The enzymatic activity of an unknown protein which cleaves the phosphodiester bond between the tyrosine residue of a viral protein and the 5' terminus of the picornavirus RNA

Introduction

Every day there are people wrestling with diseases caused by members of the picornavirus family, which includes polioviruses, hepatitis A viruses, and rhinoviruses. According to the World Health Organization, in developing countries with poor environmental hygienic conditions, nearly all children are infected with hepatitis A virus before the age of 9. In addition, poliomyelitis is still endemic in 6 countries. Even though enormous effort has been made to investigate the replication cycle of picornavirus, definitive answers still remain elusive. Among the members of the picornavirus family, the poliovirus has been best understood, thus it provides an excellent model through which to study picornavirus replication cycle.

Picornaviruses are positive-sense, single-stranded RNA viruses that code for a large polyprotein that serves as the precursor to all viral proteins. Upon entry, the virus will inhibit host cell protein synthesis to allow only the translation of picornavirus mRNA. When sufficient viral proteins are synthesized, packaged positive-sense virion RNAs are released into the extracellular environment. Previous experiments have shown that a small viral protein with a molecular weight of about 2.2 kDa is bound to the 5' terminus of picornavirus genomic RNA by a phosphodiester bond between its own tyrosine residue and the 5'-terminal uridine of the virion RNA (1, 4). VPg is now known for its critical role as a primer in virion RNA replication. Interestingly, VPg is not present on picornavirus messenger RNAs, which are used as templates for viral protein synthesis during the infectious cycle (1, 4, 5). Thus, it appears that the cleavage of the protein-RNA bond is a precursory step to translation of mRNA, carried out by an unknown enzyme in the host cell, designated "unlinkase", that specifically recognizes the tyrosyl-RNA phosphodiester bond (2, 6). The enzymatic activity of unlinkase is present in uninfected HeLa cells, suggesting the enzyme serves other purposes in normal cells (3).

Research on the structure and function(s) of unlinkase is of interest because similar enzymatic activities have not been identified in mammalian cells, except for that of tyrosyl-DNA phosphodiesterase (7). Understanding how the unknown enzyme interacts with VPg will elucidate its role in picornavirus replication, and may eventually help us to construct new drugs to fight against members of the picornavirus family. Thus, our project aims to (i) determine which, if any, human protein will interact with poliovirus VPg in yeast two-hybrid assay and (ii) develop an assay to test VPg cleavage from genomic RNA using poliovirus as our model system.

We will focus on two main hypotheses: Since VPg is absent in poliovirus messenger RNA but present in virion RNA and negative strand RNA replication intermediates, we hypothesize that uninfected host cells contain an enzyme that

cleaves VPg from poliovirus genomic RNA, a step that is vital for the translation of poliovirus mRNA. Furthermore, past data on the kinetics of unlinkase have suggested that unlinking of the phosphodiester bond between VPg and the virion RNA is only a minor activity of the enzyme (2). Thus, we hypothesize that the enzyme may have other important roles in uninfected cells. For example, other tyrosine-nucleic acid bonds may be present in the normal cell environment.

Materials and Methods

Genetic Approach: BD Matchmaker GAL4 Two-Hybrid System 3 & Pre-transformed HeLa cell cDNA library. Tests will be first carried out to verify that VPg is not toxic to yeast cells of strain AH109, which we use as the host strain, and that the reporter gene is not activated at all times. Since our HeLa cell cDNA library is already pre-transformed into Y187 yeast, we will then transform strain AH109 with the "bait" plasmid, which contains the VPg fusion insert, and mate it to our pre-transformed yeast so a single yeast cell will contain both the bait plasmid and the cDNA plasmid (8, 9). If unlinkase interacts with the single copy of VPg, or the "bait protein", it will activate transcription of four reporter genes (Figure 1). As a result, the yeast colonies will turn blue to show that positive protein-protein interaction is present *in vivo* in yeast.
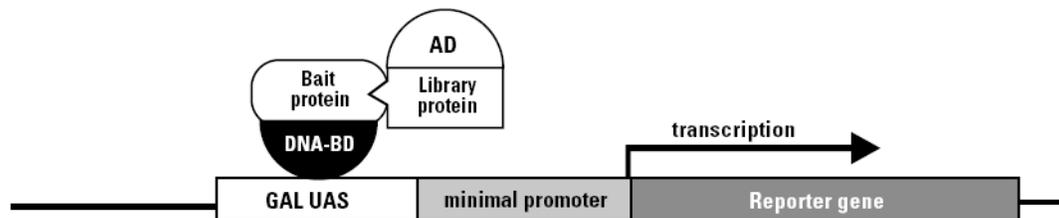

Figure 1. **The two-hybrid principle.** The DNA-BD is amino acids 1-147 of the yeast GAL4 protein, which binds to the GAL UAS upstream of the reporter genes. The AD is amino acids 768-881 of the GAL4 protein and functions as a transcriptional activator (10).

Biochemical Approach: Typical methods that are used to visualize proteins, such as Coomassie-staining and silver-staining gels, will not be able to detect VPg due to its low concentration in infected cells. High resolution gel electrophoresis will be used to detect radioactively labeled ^{35}S-methionine VPg in our project. However, wild-type VPg does not contain any methionine so it is not possible to label wt VPg with ^{35}S-methionine. To solve this problem, a mutant which contains two methionines, termed VPg 31, will be used instead (11).

First, virion RNA will be isolated from sucrose gradient purified poliovirus. Then, HeLa cells will be lysed by swelling the cells via hypotonic buffer (20mM HEPES pH 7.4, 145 mM NaCl, 11mM glucose) and disrupting the cells using a glass Dounce homogenizer. Post-lysis buffer will be immediately added and suspensions will be centrifuged to remove nuclei and any cell debris. The resulting cytoplasmic lysates for HeLa cells and rabbit reticulocytes lysates will be separated into three smaller fractions using differential ammonium sulfate precipitation. Before incubation with each ammonium sulfate fraction, VPg 31-RNA will be subjected to RNase digestion, which will leave only 9 nucleotides

attached to VPg. Thus, the entire RNA molecule will not be trapped in the well of a high percentage polyacrylamide gel and will be able to migrate into the gel.

The fractions that contain the unlinkase should be able to cleave the phosphodiester bond between the tyrosine residue in VPg and the virion RNA. As a result, when that particular truncated virion RNA incubated with a specific fraction is subjected to polyacrylamide gel electrophoresis, the protein moiety will move with an increased electrophoretic mobility relative to the other fractions due to its smaller molecular weight after cleavage. After the fraction of our interest is identified, the enzyme will be isolated and purified using biochemical methods. It can then be sequenced to compare with available protein sequences.

Future experiments beyond the immediate scope of this proposal could include Western blot analysis and other protein detection through signal amplification methods capable of detecting very small amounts of the low molecular weight VPg moiety.

Student's Responsibilities and Level of Participation

I will work closely with one of Dr. Semler's graduate students, Janet Rozovics, from which I will learn how to carry out the protocols needed to complete my project. The techniques required to carry out this project consist of two approaches.

Genetic approach:

(1) To streak out yeast colonies on YPD complete yeast media for a working stock of cells. These cells will be used for subsequent transformations.

(2) To determine if the VPg protein is toxic to transformed yeast cells.

(3) To determine if expression of VPg will activate the GAL4 promoter without the presence of the activating domain (AD).

(4) To co-transform our yeast cells with our bait domain/VPg fusion plasmid which also encodes a gene that will allow transformants to grow on –Trp dropout media and a control vector, pGADT7-T, which encodes a gene allowing transformants to grow on –Leu dropout media. Together, these plasmids, when co-transformed, will simulate a large library scale transformation. Yeast cell growth will be evaluated on low-stringency (-Trp/-Leu), medium-stringency (-Trp/-Leu/-His) and high-stringency (-Trp/-Leu/-His/-Ade) dropout media plates. We only expect growth on the low-stringency plates, unless VPg activates GAL4, which will allow these yeast cells to grow in the dropout media described.

(5) To test the interactions between pGBKT7-53 and pGADT7-T on the three dropout plates as positive control. The gene products, p53 and large T-antigen, will interact in this assay.

(6) To mate AH109 yeast cells transformed with the pGBKT7-VPg plasmid and Y187 yeast cells pre-transformed with the HeLa cell cDNA library and to screen for positive interactions determined by color change of yeast colonies.
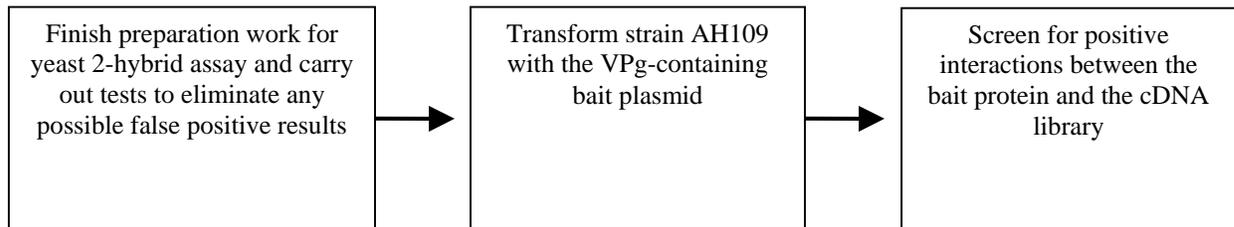
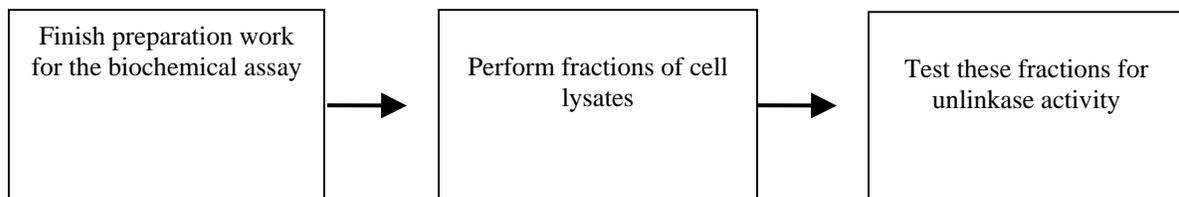Biochemical approach:

(1) To make tris-tricine buffer and gel.

(2) To prepare and fractionate uninfected HeLa cells.

(3) To isolate poliovirus RNA.

(4) To test the lysate fractions for unlinkase activity outlined in materials and
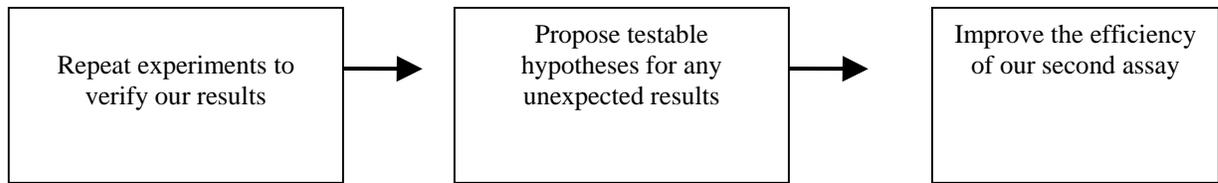
methods.

Timeline

June 20 --> July 15 (4 weeks)

Finish preparation work for yeast 2-hybrid assay and carry out tests to eliminate any possible false positive results	→	Transform strain AH109 with the VPg-containing bait plasmid	→	Screen for positive interactions between the bait protein and the cDNA library

July 18 --> Aug 12 (4 weeks)

Finish preparation work for the biochemical assay	→	Perform fractions of cell lysates	→	Test these fractions for unlinkase activity

Aug 15 --> Sept 16 (5 weeks)

Repeat experiments to verify our results	→	Propose testable hypotheses for any unexpected results	→	Improve the efficiency of our second assay

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