA region and the NS5B coding region.*

We have previously shown that the first 55 nts of X RNA, henceforth named X55, have similar interactions when isolated, or in the context of the entire 98 nt X RNA. Thus, in all further studies we have used X55 RNA as a model system for X RNA.

During the timeline of this grant there were new reports emerging in the literature indicating that the conserved segment located within the NS5B coding region (named 5BSL3.2) interacts with two other highly conserved regions from the HCV genome, namely a stem-loop fragment named IIId located in the HCV 5'-UTR, and a non-structured fragment located in the coding region, which we named Alt. Given the biological relevance of these proposed interactions for the HCV life cycle, we aimed to characterize them at the molecular level, by using native gel electrophoresis and fluorescence spectroscopy. Thus, the first specific aim has been modified to include the characterization of these interactions, in addition to those between X55 RNA and 5BSL3.2 RNA.

Given that X RNA can interact with another HCV genome, as well as with the 5BSL3.2, it is likely that it exists in more than one monomeric conformation, and this is indeed predicted when this RNA is folded with the Mfold secondary structure prediction software (figure 1). Therefore, to investigate the possible conformations adopted by X55 RNA, we analyzed it in higher percentage gels containing varying $MgCl_2$ concentrations in the range 1 mM to 10 mM. As seen in figure 2A, the monomeric form of X55 is resolved into 2 bands at lower concentrations of 1 and 5 mM $MgCl_2$, which collapse into a single wider band at 10 mM $MgCl_2$. Present also on these gels is the dimer band corresponding to the X55-X55 kissing complex intermediate (110 nt). In figure 2A (extreme right panel) it is also shown that in the absence of $MgCl_2$ in a native TBE gel, X55 exists in a single monomeric conformation. Note that the kissing dimer band is

absent in the TBE gel (figure 2A, extreme right panel), as kissing complexes require magnesium ions for stabilization and these ions are chelated in the TBE gel. As far as we know, this is the first time when the existence of two monomeric conformations of X55 RNA has been demonstrated experimentally. Based on these experiments we cannot determine unambiguously which of the two predicted conformations of the first 55 nt of X RNA (labeled X RNA-1 and X RNA-2 in figure 1) corresponds to the two monomer bands labeled as conformation 1 and conformation 2 in figure 2A. Nonetheless, it can be observed that as the $MgCl_2$ concentration increases, the intensity of the band corresponding to X55 conformation 2 decreases, with the concomitant increase in the intensity of the band corresponding to the X55-X55 homodimer (figure 2A). This suggests that the X55 monomer in conformation 2 is engaging in the X55-X55 homodimerization, corresponding most likely to the X RNA-2 structure depicted in figure 1, right.

Next we inquired if X RNA interacts with 5BSL3.2 via the formation of a kissing complex, and given that X RNA uses the same stretch of nucleotides to form a homo dimeric kissing complex with another X RNA, we set to measure its affinities for these two interactions. To analyze at the molecular level the proposed kissing interactions between 5BSL3.2 and X RNA we performed native gel electrophoresis experiments in which we used the full-length 5BSL3.2 (48 nt, spanning the sequence 9263 - 9310 in the HCV genome) and X55 RNA. Since the formation of RNA kissing complexes requires Mg^{2+} ions, we performed TBM native gel electrophoresis in the presence of various concentrations of magnesium. As shown in figure 2B, at all $MgCl_2$ concentrations investigated there is a single band for 5BSL3.2 (lane 1), whereas, as expected, X55 (lane 2) shows two lower monomeric bands (well separated at 1 mM and 5 mM $MgCl_2$) and a top band corresponding to the homodimeric X55-X55 kissing complex (110 nts). When 5BSL3.2 and X55 are mixed in equimolar concentrations (figure 2B, lane 3), in addition to the X55-X55 homodimer band (110 nt), there is a new more intense upper band that we assign to the X55-5BSL3.2 complex (103 nt). It is interesting to note that upon the formation of the X55-5BSL3.2 complex, the lower band corresponding to the monomeric X55 conformation 1 disappeared in as low as 1 mM $MgCl_2$, whereas the band corresponding to the monomeric X55 conformation 2 disappeared only in 10 mM $MgCl_2$. This result suggests that the X55 monomer in conformation 1 engages in interactions with 5BSL3.2, in agreement with our previous observation that the X55 monomer in conformation 2 engages in interactions with another X55 RNA. Although X55 was mixed in an equimolar ratio with 5BSL3.2, also present in lane 3 is a lower intensity 5BSL3.2 monomer band (48 nt; compare lanes 1 and 3), as not all of the X55 RNA is available for binding 5BSL3.2, being involved in the formation of the X55-X55 homodimer. These results clearly indicate that the X55-X55 homo dimer coexists with the 5BSL3.2-X55 complex and moreover, that the formation of the 5BSL3.2-X55 RNA hetero dimer does not require the presence of a chaperoning protein. To determine if the X55-5BSL3.2 dimer is indeed a kissing complex, we analyzed whether its formation is magnesium dependent. Since the monomeric conformations of X55 RNA can be distinctly observed only in $MgCl_2$ concentrations less than 10 mM, all further experiments analyzing the 5BSL3.2-X55 interactions were carried out in the presence of 5 mM $MgCl_2$. Two identical sets of RNA samples were prepared in the presence of 5 mM $MgCl_2$, one being analyzed on a native TBM gel containing 5 mM $MgCl_2$ (figure 3B), and the second on a native TBE gel, containing EDTA (figure 3A).

As shown in figures 3A and B, 5BSL3.2 RNA migrates as a monomer in both the TBE and TBM gel, respectively (lane 1). As discussed above, three bands are present for X55 RNA in the TBM gel, corresponding to its two monomeric conformations 1 and 2 and to the homo dimeric kissing complex (figure 3B, lane 2), all of which collapse into a single monomeric band in the TBE gel (figure 3A, lane 2). The equimolar mixture of 5BSL3.2 and X55 shows the two higher molecular weight bands corresponding to the X55-X55 homo dimer and to the X55-5BSL3.2 dimer in the TBM gel (figure 3B, lane 3). However, in a TBE gel (figure 3A, lane 3) these higher molecular weight bands disappear, the only bands present corresponding to the monomeric 5BSL3.2 and to the monomeric X55. This result indicates unambiguously that both, the X55-X55 homo dimer and the X55-5BSL3.2 hetero dimer are magnesium dependent kissing complexes.

Our earlier results indicate that the metastable magnesium dependent kissing X55 homo dimer complex gets converted to the more stable magnesium independent X55 duplex in the presence of the core protein. Given the ability of X55 RNA to also form a kissing complex with 5BSL3.2, we inquired if these interactions are affected by the presence of the core protein. As seen in figures 3A and 3B (lane 4), in both, TBM and TBE gels in the presence of the core protein, the X55-5BSL3.2 kissing complex band is absent, whereas the bands corresponding to the duplex X55-X55 homo dimer and to the monomer 5BSL3.2 become more intense (compare lanes 3 and 5 in figure 3B). Thus, the equilibrium between the X55-X55 and 5BSL3.2-X55 kissing complexes existent in the absence of the core protein, is completely shifted towards the more stable X55-X55 homodimer duplex in the presence of the core protein, resulting in the release of free monomeric 5BSL3.2. These results strongly suggest that X55 could function as a molecular switch in the HCV life cycle, wherein in the absence of the core protein the replication favourable interaction between 5BSL3.2-X55 is preferred, whereas in the presence of the core protein, X55 forms the more stable X55-X55 duplex and the replication favorable X55-5BSL3.2 heterodimer interaction is disrupted.

To obtain quantitative information about the kissing complex formed by X55 RNA and 5BSL3.2 RNAs, we used fluorescence spectroscopy. In this assay, the highly fluorescent purine analog 2-aminopurine (2AP) replaced the adenine at position 21 in the apical loop of the 5BSL3.2 sequence UC**A**CAGC (position 9283 in the full length HCV genome). Nanomolar increments of wild type X55 RNA were titrated into a fixed concentration of the 2AP-labeled 5BSL3.2, resulting in a decrease in the steady-state 2-AP fluorescence emission at 371 nm (figure 3C), due to the base stacking interactions arising when the 2AP in 5BSL3.2 (position 9283) base pairs with the uracil at position 9544 in the unlabeled X55 RNA. A dissociation constant of (42±6 nM) was determined for the kissing complex formed by X55 and 5BSL3.2 by fitting the binding curve illustrated in figure 3C with equation 1 (materials and methods). The X55-5BSL3.2 kissing complex has a much lower dissociation constant (Kd = 42 ± 6 nM) than that of the X55-X55 homo dimeric kissing complex (Kd = 318 ± 58) reported in our earlier study. This result is consistent with our native gel electrophoresis results, which showed a more intense band for the X55-5BSL3.2 kissing complex versus the X55-X55 homo dimer kissing complex (figure 3B, lane 3). To confirm the specificity of the fluorescence spectroscopy assay, in a separate control experiment we titrated the 2AP labeled 5BSL3.2 with the 37 nt RNA IIId derived from the HCV IRES (figure 3C, red points). IIId has complementarity for the internal loop of 5BSL3.2, but not for its apical loop where the 2AP reporter is located. As expected, no significant quenching was observed for the 2-AP steady-state fluorescence. Although genetic evidence has been obtained

for X55 – 5BSL3.2 interaction, our results provide first time direct experimental evidence and quantitative information about this interaction *in vitro* in the absence of any chaperoning protein.

Taken together these results indicate that X RNA exists in more than conformation and can engage in both, X55-X55 homo dimer interactions, as well as in X55-5BSL3.2 kissing complex interactions.

Once we characterized the interactions involving the apical loop of 5BSL3.2, we performed native gel electrophoresis experiments to analyze the proposed interactions engaging its highly conserved GCCCG internal loop motif and the IIId domain and Alt sequence, respectively. For this purpose, we have performed TBM gels in the presence of as low as 0.5 mM Mg^{2+} ions, and TBE gels in which these ions are chelated by EDTA. Figures 4A and B show the interactions between full-length 5BSL3.2 and IIId in a TBE and TBM gel respectively. As expected, both IIId (37 nt) and 5BSL3.2 (48 nt) individually migrate as monomers (figure 4A and 4B, lanes 1 and 2). When mixed in a 1:1 ratio, a higher band appears in the TBM gel (figure 4B, lane 3), corresponding to the 5BSL3.2-IIId complex, but this band is absent in the TBE gel in which the Mg^{2+} ions are chelated by EDTA (figure 4A, lane 3). To test whether the metastable magnesium dependent 5BSL3.2-IIId kissing complex gets converted to a stable duplex in the presence of a chaperoning protein we treated the 5BSL3.2-IIId kissing complex (in the presence of magnesium) with the HCV chaperoning core peptide and analyzed the peptide-treated 5BSL3.2-IIId mixture in both TBM and TBE gels (figures 4A and B, lane 4). The kissing complex conformation cannot be distinguished from the duplex conformation on a TBM gel, as they migrate similarly due to their identical size. However, in a TBE gel, the 5BSL3.2-IIId kissing complex will dissociate into monomers due to its dependence on Mg^{2+}, but the duplex will remain in the dimeric conformation, as it is Mg^{2+} independent. As seen in figure 4B, lane 4, the core treated 5BSL3.2-IIId kissing complex dissociates into monomers in the absence of Mg^{2+}, indicating that the kissing complex does not get converted to the stable duplex conformation.

In a similar manner using TBM and TBE native gel electrophoresis, we analyzed the proposed interactions between 5BSL3.2 and Alt, the sequence located 200 nt upstream from it. The Alt sequence used in this study is 31 nt long and contains the invariant sequence CGGGC, proposed to bind to the GCCCG internal loop of 5BSL3.2. In a TBM gel, (figure 5A, lanes 1 and 2) both Alt and 5BSL3.2 RNAs migrate as monomers, whereas when mixed in equimolar ratios, a higher band corresponding to the 5BSL3.2-Alt complex is present (figure 5A, lane 3). However, when the 1:1 5BSL3.2-Alt complex was analyzed in a TBE gel (figure 5B, lane 3), it dissociated into its respective monomers, indicating that the 5BSL3.2-Alt complex is magnesium dependent, being thus, a kissing complex.

In summary, we have accomplished the goal originally set up under this specific aim to characterize the interactions of X RNA with the 5BSL3.2 RNA, as well as the new goals emerging during the duration of the grant, to characterize the interactions of 5BSL3.2 with the Alt and IIId regions of the HCV RNA. Since all these interactions are vital for the HCV life cycle, the results of this study make now possible for the design and in vitro testing of peptide-nucleic acids with antiviral activity against HCV, which aim to disrupt these interactions.

Specific aim 2. Screening for nucleic acid based aptamers that block the formation of the long range kissing complex between the HCV 3'-UTR highly conserved X RNA region and the NS5B coding region, preventing the HCV replication.

In this study we investigated a PNA that was designed against the HCV X RNA sequence 9539 – 9548, that has been shown to be involved in *in vitro* RNA homodimerization and also in interactions with the 5BSL3.2 apical loop in the coding region. Given this dual interaction of X RNA, a 10 base PNA was designed against X RNA, such that both the homo- and hetero-interactions of X55 RNA with another X55 or with 5BSL3.2 would be disrupted. This PNA was synthesized in the laboratory of Dr. Danith Ly of Carnegie Mellon University. Since this was a first study to investigate the interactions of the PNA with X55 RNA, several experimental conditions such as PNA specificity, temperature, PNA concentration and incubation time were analyzed.

i. PNA Specificity: Initially we analyzed the specificity of the PNA developed against X55 RNA. All RNA samples were pretreated with 5 mM $MgCl_2$ and their interactions with the PNA were analyzed by TBM gel electrophoresis (figure 6). Lanes 1, 2 and 3 in figure 6 contain free 5BSL3.2 RNA, free X55 RNA, and their 1:1 mixture, whereas lanes 4-6 contain identical samples which were incubated for 2 hours at 37°C with 2-fold PNA. When 5BSL3.2 was incubated with PNA (lane 5) a single band corresponding to the monomeric 5BSL3.2 (compare with lane 1) was observed in the gel, indicating the absence of any reactivity of 5BSL3.2 RNA with PNA. However, when the PNA was added to its target X55 RNA (lane 6), the monomer and dimer bands of X55 RNA disappeared, with the concomitant appearance of three new bands (compare with lane 2), which were labeled as Band A (lowermost), Band B (middle) and Band C (topmost). Our results that the PNA binds to X55, but not to 5BSL3.2 indicate that the PNA is specific for X55 RNA. The origin of the bands A, B, and C is not clear, initially we assumed that bands A and B correspond to the PNA bound to the two monomeric conformations of X55 RNA, whereas band C corresponds to a dimer of X55 bound by PNA. However, this hypothesis was not confirmed experimentally, as these bands are also present in the absence of $MgCl_2$, when X55 exists in a single monomeric conformation (data not shown). Further studies as needed to elucidate the origin of the A, B and C bands.

ii. Concentration Dependence: To narrow down the effective required PNA concentration, we treated the target X55 RNA with increasing concentrations of PNA (0.25-fold, 0.5-fold and 1-fold PNA). As seen in figure 7, a single band is present in lane 1 which contains free 5BSL3.2, two monomeric and one dimer band are present in lane 2 which contains free X55 RNA. As expected, both the X55-X55 homodimer and the X55-5BSL3.2 heterodimer bands are present in lane 3, as well as some unreacted free 5BSL3.2.

As a 0.25-fold PNA is added (figure 7, lane 4), both the monomeric X55 bands and the X55 homodimer band disappear with the concomitant apparition of bands A, B and a faint band C due to the interactions of X55 with PNA. The X55-5BSL3.2 heterodimer band though weaker than in lane 3, is still present in lane 4. In the presence of 0.5-fold PNA (lane 5), the X55-5BSL3.2 heterodimer band gets weaker while the bands A and B become more intense. Finally, upon the addition of 1-fold PNA (lane 6), both the X55 homodimer and the heterodimer bands disappear, while the monomeric 5BSL3.2 band and the bands A, B and C caused due to X55-PNA

interactions are more intense. These results clearly indicate that at 37°C, the treatment of X55 RNA with 1-fold PNA for 2 hours is effective in disrupting both, the X55-X55 homodimer, as well as the X55-5BSL3.2 heterodimer interaction.

Similar experiments were performed to determine the optimum temperature and time of incubation, as well as PNA concentration (data not shown). Based on these results we can definitely confirm that the PNA we designed is highly specific to its target X55 RNA at concentrations up to 8 fold to target RNA. The efficiency of PNA appears to be similar at both 21°C and 37°C, with as little as 1-fold PNA being efficient in completely disrupting both the homo- and hetero-interactions of X55 in a time period of an hour. In addition, even at lower than 1 fold concentrations, the PNA affected the homo and hetero interactions of X55.

In summary, we have accomplished the goal of specific aim 2, as the single PNA designed in this study, possesses this dual ability to disengage two different interactions of X55. Though the biological significance of X55 dimerization remains to be elucidated, the 5BSL3.2-X55 interaction has been demonstrated to be essential for replication. Thus at least one of the known biological significant interactions of X55 RNA is disrupted by the PNA designed in this study. Furthermore, even assuming that the HCV genomic dimerization is not of biological importance, the ability of PNA to stabilize the X55 monomer and homodimer might make it functionally unavailable for other essential interactions within the HCV genome. Thus, this PNA is promising as an effective therapeutic agent, and currently we are exploring its antiviral activity against HCV in cell-culture systems, through our research collaboration with Drs. Yan Yang and Stanley Lemon at UNC at Chapel Hill.

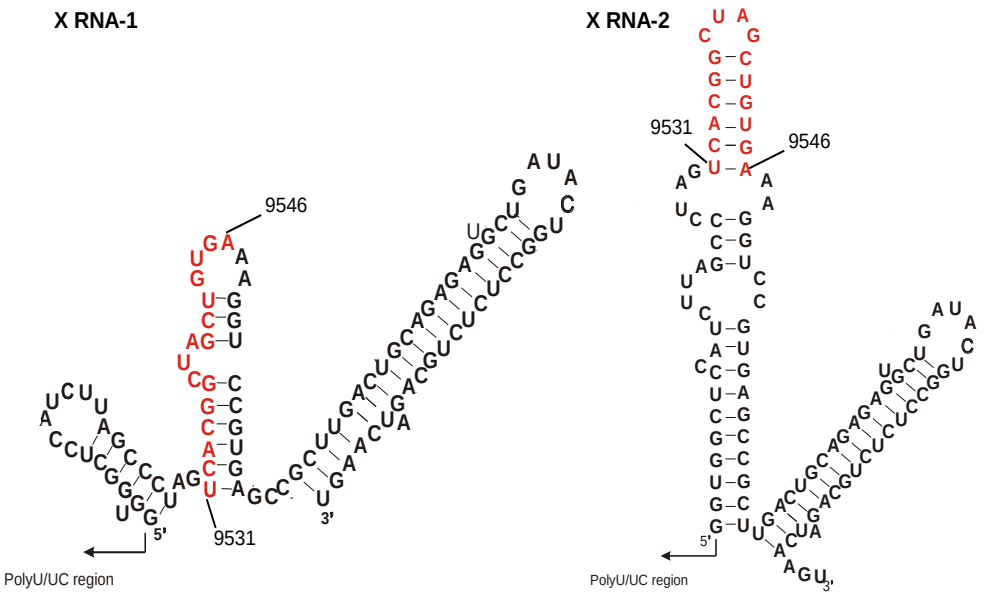

Figure 1. Alternate conformations of HCV X RNA region as predicted by the Mfold software. Shown in red is the 16 nt palindromic sequence named the dimer linkage sequence (DLS).

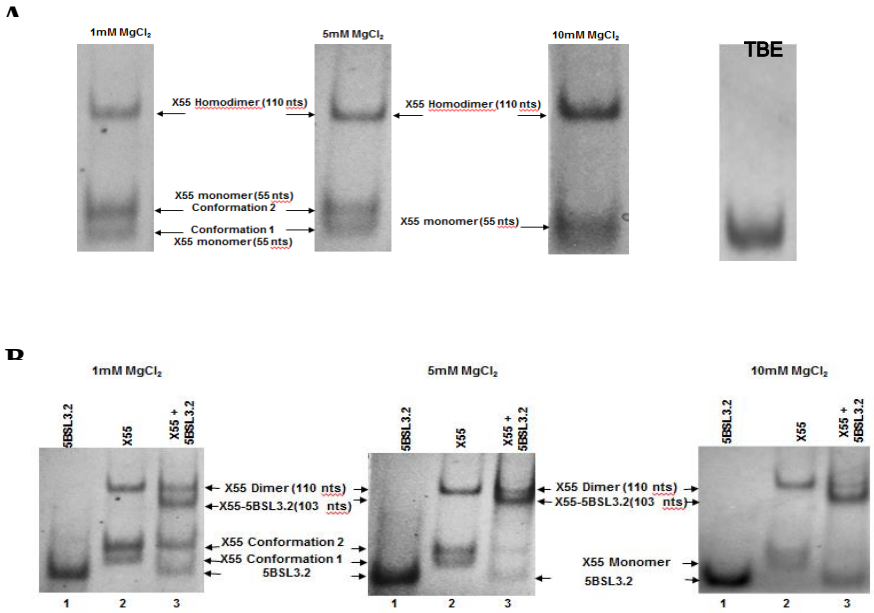

Figure 2. (A). *Left:* 12% TBM gels of X55 RNA in the presence of 1 mM, 5 mM and 10 mM $MgCl_2$. *Extreme right:* 12% TBE gel of X55 RNA. **(B).** 12% TBM gel in the presence of 1 mM, 5 mM and 10 mM $MgCl_2$. Lane 1: 5BSL3.2 RNA (48 nt); lane 2: X55 RNA (55 nt); lane 3: 5BSL3.2 and X55 RNAs in a 1:1 ratio. The gels were visualized by UV shadowing at 254 nm.

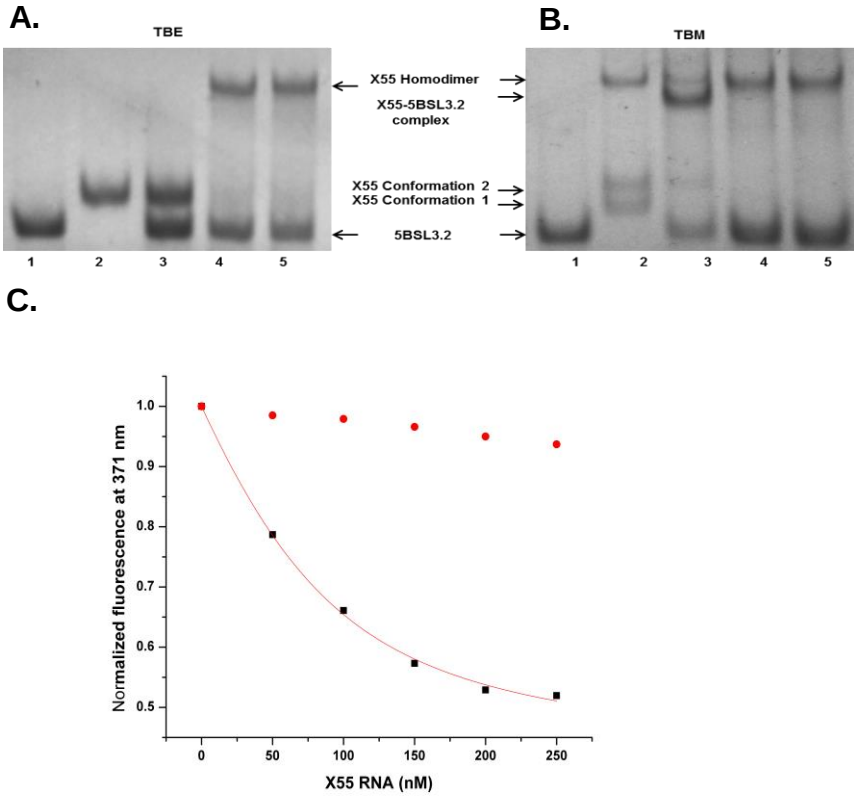

Figure 3. 12% TBE gel **(A).** and 12% TBM gel **(B).** The RNA samples (15 μM) were prepared similarly for both gels, being pre-incubated in the presence of 5 mM $MgCl_2$ at 22°C unless otherwise specified. The gels were visualized by UV shadowing at 254 nm. Lane 1: 5BSL3.2 RNA (48 nt); lane 2: X55 RNA (55 nt); lane 3: 5BSL3.2 and X55 RNAs in a 1:1 ratio; lane 4: 5BSL3.2 and X55 RNAs in a 1:1 which following incubation were treated with core 2BD and proteinase K; lane 5: 5BSL3.2 and X55 RNAs pre-incubated in the presence of 5 mM $MgCl_2$ at 55°C for 2.5 hrs. **(C).** Fluorescence spectroscopy: a solution of 76 nM 5BSL3.2_21AP in 1 mM cacodylic acid pH 6.5 containing 5 mM $MgCl_2$ was titrated with increasing concentrations of wild type X55 (black squares). The kissing complex dissociation constant was determined by fitting the binding curve to equation 1. In a control experiment, 5BSL3.2_21AP (76 nM) was titrated with IIId, which does not bind in the apical loop of 5BSL3.2 (red circles).

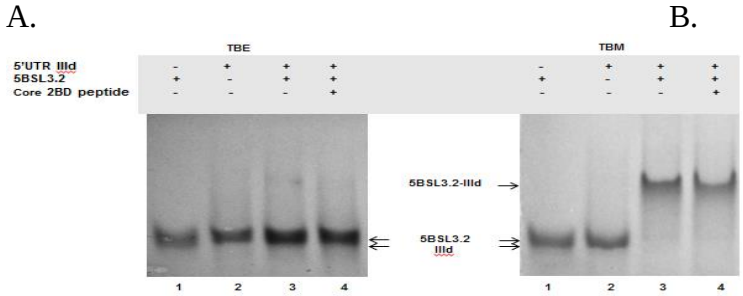

Figure 4. 12% TBE gel **(A).** and 12% TBM gel **(B).** The RNA samples (15 μM) were prepared similarly for both gels, being pre-incubated in the presence of 0.5 mM $MgCl_2$. Lane 1: IIId RNA (37 nt); Lane 2: 5BSL3.2 RNA (48 nt); Lane 3: 5BSL3.2 and IIId RNAs in a 1:1 ratio; lane 4: 5BSL3.2 and IIId RNAs in a 1:1 ratio, which following incubation were treated with core 2BD and proteinase K. The gels were visualized by UV shadowing at 254 nm.

A. B.

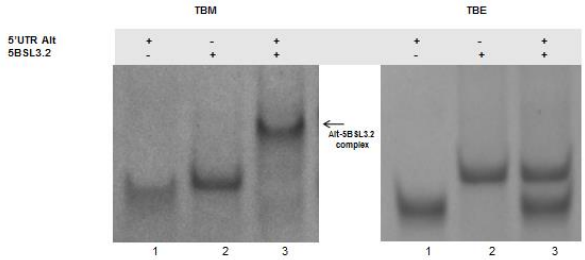

Figure 5. 12% TBM gel **(A).** and 12% TBE gel **(B).** The RNA samples (15 μM) were prepared the same for both gels, being pre-incubated in the presence of 0.5 mM $MgCl_2$. Lane 1: Alt RNA (31 nt); lane 2- 5BSL3.2 RNA (48 nt); lane 3: 5BSL3.2 and Alt RNAs in a 1:1 ratio. The gels were visualized by UV shadowing at 254 nm.

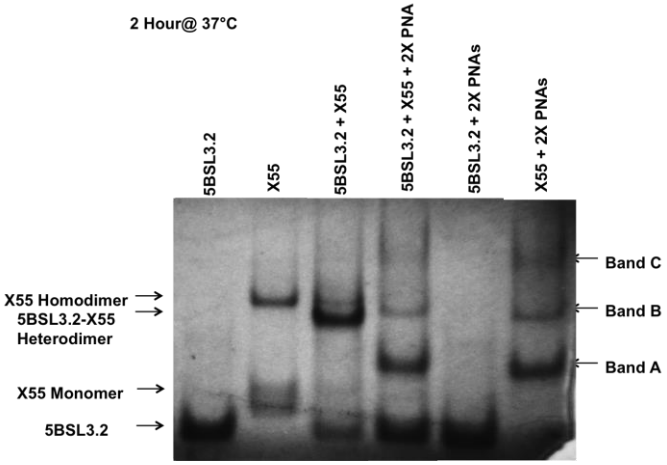

Figure 6: 12% TBM gel in the presence of 5 mM $MgCl_2$. Lane 1- 5BSL3.2 (15μM) Lane 2-X55 (15μM), Lane 3-5BSL3.2 (15μM) +X55 (15μM), Lane 4-X55 (15μM) +5BSL3.2 (15μM) +2-fold PNA (30μM) for 2 hours at 37°C), Lane 5- 5BSL3.2 (15μM) +2-fold PNA (30μM) for 2 hours at 37°C, Lane 6-X55 (15μM) +2-fold PNA (30μM) for 2 hours at 37°C.

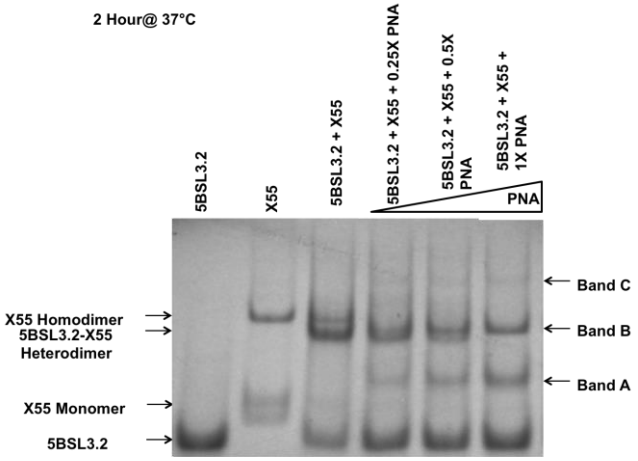

Figure 7:12% TBM gel in the presence of 5mM magnesium. All samples were pretreated with magnesium. Lane 1: 5BSL3.2, Lane 2: X55, Lane 3:5BSL3.2+X55, Lanes 4 to 6-X55+5BSL3.2+PNA (0.25 to 1 fold for 2 hours at 37°C).

Conference Proceedings

1. Shetty S. & Mihailescu M.R., Hepatitis C virus (HCV)–3'UTR: A kissing complex dependent molecular switch?, *Biophys. J.* 2010, 98(3), pp 472a.

Competitive Poster Presentations at National and International Meetings

* Indicates the presenting author.

2. *International, poster presentation*
   Mihailescu, Mihaela Rita*, Shetty, Sumangala, Ly, Danith & Armitage, Bruce, "Short peptide nucleic acids (PNA) inhibit long range kissing interactions essential for Hepatitis C virus replication", *Pacifichem* 2010, Hawaii, December 2010

3. *National, poster presentation*
   Shetty Sumangala* & Mihailescu Mihaela Rita, "HCV 3' UTR: A molecular switch?" 238th*ACS National Meeting,* Washington D.C., August 2009

18. Extent of Clinical Activities Initiated and Completed. Items 18(A) and 18(B) should be completed for all research projects. If the project was restricted to secondary analysis of clinical data or data analysis of clinical research, then responses to 18(A) and 18(B) should be "No."

18(A) Did you initiate a study that involved the testing of treatment, prevention or diagnostic procedures on human subjects?

_____Yes
__x___No

18(B) Did you complete a study that involved the testing of treatment, prevention or diagnostic procedures on human subjects?

_____Yes
__x___No

If "Yes" to either 18(A) or 18(B), items 18(C) – (F) must also be completed. (Do NOT complete 18(C-F) if 18(A) and 18(B) are both "No.")

18(C) How many hospital and health care professionals were involved in the research project?

_____Number of hospital and health care professionals involved in the research project

18(D) How many subjects were included in the study compared to targeted goals?

_____Number of subjects originally targeted to be included in the study
_____Number of subjects enrolled in the study

Note: Studies that fall dramatically short on recruitment are encouraged to provide the details of their recruitment efforts in Item 17, Progress in Achieving Research Goals, Objectives and Aims. For example, the number of eligible subjects approached, the number that refused to participate and the reasons for refusal. Without this information it is difficult to discern whether eligibility criteria were too restrictive or the study simply did not appeal to subjects.

18(E) How many subjects were enrolled in the study by gender, ethnicity and race?

Gender:
_____Males
_____Females
_____Unknown

Ethnicity:
_____Latinos or Hispanics
_____Not Latinos or Hispanics
_____Unknown

Race:
_____American Indian or Alaska Native
_____Asian
_____Blacks or African American
_____Native Hawaiian or Other Pacific Islander
_____White
_____Other, specify:_____
_____Unknown

18(F) Where was the research study conducted? (List the county where the research study was conducted. If the treatment, prevention and diagnostic tests were offered in more than one county, list all of the counties where the research study was conducted.)

19. Human Embryonic Stem Cell Research. Item 19(A) should be completed for all research projects. If the research project involved human embryonic stem cells, items 19(B) and 19(C) must also be completed.

19(A) Did this project involve, in any capacity, human embryonic stem cells?
_____Yes
___x__ No

19(B) Were these stem cell lines NIH-approved lines that were derived outside of Pennsylvania?
_____Yes
_____ No

19(C) Please describe how this project involved human embryonic stem cells:

20. Articles Submitted to Peer-Reviewed Publications.

20(A) Identify all publications that resulted from the research performed during the funding period and that have been submitted to peer-reviewed publications. Do not list journal abstracts or presentations at professional meetings; abstract and meeting presentations should be listed at the end of item 17. **Include only those publications that acknowledge the Pennsylvania Department of Health as a funding source** (as required in the grant agreement). List the title of the journal article, the authors, the name of the peer-reviewed publication, the month and year when it was submitted, and the status of publication (submitted for publication, accepted for publication or published.). Submit an electronic copy of each publication or paper submitted for publication, listed in the table, in a PDF version 5.0.5 (or greater) format, 1,200 dpi. Filenames for each publication should include the number of the research project, the last name of the PI, the number of the publication and an abbreviated research project title. For example, if you submit two publications for PI Smith for the "Cognition and MRI in Older Adults" research project (Project 1), and two publications for PI Zhang for the "Lung Cancer" research project (Project 3), the filenames should be:

Project 1 – Smith – Publication 1 – Cognition and MRI
Project 1 – Smith – Publication 2 – Cognition and MRI
Project 3 – Zhang – Publication 1 – Lung Cancer
Project 3 – Zhang – Publication 2 – Lung Cancer

If the publication is not available electronically, provide 5 paper copies of the publication.

Note: The grant agreement requires that recipients acknowledge the Pennsylvania

Department of Health funding in all publications. Please ensure that all publications listed acknowledge the Department of Health funding. If a publication does not acknowledge the funding from the Commonwealth, do not list the publication.

Title of Journal Article:	Authors:	Name of Peer-reviewed Publication:	Month and Year Submitted:	Publication Status (check appropriate box below):
1. Hepatitis C virus genomic RNA dimerization is mediated via a kissing complex intermediate	Shetty S., Kim S., Shimakami T., Lemon S.M. & Mihailescu M.R.	RNA Journal	10/2009	☐Submitted ☐Accepted ☒ Published
2. Hepatitis C Virus RNA: Molecular Switches Mediated by Long Range RNA-RNA Interactions?	Shetty S. and Mihailescu M.R.	Nucleic Acids Research	08/2012	☒ Submitted ☐Accepted ☐Published

20(B) Based on this project, are you planning to submit articles to peer-reviewed publications in the future?

Yes___x_____ No_____

If yes, please describe your plans:

After work with the peptide nucleic acids is completed, we will submit it for publication.

21. Changes in Outcome, Impact and Effectiveness Attributable to the Research Project. Describe the outcome, impact, and effectiveness of the research project by summarizing its impact on the incidence of disease, death from disease, stage of disease at time of diagnosis, or other relevant measures of outcome, impact or effectiveness of the research project. If there were no changes, insert "None"; do not use "Not applicable." Responses must be single-spaced below, and no smaller than 12-point type. DO NOT DELETE THESE INSTRUCTIONS. There is no limit to the length of your response.

None yet.

22. Major Discoveries, New Drugs, and New Approaches for Prevention Diagnosis and Treatment. Describe major discoveries, new drugs, and new approaches for prevention, diagnosis and treatment that are attributable to the completed research project. If there were no major discoveries, drugs or approaches, insert "None"; do not use "Not applicable." Responses must be single-spaced below, and no smaller than 12-point type. DO NOT DELETE THESE INSTRUCTIONS. There is no limit to the length of your response.

None yet.

23. Inventions, Patents and Commercial Development Opportunities.

23(A) Were any inventions, which may be patentable or otherwise protectable under Title 35 of the United States Code, conceived or first actually reduced to practice in the performance of work under this health research grant? Yes_____ No___x_____

If "Yes" to 23(A), complete items a – g below for each invention. (Do NOT complete items a - g if 23(A) is "No.")

a. Title of Invention:

b. Name of Inventor(s):

c. Technical Description of Invention (describe nature, purpose, operation and physical, chemical, biological or electrical characteristics of the invention):

d. Was a patent filed for the invention conceived or first actually reduced to practice in the performance of work under this health research grant?
Yes_____ No____

If yes, indicate date patent was filed:

e. Was a patent issued for the invention conceived or first actually reduced to practice in the performance of work under this health research grant?
Yes_____ No____
If yes, indicate number of patent, title and date issued:
Patent number:
Title of patent:
Date issued:

f. Were any licenses granted for the patent obtained as a result of work performed under this health research grant? Yes_____ No____

If yes, how many licenses were granted?_____

g. Were any commercial development activities taken to develop the invention into a commercial product or service for manufacture or sale? Yes___ No____

If yes, describe the commercial development activities:

23(B) Based on the results of this project, are you planning to file for any licenses or patents, or undertake any commercial development opportunities in the future?

Yes_____ No____x_____

If yes, please describe your plans:

24. Key Investigator Qualifications. Briefly describe the education, research interests and experience and professional commitments of the Principal Investigator and all other key investigators. In place of narrative you may insert the NIH biosketch form here; however, please limit each biosketch to 1-2 pages. *For Nonformula grants only – include information for only those key investigators whose biosketches were not included in the original grant application.*

BIOGRAPHICAL SKETCH

Provide the following information for the key personnel and other significant contributors in the order listed on Form Page 2.
Follow this format for each person. **DO NOT EXCEED FOUR PAGES.**

NAME	POSITION TITLE
Mihailescu, Mihaela Rita	Associate Professor of Biochemistry
eRA COMMONS USER NAME	

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	YEAR(s)	FIELD OF STUDY
University of Bucharest, Romania	B.S., M.S.	1987-1992	Physics, Biophysics
Wesleyan University, Middletown, CT	Ph.D.	1995-2001	Mol.Biol. & Biochem.
Center for Advanced Research in Biotechnology/University of Maryland Biotechnology Institute, Rockville, MD	Postdoctoral	2001-2003	Biophysical Chemistry

A. Personal Statement

One of my research interests is the development of γ-modified peptide nucleic acids (γPNAs) with antiviral activity against Hepatitis C viral (HCV) infection. To pursue these interests I have established a collaboration with Dr. Danith Ly at Carnegie Mellon University and Drs. Stanley Lemon and Yan Yang at the University of North Carolina at Chapel Hill, in which we recognized the potential of the γPNAs as therapeutic agents against DNA/RNA viruses. My expertise is in biophysical chemistry and molecular biology and biochemistry methods are employed in my lab to produce the DNA/RNA and proteins of interest and biochemical and biophysical techniques that include fluorescence, UV-Vis, circular dichroism and nuclear magnetic resonance (NMR) spectroscopy, are employed to study these biomolecules and their interactions. These methods have been applied successfully in the two major projects underway in my lab, one dealing with the HCV system, and the second with the fragile X syndrome, the most common form of inherited mental retardation.

B. Positions and Honors.

Professional positions

1987-1992 Undergraduate and Graduate (M.S.) Research Assistant (Dr. Aurel Popescu), University of Bucharest, Romania

1992-1995 Research Assistant (Dr. Sezgli Geza), National Institute of Research and Development for Microbiology and Immunology "Cantacuzino", Bucharest, Romania

1995-2001 Graduate Research Assistant (Dr. Irina M. Russu), Wesleyan University, Middletown, CT

2001-2003 Postdoctoral Fellow (Dr. John Marino), Center for Advanced Research in Biotechnology/University of Maryland Biotechnology Institute, Rockville, MD

2003-2004 Assistant Professor, Department of Chemistry, Acadia University, Wolfville, NS, Canada

2004-2010 Assistant Professor, Department of Chemistry & Biochemistry, Bayer School of Natural Environmental Sciences, Duquesne University, Pittsburgh, PA

2010- Associate Professor, Department of Chemistry & Biochemistry, Bayer School of Natural Environmental Sciences, Duquesne University, Pittsburgh, PA

Honors

1993 Fellowship at the "Biophysics of Proton and Ion Transport"- International Summer School, Brasov, Romania

2001 Peterson Fellowship (graduate award in biochemistry), Wesleyan University

2002 Competitive Travel Award to the XXth International Conference of Magnetic Resonance in Biological Systems, Toronto, Canada

2008 Teacher of the Year Award by Duquesne University's Chapter of Omicron Delta Kappa National Leadership Honor Society

2008 Bayer School of Natural and Environmental Sciences Excellence in Teaching Award, Duquesne University.

2012 Ruth L. Kirschstein National Research Service Awards, National Institute of Allergy and Infectious Diseases

C. Selected Peer-reviewed Publications.

* Undergraduate student names are bolded.

1. Evans, T.L., Blice-Baum A.C. & Mihailescu M.R. (2012) Analysis of the Fragile X Mental Retardation Protein Isoforms 1, 2 and 3 Interactions with the G-quadruplex Forming Semaphorin 3F mRNA, *Molecular BioSystems.* **8(2):** 642-9. PMID: 22134704

2. Fisette, J.F., Montagna, D.R., Mihailescu, M.R. & Wolfe, M.S. (2012) A G-Rich element forms a G-quadruplex and regulates BACE1 mRNA alternative splicing. *J. Neurochem.* **121(5)**: 763-73. PMID: 22303960

3. Shetty S., Kim S., Shimakami T., Lemon S.M. & Mihailescu M.R. (2010), Hepatitis C virus genomic RNA dimerization is mediated via a kissing complex intermediate, *RNA,* 16(5): 913-25. PMID: 20360391

4. Evans TL & Mihailescu M.R. (2010), Recombinant bacterial expression and purification of human fragile X mental retardation protein isoform 1, *Protein Expr. Purif.* 74(2):242-247.PMID: 20541608

5. **Lipay, J.M.** & Mihailescu, M.R. (2009), "NMR spectroscopy and kinetic studies of the quadruplex forming RNA r(UGGAGGU)", *Mol. BioSyst.*, 5:1347–1355. PMID 19823751.

6. **Bole, M.,** Menon, L. & Mihailescu, M. R. (2008) Fragile X Mental Retardation Protein Recognition of G Quadruplex Structure *per se* is Sufficient for High Affinity Binding to RNA, *Mol. BioSyst.* 4(12):1212-1219. PMID: 19366385.

7. Menon, L, Mader, S.A. & Mihailescu, M. R. (2008) Fragile X Mental Retardation Protein Interactions with the Microtubule Associated Protein 1B RNA. *RNA*, 14(8):1644-55. PMID: 18379868.

8. Menon L. & Mihailescu M. R. (2007). Interactions of the G quartet forming Semaphorin 3F RNA with the RGG box Domain of the fragile X protein family. *Nucleic Acids Research*, 35(16): 5379-5392. PMID: 17693432.

9. **Zanotti, K.J., Lackey, P.E., Evans, G. L.** & Mihailescu M. R (2006). Thermodynamics of the fragile X mental retardation protein RGG box interactions with G quartet forming RNA. *Biochemistry*, 45(27): 8319-8330. PMID: 16819831.

10. Mihailescu M.R. & Marino J.P. (2004). A proton-coupled dynamic conformational switch in the HIV-1 dimerization initiation site kissing complex. *Proc. Natl. Acad. Sci. USA.* 101(5):1189-94. PMID: 14734802.