# Approaches to Localizing Small RNAs and Proteins in the Cell

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By

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# ABSTRACT

This project focused on the localization of small RNAs as well as certain proteins in mammalian cells grown in culture. Immunofluorescence and confocal microscopy were used to locate signal recognition particle (SRP) RNA and three associated protein subunits, and also telomerase RNA and two associated proteins. Several different cell types were used to study the molecules, as well as multiple techniques for locating these molecules including fluorescence *in situ* nucleic acid hybridization, immunostaining, and fluorescent protein gene transfection and expression. The results from the SRP study demonstrate that there is colocalization in the nucleolus between the SRP RNA and the three protein subunits studied. The results from the study on telomerase demonstrate that further modifications need to be made to the techniques used because of the difficulty of localizing this small RNA and its associated proteins in the cell.

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## ACKNOWLEDGEMENTS

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# BACKGROUND

## Introduction

Molecular biology can be described as the study of Biology at the molecular level and is associated with many fields like chemistry, biochemistry, and genetics. The molecules of significant importance to molecular biology are DNA, RNA, and proteins. These molecules are studied to a great extent with respect to their assembly, structure, and function. The roles of many molecules of the cell and the relationships between them are still poorly understood. Understanding the structure and interaction among certain molecules will aid in the understanding of their roles in the context of a living organism (1).

There are many different stable cellular structures that need to be assembled, disassembled, moved, and reorganized during different stages of the cell cycle. There are also structures that rapidly move and reorganize themselves in response to the cell's environment, independent of the cell cycle. Some molecules have the complex task of moving other molecular components around the cell, in and out of the nucleus, from one organelle to another, and in and out of the cell. There are very dynamic processes in cells that are well understood, while there are other processes that are poorly understood. One major reason why certain processes are still poorly understood is because of the available techniques. As more and more new techniques are discovered for studying the dynamic processes of the cell, roles of certain molecules in the context of a living organism will be better understood (2).

## Signal Recognition Particle

### Function

The signal recognition particle (SRP) is a protein/RNA complex, a ribonucleprotein, and it recognizes certain nascent polypeptides and transports the polypeptide bound to a ribosome to the endoplasmic reticulum (ER) in eukaryotes and the plasma membrane in prokaryotes. In humans, the SRP translocates only secretory or membrane proteins into the ER (3). As seen in Figure 1, the SRP starts by associating with a ribosome in the cytosol that is in the process of translating an mRNA that encodes for a secretory or membrane protein. The SRP is able to recognize these types of proteins because of a short signal sequence at the N-terminus of the nascent peptides. The SRP bound to the ribosome travels and interacts with a SRP receptor that is located in the membrane of the ER. The ribosome then continues translation of the protein through a channel called the translocon and into the endoplasmic reticulum. The released SRP is then able to go and find another protein destined for the ER (4).

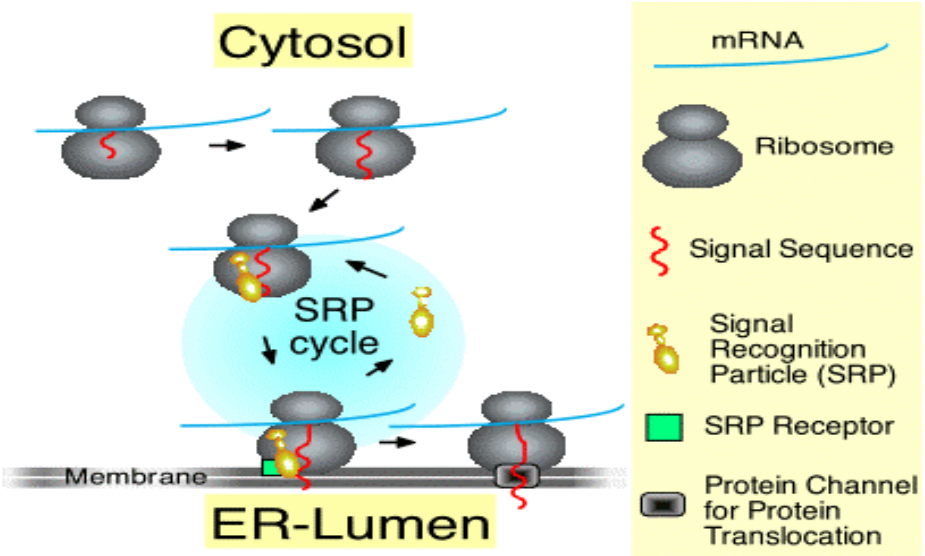


Figure 1. Diagram of SRP function (4).

**Structure**

The human SRP is composed of six different proteins that are bound to one RNA. Interestingly, the SRP RNA has a secondary structure that has been conserved during the evolution of bacteria, archaea, and eukarya. The human SRP RNA contains two domains, the translational arrest activity located in the *Alu* domain and the nascent polypeptide signal sequence recognition and protein translocation activity located in the S domain. This RNA is bound to the proteins: SRP9, SRP14, SRP19, SRP54, SRP68, and SRP72, named according to their approximate  in kDa. Two pairs of proteins, SRP9/SRP14 and SRP68/SRP72, are present as heterodimers in the SRP (3). Shown in Figure 2 is a basic diagram of the components of the eukaryotic SRP. The  of the RNA molecule  shown in light blue, the six proteins are numbered in red, and the signal peptide of the protein is shown in pink. The overall structure of the SRP particle resembles a dumbbell and is roughly 240 Å long (4). Remarkably, purified SRP can be disassembled into its constituents and subsequently reassembled to form a functional particle. It has been demonstrated that the constituents of the SRP come together in a step-wise pathway in which different conformational changes allow subsequent binding of additional proteins (3).

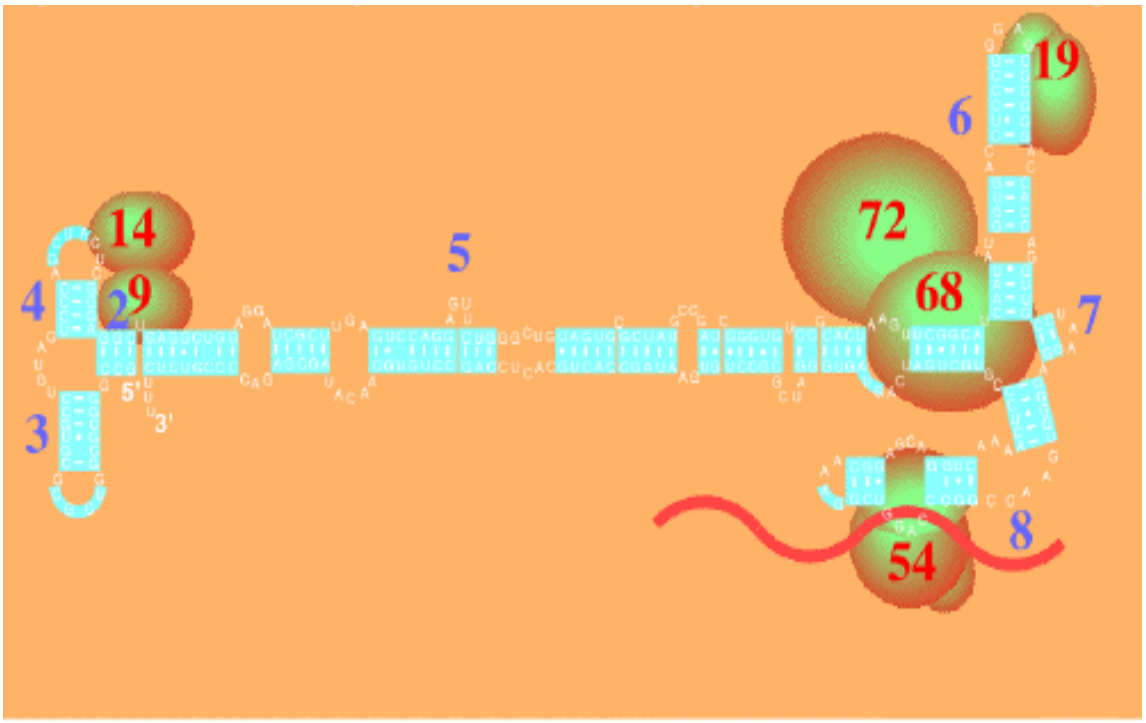


Figure 2. Components of the eukaryotic SRP (4).

Interestingly, though the SRP complex has been studied in great detail and much is known about its structure and function, much less is known about how the complex is assembled in the cell. Studies using yeast and mammalian cells have shown that the nucleolus might be the initial site of SRP assembly, as SRP components have been located in the nucleoli of these cells (5). It has been shown that when fluorescent SRP RNA is microinjected into the nucleus of mammalian cells, the RNA rapidly localizes in the nucleolus. It was also shown that through the microinjection of mutant SRP RNAs, the *Alu* domain and helix 8 are required for nucleolar localization (3).

## Telomerase

### Telomeres

Telomeres are special DNA sequence elements at the termini of eukaryotic chromosomes. In vertebrates, telomeres consist of DNA repeats of the nucleotides TTAGGG. Telomere repeats have been observed to be between 10-50 kilobases in

different organisms (6). There are telomere specific proteins that bind directly to the telomeres to form a complex that caps the end of chromosomes and protects them from degradation, recombination, and end-joining reactions. Some examples of telomere specific proteins are protection of telomeres-1 (POT1), and telomeric repeat factors 1 and 2 (TRF-1 and TRF-2). If the structure or the DNA sequence of telomeres is distorted in some way or the telomere specific proteins are altered or lowered, chromosomes could possibly undergo end associations and fusions that could result in cell senescence or apoptosis (7). There because DNA replication (8). Figure 3 is a diagram of telomere shortening during DNA replication. It can be noticed that during replication, an RNA primer is left at the 5' end of each RNA strand being replicated. This primer then will get degraded by a 5' – 3' exonuclease, which results in the formation of a 3' overhang structure that is rich in the nucleotide guanine. This resulting 3' overhang structure plays an important role in the formation of certain DNA structures at the termini (9).

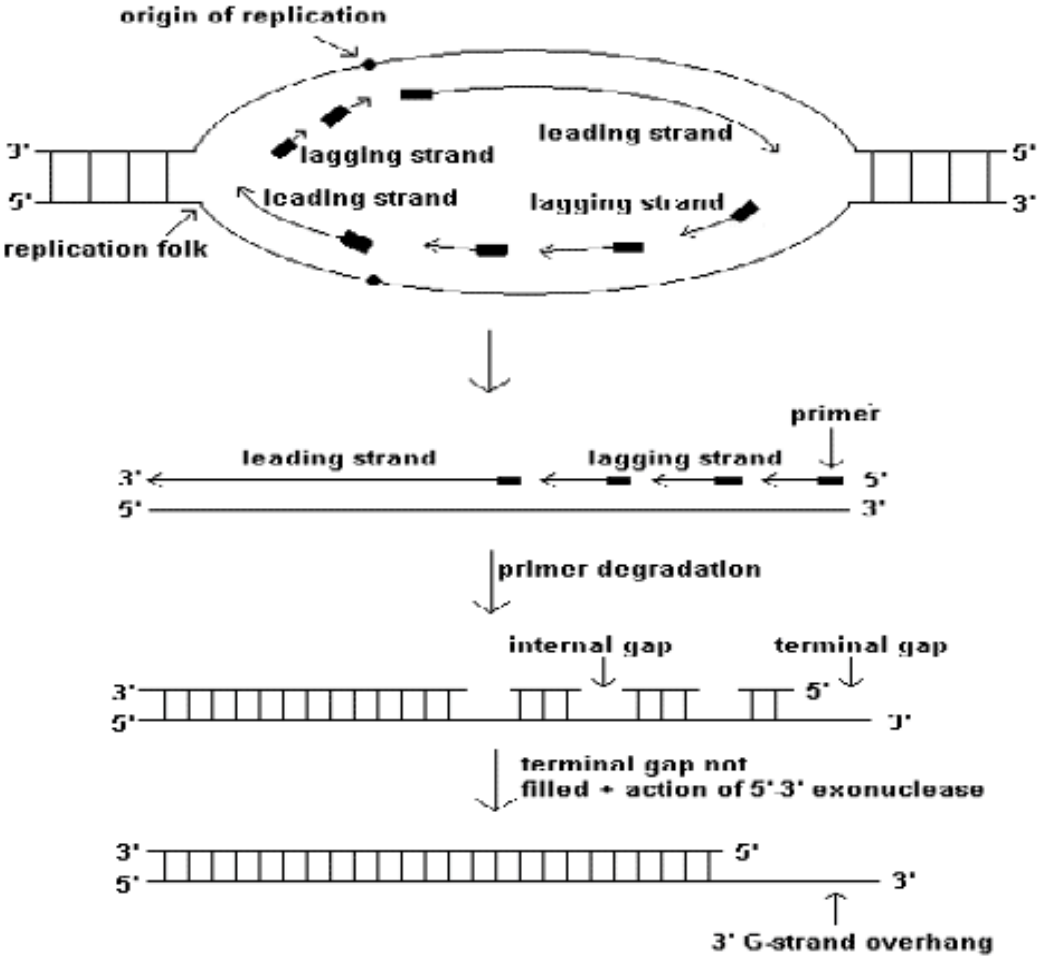


Figure 3. Telomere shortening during DNA replication (9).

Human telomeres are approximately 15-20 kbp of the nucleotides TTAGGG, followed by area on the 3' overhang that is very Guanine-rich. A structure that resembles a lariat, the telomere loop (T – loop), is formed by the double-stranded region and the 3' overhang (7). Figure 4 below is a diagram of a mammalian telomere structure with various telomere binding proteins. As shown in the Figure, there is a large telomere T – loop that forms from the telomere region folding back on itself and a small displacement loop (D –loop) that forms when the 3' overhang binds to the double-stranded telomere repeat sequence of the 5' end strand (9).

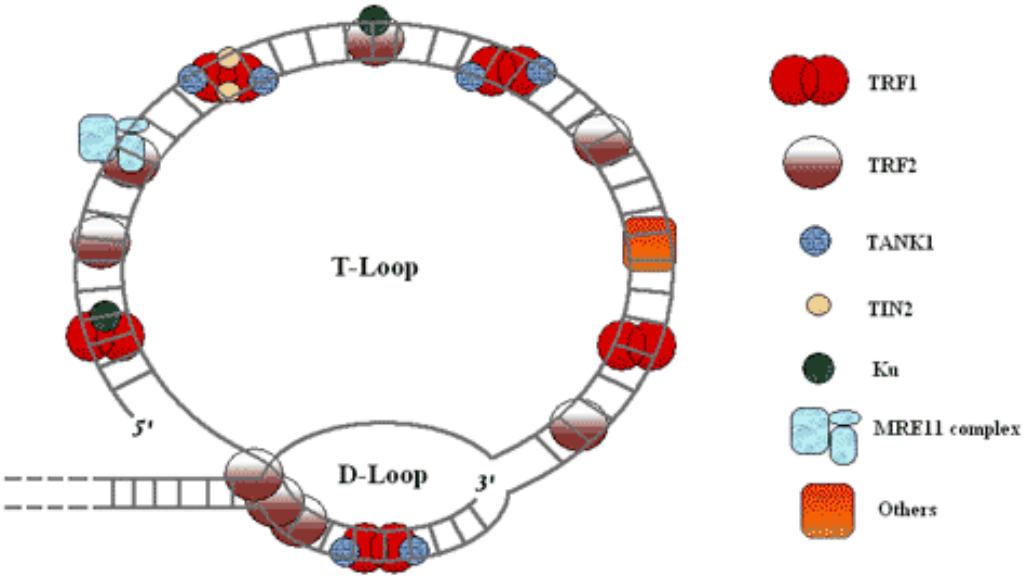


Figure 4. Diagram of mammalian telomere structure with various telomere binding proteins (9).

Telomere length can play a significant role in how a cell functions. For example, if telomeres are shortened to a certain critical length, the cell could be triggered to enter a state of cellular senescence or apoptosis. That is why telomeres can be described as molecular clocks that count the number of times a cell divides and determines the occurrence of the mortality stages M1 (cellular senescence) and M2 (crisis). The pathways that are initiated by telomere shortening can be seen in Figure 5. Figure 5 a cell that does not have any telomerase activity, which means that the amount of telomere sequence is lowered during each round of replication. Once the telomere repeats are shortened to a certain amount, the cell will enter crisis stage involving cellular senescence, meaning that the cell will not be able to divide. The cell could also enter a another crisis stage, where the cell will enter cellular apoptosis or, rarely, become cancerous, if the telomere repeats shorten to another certain critical amount (10).

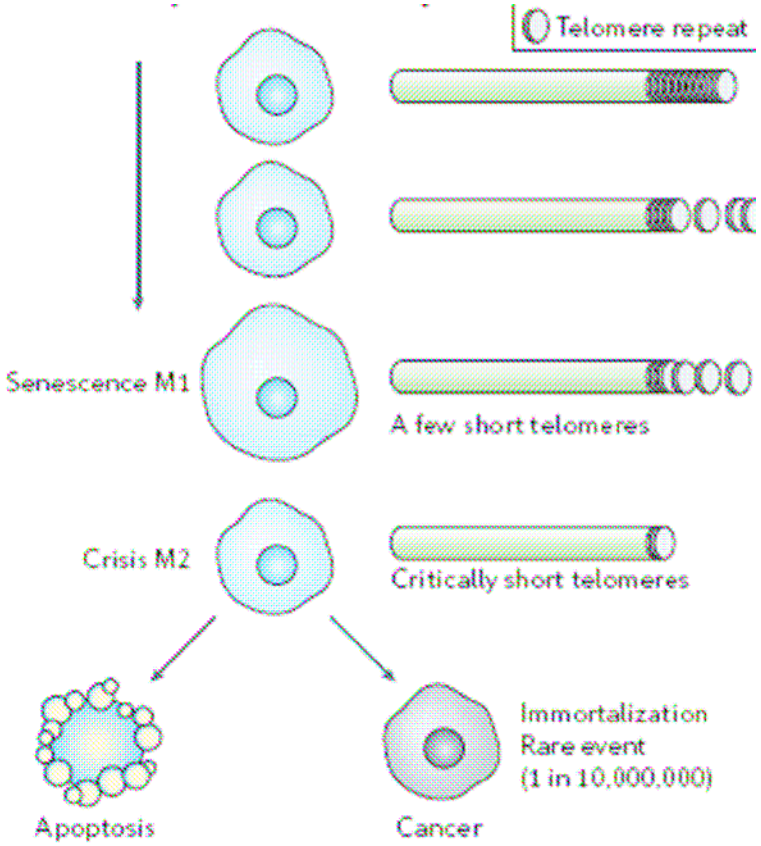

Figure 5. The pathways initiated by telomere shortening that leads to the M1 or M2 stage (7).

**Structure**

Telomerase is a ribonucleoprotein (RNP) enzyme that synthesizes telomeres (11). Human telomerase has a reverse transcriptase (hTERT) component that catalyzes the repeated addition of telomeres *de novo*, and also has a RNA (hTR) component that is used as a template to synthesize the telomeres. These two components of telomerase are essential for synthesis of telomeres, which starts early in development (8). The hTERT component of telomerase contains reverse transcriptase motifs that are essential for enzymatic activity as well as a T motif specific for telomerase. Several motifs near the N-terminus of hTERT are conserved across species. It has also been observed that both hTERT and hTR localize to nucleolus in certain cell lines (12).

Even though sequences and lengths of telomerase RNA may vary considerably across species, there are many features of the structure that appear to be conserved. In vertebrates, there are four highly conserved telomerase RNA domains that contribute to very similar secondary structures. These domains are essential for the 3' terminal formation of the established telomerase RNA, nucleolar localization of telomerase RNA, and catalytic activity. Through phylogenic and biochemical footprinting analysis in human cells, the secondary structure of the 451 nucleotide hTR was recognized. The templating region is 11 nucleotides (5'-CUAACCC*UAAC*-3') and is located near the 5' terminus. The region is comprised of the template sequence for the synthesis of telomeres and has an alignment domain. Certain nucleotides on hTR contain hTERT binding sites are essential for telomerase activity. The stable association between hTR and hTERT is also a unique feature (13).

Interestingly, nearly all adult human somatic cells have no significant telomerase activity because of the transcriptional silencing of its protein component. The silencing of telomerase components leads to the shortening of telomeres. The shortening of telomeric sequences to certain critical points can lead to a decrease in the ability of a cell to go through cell division. So when and if telomeres are synthesized, it is during the S phase in the human cell cycle, although it is not known how the activity of telomerase is constrained to the specific stage of the cell cycle (8). It is also interesting that the introduction of the protein component (hTERT) into cells that are telomerase inactive but have telomerase RNA, can lead to the reactivation of telomerase, which results in the bypass of the mortality stages M1 and M2 (7).

It is thought that the regulation of telomere synthesis through telomerase activity could serve as a very powerful tumor suppressor mechanism. If there were a way to block the production of telomeres in just cancerous cells and not the surrounding tissue, then the cancerous cells could possibly be triggered to enter cellular senescence or apoptosis. There are also several diseases involving telomere dysfunction, and research has shown that individuals born with reduced levels of telomerase activity have a shorter telomeric sequence, which leads to telomere dysfunction in highly proliferating cells. Telomeres are very vital structures inside the cell and that means knowledge about telomerase and telomeres could provide insights into several human diseases (7).

**Cancer Cells, Stem Cells, Progenitor Cells**

In the majority of human cancers, telomerase activity is amplified and provides an increase in proliferation capacity for the cancerous cells. Thus, telomerase biology has significant implications for cancer (8). In advanced stages of most cancers, telomerase allows proliferation of transformed cells by replenishing telomeres. There are also possibilities that telomerase could contribute to tumor growth through other unknown functions that promote cell growth (14).

The activity of telomerase could be the rate-limiting step required for the continued proliferation of progressing cancers. It is known that the majority of human tumors have telomerase activity and nearby normal human somatic cells do not. So, since cancer cells have considerably shorter telomeres than the surrounding normal telomerase-expressing cells, such as stem and germline cells, there is a possibility that cancer cells could be effectively targeted by telomerase inhibitors. This is because normal telomerase-expressing cells have longer telomere lengths and divide at slower rates than cancer cells.

The cancer cells would eventually enter a crisis stage if their telomeric sequence shortens enough. The treatment would have to be extensively monitored so that when the cancer cells die, the treatment can be stopped immediately so that normal telomerase-expressing cells remain unaffected. An overview of telomerase inhibition in normal telomerase-expressing cells and cancer cells is shown in Figure 6. The figure shows how only cancer cells will be triggered to enter apoptosis while normal telomerase-expressing cells remain healthy (7).

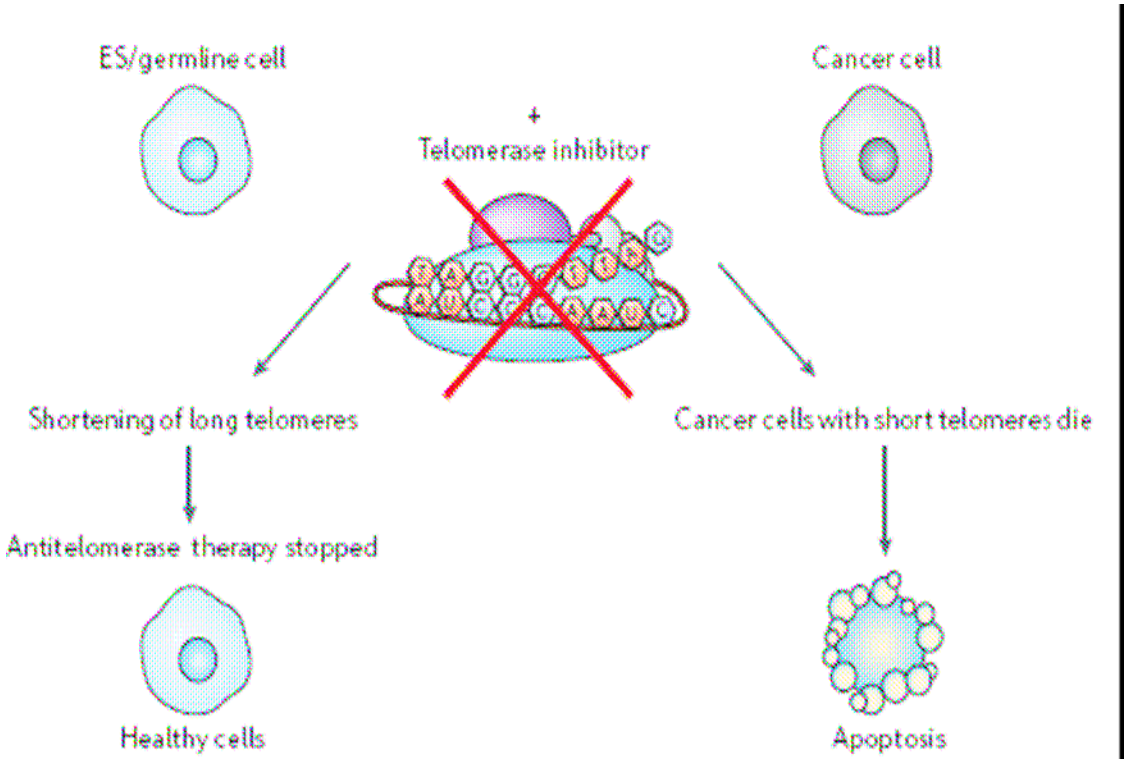


Figure 6. Telomerase inhibition in normal telomerase expressing cells and cancer cells (7).

A study was performed that involved inhibiting telomerase activity through RNA interference (RNAi) of hTERT in two human glioblastoma cell lines (10). Although the results showed that there was no effect on cell growth *in vitro*, tumors subcutaneously and intracranially grafted mice were considerably inhibited by anti-telomerase RNAi. Even though telomerase inhibition is strongly dependent on the context of the cell, the

inhibition is a potential therapeutic approach for cancer (10). There have also been investigations into the localization of telomerase hTR in several cancer cell lines. Studies using fluorescence in situ hybridization (FISH) on several cancerous cells and normal somatic cells were performed. Accumulation of hTR at nuclear foci was observed in the cancerous cells, but not in the normal cell lines that were studied (15).

In normal regenerating tissues, telomerase is expressed by stem cells and progenitor cells. The activity is required for intact telomere function and tissue homeostasis. It has been observed that in telomerase-knockout mice, the shortening of telomeres to a critical length induces programmed cell death. It was also noted that the shortening impairs the function of actively dividing tissue such as bone marrow, testis, and the gastrinointestinal tract. The requirement of telomeres in tissue maintenance could possibly be related to the role of intact telomeres in stem cell self-renewal, and this idea has been most evidently demonstrated through transplantation experiments using hematopoietic stem cells. It also has been found that telomerase was active in stem cells through a mechanism not related to the maintenance of telomeres (14).

## Techniques

### Fluorescence *In Situ* Nucleic Acid Hybridization

One approach to localizing small RNAs in a cell culture is through fluorescence *in situ* hybridization (FISH). FISH can be described as the labeling of a piece of a nucleic acid strand, called a probe, that is complementary to a specific DNA or RNA strand of interest, and then the exposure of that complementary nucleic acid strand to a specific cell sample, hoping that it hybridizes and shows the localization of a molecule of interest. The hybridized probe can be observed by using a fluorescent microscope or by using a

higher resolution microscope and a technique called confocal microscopy which allows for three-dimensional imaging (16). FISH using complementary nucleic acid strands to DNA can be used to analyze chromosomes, while complementary nucleic acid strands to RNA can be used to analyze a wide variety of types of RNA in the cell (17).

Before FISH can be applied to samples, the cell usually go through a chemical fixation procedure to keep the target DNAs and RNAs in place, as well as a permeabilization step to allow access for the probe. During FISH, when the labeled probe is exposed to the sample, the temperature is usually increased to allow denaturation of base-paired DNA or RNA to occur, and then the temperature is decreased to allow hybridization to occur. The sample is then washed so that the excess probe is washed away. Interestingly, two or more probes can be labeled with dyes of different colors and used on one sample to show the localization of two or more molecules of interest. FISH can also be used in combination with a technique called immunostaining, which is used mostly for the detection of protein, to show the colocalization of a protein and RNA or DNA of interest. Colocalization studies can be performed on molecules that have a protein component and an RNA component (16).

**Immunostaining**

Immunostaining is an approach to localizing protein components in a cell. It can be described as the labeling of antibodies with fluorescent molecules, and then the exposure of those antibodies to a sample of interest hoping that they show the localization of a molecule of interest. There are two main types of antibodies used during immunostaining. The first type is the primary antibody and it is specific for the antigen of interest. The second antibody, called the secondary antibody, is fluorescently labeled and

is specific for the primary antibody. That way, researchers can choose to show the localization of a certain molecule, obtain a primary antibody to bind to that molecule, and then obtain a fluorescently labeled secondary antibody to bind to the primary antibody to show the localization of that molecule (18). Figure 7 is the general structure of an antibody, showing the stem and the arms of the molecule. The stem of the antibody is a so-called "constant" region that is composed of heavy chains, and it defines the class of antibody and organism it is present in. The arms of the antibody are the so-called "variable" regions and are part heavy and part light chains. Each arm has the ability to bind an antigen, so one antibody has the ability to bind two antigens (19).

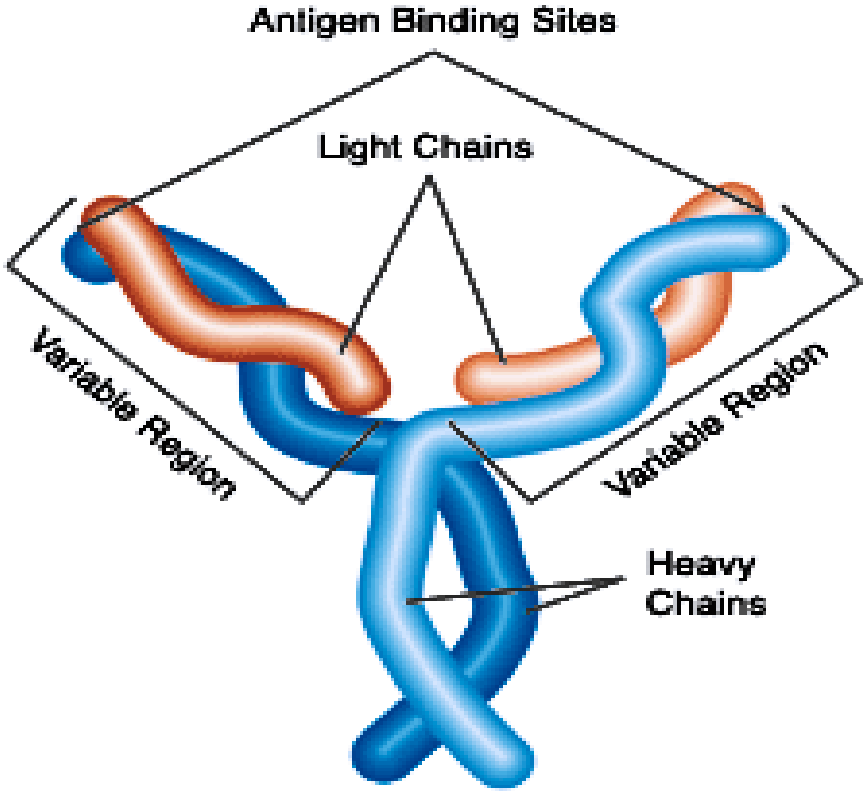


Figure 7. The structure of an antibody showing: Heavy and light chains, variable regions, and antigen binding sites (19).

Before immunostaining can be performed on a sample, the cells usually go through a fixation and permeabilization procedure to keep proteins in place and to allow

access for the antibodies. The primary antibody is then applied to the sample and allowed to incubate, and the sample is washed so that the excess antibody is washed away. The secondary antibody is then applied and allowed to incubate, and the samples are then washed again so that the excess antibody is washed away. The labeled samples can then be observed by using a fluorescent microscope or by using confocal microscopy (18).

**Fluorescent Protein Gene Transfection and Expression**

Fluorescent protein gene transfection and expression is also an approach for localizing protein components in a cell and it involves incorporating a piece of foreign DNA that encodes a fluorescent protein fused to the coding region of the protein of interest. The techniques that are used to allow foreign DNA uptake are lipofection, virus mediated delivery, electroporation, and microinjection. The foreign DNA does not have to get incorporated into the host genome as long as the foreign DNA has specific control and coding regions that allow for transient expression. Figure 8 shows how nanoparticles, typically liposomes are used, through the cell membrane and either release foreign DNA into the cell cytoplasm or fuse with the nucleus of the cell and thereby the DNA into it. The foreign DNA can then be expressed and studies performed (20).

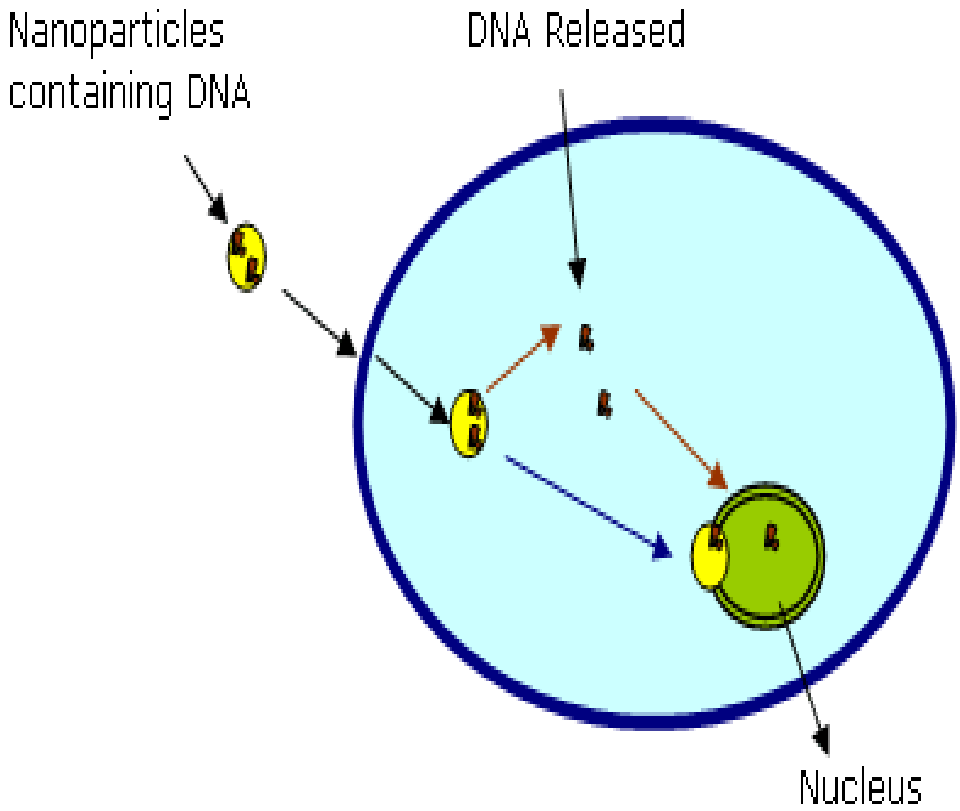


Figure 8. Mechanisms for incorporating and transiently expressing foreign DNA in a host cell (21).

The cell culture sample that gets transfected is left to sit for a number of hours to allow for the expression of the fluorescent protein. The cell sample then goes through a fixing procedure to keep the fluorescent proteins in their place. The labeled samples can be observed by using a fluorescent microscope or by confocal microscopy. Transfection can also be used with FISH, to show the colocalization of a protein and RNA or DNA of interest. Colocalization studies can be performed on molecules that have a protein component and an RNA component (20).

## MATERIALS AND METHODS

### Cell Lines

The cell lines used in this project were Normal Rat Kidney (NRK) cells, HeLa cells, U2OS cells, GM00847 cells, and VA13 cells. All of the cells except for the GM00847 cells were grown using DMEM low glucose 1X medium with 10% fetal bovine serum added. The GM00847 cell line was grown using MEM 1X medium with 10% fetal bovine serum added. The NRK cells were used for the study on the signal recognition particle, and the HeLa, U2O3, VA13, and GM00847 cell lines were used for the study on telomerase. Table 1 below shows descriptions of all the cell lines used in this report and the presence or absence of the cell component of interest. This information was gathered from the cell line characteristics and information guide that came with the cell lines from various resources.

| **Signal Recognition Particle** | | | |
|---|---|---|---|
| Cell line | Description | RNA | Proteins |
| NRK | Normal rat epithelial kidney cells | Present | Present |
| **Telomerase** | | | |
| Cell line | Description | RNA | Proteins |
| HeLa | Human, 31 year old African American female, epithelial cervical cancer cells | Present | Present |
| U2OS | Human, 15 year old Caucasian female, epithelial-like osteosarcoma cells | Present | Present |
| VA13 | Human, 3 months gestation Caucasian female, normal lung fibroblast cells | Not present | Not Present |
| GM00847 | Human, 5 year old African American male, skin fibroblast cells | Not present | Not present |

Table 1. Descriptions of all the cell lines used in this report.

**Plasmids, Probes, Antibodies**

There were many plasmids, probes, and antibodies used in this report and a summary can be viewed in Table 2. The plasmids that were used in this report encode an SRP protein as a green fluorescent protein (GFP) fusion molecule. There were two types of probes used in this report, LNAs and PNAs. Locked nucleic acids (LNAs) are modified RNAs that have an extra bridge connecting 2' and 4' carbons, which allows for enhanced base stacking and backbone pre-organization for enhanced specificity. All of the LNAs used in this report are approximately 15 nucleotides long. Peptide nucleic acids (PNAs) are nucleic acids with repeating N-(2-aminoethyl)-glycine units linked by peptide bonds in place of the usual phosphodiester backbone. Thus the PNA contain no charged phosphate groups which allows for enhanced binding due to less electrostatic . The PNAs used in this report are a little longer averaging 20 nucleotides. Along with the primary probes in Table 2, there were also scrambled sense and scrambled anti-sense probes that were used as control probes and are approximately the same length with respect to the primary probe. There were also two types of antibodies used, primary and secondary antibodies. The two types of primary antibodies used were the telomerase protein (hTERT) specific antibody and thetelomeric repeat factor-2 (TRF-2) specific antibody. The one secondary antibody that was used was a Cy3 – conjugated donkey anti-rabbit antibody.

| Plasmids | | |
|---|---|---|
| Name | Description | Obtained From |
| SRP-19 GFP | 4,642 base pair plasmid that encodes for human SRP-19 fused to a green fluorescent molecule | University of Texas Health Center, Department of Molecular Biology |
| SRP-68 GFP | 6,633 base pair plasmid that encodes for human SRP-68 fused to a green fluorescent molecule | University of Texas Health Center, Department of Molecular Biology |
| SRP-72 GFP | 6,773 base pair plasmid that encodes for human SRP-72 fused to a green fluorescent molecule | University of Texas Health Center, Department of Molecular Biology |
| **Probes** | | |
| SRP LNA | A LNA probe specific for a site on SRP RNA | Pederson Lab, University of Massachusetts Medical School, Department of Biology and Molecular Pharmacology |
| SRP-2 PNA | A PNA probe specific for a site on SRP RNA | |
| TH1 PNA | A PNA probe specific for a site on telomerase RNA | |
| TH4 PNA | A PNA probe specific for a site on telomerase RNA | |
| TH1 LNA | A LNA probe specific for a site on telomerase RNA | |
| TH4 LNA | A LNA probe specific for a site on telomerase RNA | |
| TERNS | A LNA probe specific for a site on telomerase RNA | |
| Yeast PNA | A PNA probe specific for a yeast cell component | |
| **Antibodies** | | |
| TRF-2 | Primary telomeric repeat factor-2 (TRF-2) specific antibody | Calbiochem Inc. |
| hTERT | Primary telomerase protein (hTERT) specific antibody | Rockland Immunochemicals Inc. |
| DAR | Secondary Cy3 – conjugated donkey anti-rabbit antibody | Jackson ImmunoResearch Laboratories Inc. |

Table 2. Summary of all the plasmids, probes, and antibodies used in this report.

**Cell Restoring**

The NRK, HeLa, U20S, and Va13 cell lines were thawed from a stock in a liquid nitrogen tank, and the GM00847 cell line was obtained as a live culture from Coriell Cell Repositories. Frozen cell lines were taken out of a liquid nitrogen tank and immediately placed on a floating rack in a 37°C water bath for 1-2 minutes. During that time, 5 m of the growing medium was added into new 25cm$^2$ flasks. Once the cells were thawed, 1 mL of medium with cells was transferred into the new flasks and observed under a microscope. Once the cells were observed, the flasks were labelled and placed into a 37°C, 5% $CO_2$ incubator.

**Cell Culture**

When cells needed to be split, the media and STE buffer for the cells were warmed in a 37°C water bath for 15 – 60 minutes before the cells were cultured. The trypsin buffer, which is used to release the cells that adhered to the flasks, was allowed to warm in the cell culture hood. Media from the old growing cell flask was removed and 2.5 m of STE buffer was added, covering the cells. The STE buffer contained 10mM Tris-HCl at pH7.5, 10mM NaCl, and 1mM EDTA. The STE buffer was then removed and 0.5 m of 2.5% (w/v) trypsin in PBS was added. The flask was then incubated in a 37°C, 5% $CO_2$ incubator for 1 – 2 minutes. During that time, 5 m of medium was added to the new 25$cm^2$ flask. The old flask was then observed under a microscope to make sure the cells had come off the surface. Once the cells looked ready to be divided, 5 m of medium was added to the flask and pipetted over the area where the cells had been growing 7 – 8 times. An average of 0.5 m of medium from the old flask was transferred to the new flask. The new flasks were observed under a microscope, labelled, and placed into a 37°C, 5% $CO_2$ incubator until the cultures needed to be split again.

**Cell Plating**

2.0 m of ted cells was added to 18 msof medium and that mixture was used to seed coverslips in 6-well culture dishes. 3 m of the diluted cell resuspension was added to each well. The 6-well culture dishes were placed in a 37°C, 5% $CO_2$ incubator until cells were ready to be fixed.

**Fluorescent Protein Gene Transfection and Expression**

Cells were transfected 24 hours after the cells were plated in a sterile environment. There are several mixtures that were needed for the transfection. There is a plasmid

mixture, which contained 2μg/well plate of the plasmid of interest. There is a control mixture, which contained RNAse-free $H_2O$ instead of the plasmid. There is also a lipofectamine mixture, which contained 5μL/well plate of lipofectamine. All these mixtures contained Opti-MEM in them, which is a medium that optimizes the transfection procedure so that the plasmid can get incorporated into the cells. Once the cells were ready to be transfected, equal amounts of both the plasmid mixture and control mixture were mixed separately with the lipofectamine mixture and allowed to sit for 20 minutes. The medium was removed from the culture dishes that contained the cells, and 2.5 m of Opti-MEM was added to each well. Then, 500 μL of the plasmid or control mixture with the lipofectamine was added to the corresponding wells, and the culture dishes were then swirled. The dishes were placed in a 37°C, 5% $CO_2$ incubator for 3 hours. The medium was then changed back to the normal growth medium and the cells were allowed to incubate in a 37°C, 5% $CO_2$ incubator until they were ready to be fixed.

**Cell Fixation**

Cells were fixed depending on what type of experiment they were going to be used in, and this procedure did not require a sterile environment. For cells that were transfected, the cells were fixed 24 hours after transfection. For fluorescence *in situ* hybridization experiments, the cells needed to be fixed before the cells occupied more than 50% of the total area. For immunostaining experiments, cell occupancy of the total area should be between 50% and 90% for optimal results.

Cells used for immunostaining experiments were first washed with 1X PBS, and then incubated with a 4% paraformaldehyde solution for 12 minutes at room temperature. The 4% paraformaldehyde solution was then removed and the cells were washed twice

with 1X PBS. The cells could then be used immediately for an experiment, or stored in a 1X PBS/5mM $MgCl_2$ solution in a 4°C room for no more than a month before performing an experiment.

Cells used for *in situ* hybridization experiments were first washed with 1X PBS, and then were incubated with a 4% paraformaldehyde solution for 15 minutes at room temperature. The 4% paraformaldehyde solution was then removed and the cells were washed twice with 70% ethanol. The cells then had to be stored overnight in 70% ethanol in a 4°C room before being used in an experiment, and had to be used within a month after the cells were fixed.

**Fluorescence *in Situ* Nucleic Acid Hybridization**

First, cells that were fixed for the *in situ* hybridization procedure were taken out of the 4°C room and the 6-well culture dishes were marked according to what probe were going to be added to each well. The 70% ethanol was then removed, and 1X PBS/5mM $MgCl_2$ was added to each well and the culture dishes were allowed to gently be stirred on a gyratory platform for 10 minutes at room temperature. During that time, the probes, hybridization buffer, and 2X SSC/40% formamide solution were prepared. 1X SSC is 0.15M NaCl and 0.015M sodium citrate at pH 7.0. All of the probes for the *in situ* hybridization experiments were used at a 20 ng/uL concentration. The 1X PBS/5mM $MgCl_2$ was then removed and 2X SSC/40% formamide was added to each well and the culture dishes were allowed to stir for 10 minutes at room temperature. During that time, a layer of parafilm was placed on a coverglass (12" X 12"). When there was two minutes left, the probe solutions were placed in a 95°C heat block for at least two minutes and no longer than ten minutes. When the cells were done rotating in 2X SSC/40% formamide

and the probes were in the heat block for at least two minutes, the probe solutions were mixed with equal amounts of hybridization buffer. Those solutions were then pipetted at 20 μ per coverslip onto the corresponding area of the first layer of parafilm on the coverglass. The coverslips from the culture dishes were then immediately placed face down with tweezers onto the corresponding mixture. The first layer of parafilm was then covered with another layer of parafilm and the coverglass was placed into a 37°C incubator for 3 hours, along with the well plates that contain the 2X SSC/40% formamide solution. During that time, a 1X SSC/40% formamide solution was made and placed in a 37°C water bath until needed. When the 3 hours expired, tweezers were used to place the coverslips back into their original wells with the 2X SSC/40% formamide, cells facing up. The plate was allowed to sit for 10 minutes in a 37°C incubator. The 2X SSC/40% formamide solution was then removed, and the cells were washed twice with 1X SSC/40% formamide for 30-45 minutes in a 37°C incubator. The 1X SSC/40% formamide was then removed and 1X SSC was added to each well and allowed to rotate for 15 minutes at room temperature. The 1X SSC was then removed and 1X PBS was added to each well and allowed to rotate for 15 minutes at room temperature. The coverslips were then mounted onto slides using tweezers and a solution called a "prolonged antifade reagent" that acts like a seal and preserves fluorescence by excluding air (oxygen promotes fading). The slides were kept in a folder in a dark drawer until observed with a microscope.

**Immunostaining**

First, the 6-well culture dishes that contained cells that were fixed for the immunostaining procedure were either taken right after the fixing procedure or taken out

of the 4°C room. The culture dishes were then marked according to what antibodies were going to be added to each well. If the cells were stored in the 4°C room, the 1X PBS/5mM $MgCl_2$ was removed and the cells were washed twice with 1X PBS. If the cells were taken right after the fixing procedure, the cells were already washed with 1X PBS and were ready to be subjected to the rest of the procedure. The cells then had to be washed twice with 1X PBS/1 % BSA for 5 minutes at room temperature with rotation. The cells were then permeabilized with 0.5% Triton-100X for 5 min at room temperature. The cells were then washed twice again with 1X PBS/1 % BSA for 5 minutes at room temperature with rotation. During that time, a layer of parafilm was placed on a coverglass (12" X 12"). The primary antibodies were then applied to the first layer of parafilm, at different dilutions depending on which antibody was used. The coverslips from the culture dishes were then immediately placed face down with tweezers onto the corresponding antibody. The first layer of parafilm was then covered with another layer of parafilm and the coverglass was placed into a humidity chamber for 1 hour. After the incubation, tweezers were used to place the coverslips back into their original wells with the 1X PBS/1 % BSA, cells facing up, and allowed to rotate at room temperature for 7 minutes. The cells were then washed twice again with 1X PBS/1 % BSA for 7 minutes at room temperature with rotation. During that time, a layer of parafilm was placed on a coverglass. The secondary antibodies were then applied to the first layer of parafilm. The coverslips from the culture dishes were then immediately placed face down with tweezers onto the corresponding antibody. The first layer of parafilm was then covered with another layer of parafilm and the coverglass was placed into a humidity chamber for 1 hour. After the incubation, tweezers were used to place the coverslips back into their

original wells with the 1X PBS/1 % BSA, cells facing up, and allowed to rotate at room temperature for 7 minutes. The cells were then washed twice again with 1X PBS/1 % BSA for 7 minutes at room temperature with rotation. The cells were then washed twice with 1X PBS and the coverslips were mounted onto slides using tweezers and the "prolonged antifade reagent." The slides were kept in a folder in a dark drawer until observed with a microscope.

**Imaging**

All of the slides in this report were imaged using a fluorescence microscope and a 100X objective lens. The observed fluorescent molecules either showed green or red fluorescence. For the SRP colocalization analysis, three-dimensional images were generated by capturing different planes of focus through the cell. These images had their background light subtracted and had their green (protein) and red (RNA) fluorescence combined. A computer program was used to register as yellow those sites at which both red and green signal were present, as would result from any overlap of the SRP RNA and SRP protein components. Different planes could then be analyzed to show the spatial arrangement of the SRP RNA and protein components in the cell. For the telomerase localization studies, only one plane of the cell was imaged to show the localization of either RNA or protein.

## RESULTS

The SRP was the first molecule examined in this report. NRK cells were chosen as a cell line for this study because they are easily cultured, can endure the experimental techniques applied to them, and have known SRP components. To localize the SRP proteins (SRP19, SRP68, SRP72), plasmids that encode each SRP protein as a green fluorescent protein (GFP) fusion protein were transfected into the NRK cells and were expressed. To localize the SRP RNA, a LNA probe was used during the FISH procedure. Images were captured using a using a fluorescence microscope and a 100X objective lens. Multiple planes of the cell were captured so a three dimensional construct could be generated and background light subtracted, to analyze the spatial arrangement of the SRP proteins and the SRP RNA.

The colocalization of SRP19 and SRP RNA was studied first. The SRP19-GFP fusion proteins showed prominent nucleolar signal and also more diffuse nucleoplasmic and cytoplasmic signal, indicating that the nucleolus is a spot where SRP19 localizes and that it also exists in the nucleoplasm and cytoplasm (Figure 9A and 9E). The SRP RNA probes also showed prominent nucleolar signal and also diffuse nucleoplasmic and cytoplasmic signal, which also indicates that the nucleolus is a spot where SRP RNA localizes and that it also exists in the nucleoplasm and cytoplasm (Figure 9C and 9G). When the fluorescence signals from Figure 9A and 9C were combined to examine colocalization, the nucleolus showed the strongest signal overlap while there was also signal overlap in the nucleoplasm and cytoplasm (Figure 9B and 9F).

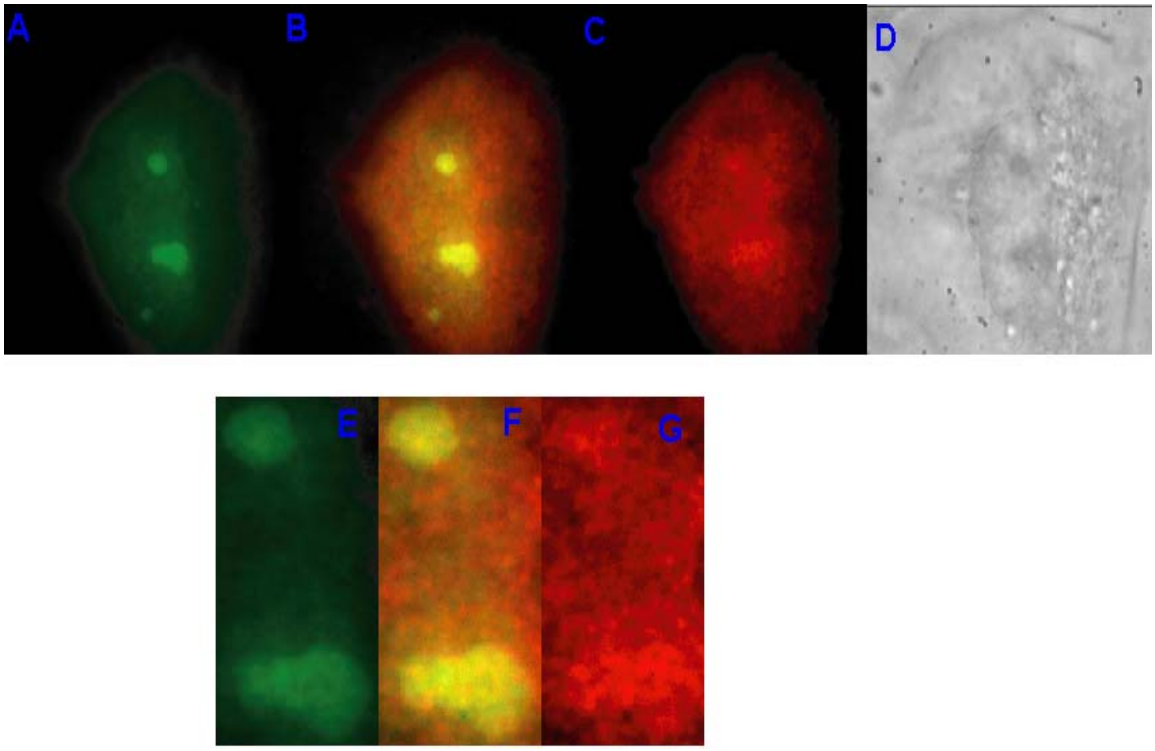


Figure 9. Results from the SRP19 and SRP RNA colocalization study in a NRK cell. This figure displays an example of an NRK cell on a slide that has gone through transfection with SRP19 GFP plasmids and then FISH using LNA probes for SRP RNA. The images have their intensity scaled the same as the controls in Figure 10 and are in the middle plane of the cell. (A) displays green fluorescence from SRP19-GFP fusion proteins. (C) displays red fluorescence from SRP RNA probe. (B) displays the combination of the green fluorescence from (A) and red fluorescence from (C). (D) displays the phase image from the NRK cell. (E), (F), and (G) display magnifications of the nucleoli in the cell in (A), (B), and (C) respectively.

Similarly to the results in Figure 9A and 9E, SRP19-GFP fusion proteins again showed prominent nucleolar signal, moderate nucleoplasmic signal, and some cytoplasmic signal (Figure 10A and 10D). Similarly to the results in Figure 9C and 9G, SRP RNA probes showed prominent nucleolar and cytoplasmic signal and some nucleoplasmic signal (Figure 10H and 10N). There was only autofluorescence detected in cells that were transfected with control plasmids (Figure 10G and 10J) and cells that were not transfected (Figure 10M and 10P). There was also only autofluorescence detected in cells that went through FISH using SRP RNA anti-sense scramble probes (Figure 10B and 10K) and in cells that went through FISH using control probes (Figure 10E and 10Q).

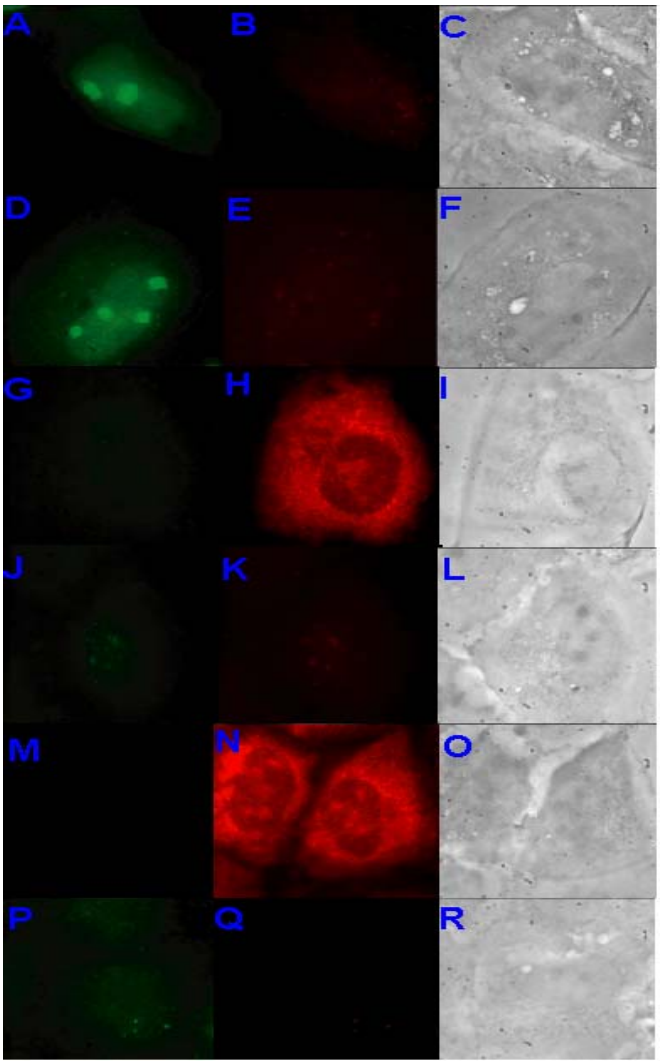


Figure 10. Controls from the SRP19 and SRP RNA colocalization study in NRK cells. Each row in the figure displays an example of an NRK cell on a slide that was either not transfected or transfected with a SRP19 GFP plasmid or control mixture and then had FISH performed using different types of probes. (A) and (D) display green fluorescence from SRP19 GFP plasmid transfected cells, (G) and (J) display green fluorescence from control mixture transfected cells, and (M) and (P) display green fluorescence from non-transfected cells. (H) and (N) display red fluorescence from FISH using SRP RNA probes, (B) and (K) display red fluorescence from FISH using SRP RNA anti-sense scrambled probes, and (E) and (Q) display red fluorescence from FISH using a control mixture. (C), (F), (I), (L), (Q), and (R) are the phase images of the control NRK cells used.

The colocalization of SRP68 and SRP RNA was studied next. The SRP68-GFP fusion proteins showed prominent nucleolar signal and also nucleoplasmic and cytoplasmic signal, which was very similar to the signal pattern from the SRP19-GFP fusion proteins (Figure 11A and 11E). As seen previously, the SRP RNA probes displayed a prominent nucleolar signal and also nucleoplasmic and cytoplasmic signal

(Figure 11C and 11G). When the fluorescence from Figure 11A and 11C were combined to examine colocalization, the nucleolus showed the strongest signal overlap while there was also signal overlap in the nucleoplasm and cytoplasm (Figure 11B and 11F).

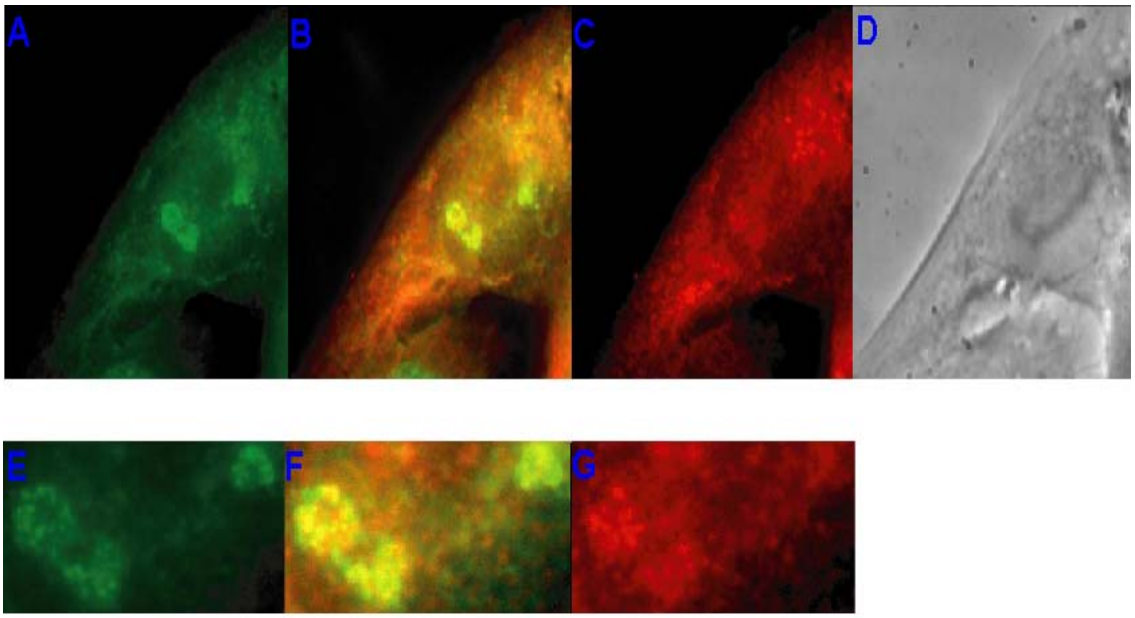


Figure 11. Results from the SRP68 and SRP RNA colocalization study in a NRK cell. This figure displays an example of an NRK cell on a slide that has gone through transfection with SRP68 GFP plasmids and then FISH using LNA probes for SRP RNA. The images have their intensity scaled the same as the controls in Figure 12 and are in the middle plane of the cell. (A) displays green fluorescence from SRP68 GFP fusion proteins. (C) displays red fluorescence from SRP RNA probes. (B) displays the combination of the green fluorescence from (A) and red fluorescence from (C). (D) displays the phase image from the NRK cell. (E), (F), and (G) display magnifications of the nucleoli in the cell in (A), (B), and (C) respectively.

Similarly to the results in Figure 11A and 11E, the SRP68 GFP fusion proteins again showed prominent nucleolar signal and also nucleoplasmic and cytoplasmic signal (Figure 12A and 12D). Similarly to the results in Figure 11C and 11G, SRP RNA probes showed prominent nucleolar and cytoplasmic signal and some nucleoplasmic signal (Figure 12H and 12N). There was onlyautofluorescence detected in cells that were transfected with control plasmids (Figure 12G and 12J) and cells that were not transfected (Figure 12M and 12P). There was also only autofluorescence detected in cells that went through FISH using SRP RNA anti-sense scramble probes (Figure 12B and 12K) and cells that went through FISH using control probes (Figure 12E and 12Q).

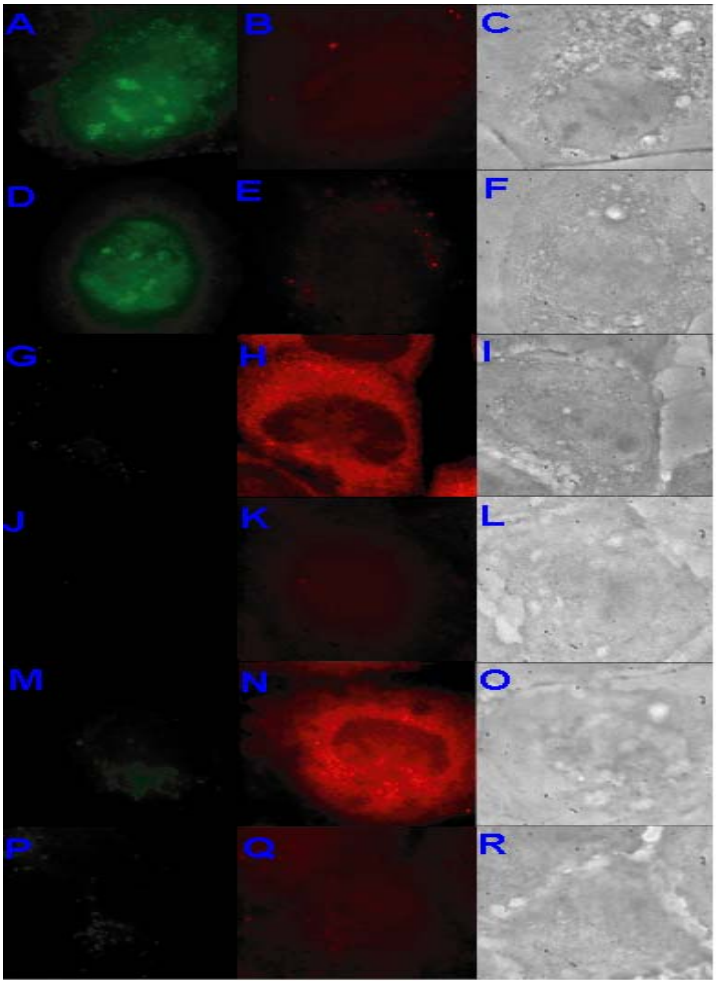


Figure 12. Controls from the SRP68 and SRP RNA colocalization study in NRK cells. Each row in the figure displays an example of an NRK cell on a slide that was either not transfected or transfected with a SRP68-GFP plasmid or control mixture and then had FISH performed using different types of probes. (A) and (D) display green fluorescence from SRP68-GFP plasmid transfected cells, (G) and (J) display green fluorescence from control mixture transfected cells, and (M) and (P) display green fluorescence from non-transfected cells. (H) and (N) display red fluorescence from FISH using LNA probes for SRP RNA, (B) and (K) display red fluorescence from FISH using SRP RNA scrambled anti-sense probes, and (E) and (Q) display red fluorescence from FISH using a control mixture. (C), (F), (I), (L), (Q), and (R) are the phase images of the control NRK cells used.

The colocalization of SRP72 and SRP RNA was the last localization study performed on SRP components. Like the SRP 19 GFP and SRP 68 GFP fusion proteins, the SRP 72 GFP fusion proteins showed prominent nucleolar signal and also some cytoplasmic and nucleoplasmic signal (Figure 13A and 13E). Also like the SRP RNA probes used in the previous experiments, SRP RNA probes showed prominent nucleolar signal and also nucleoplasmic and cytoplasmic signal (Figure 13C and 13G). When the

fluorescence from Figure 13A and 13C were combined to examine colocalization, the nucleolus showed the strongest signal overlap while there was also signal overlap in the cytoplasm and nucleoplasm (Figure 13B and 13F).

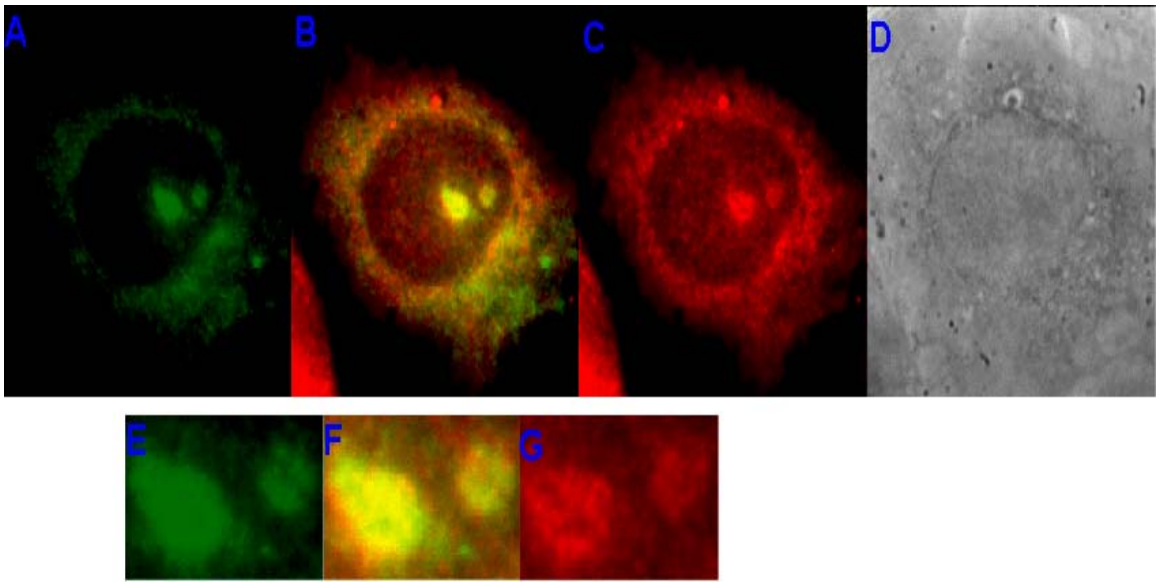


Figure 13. Results from the SRP72 and SRP RNA colocalization study in a NRK cell. This figure displays an example of an NRK cell on a slide that has gone through transfection with SRP72-GFP plasmids and then FISH using SRP probes for SRP RNA. The images have their intensity scaled the same as the controls in Figure 14 and are in the middle plane of the cell. (A) displays green fluorescence from SRP72-GFP fusion proteins. (C) displays red fluorescence from SRP RNA probes. (B) displays the combination of the green fluorescence from (A) and red fluorescence from (C). (D) displays the phase image from the NRK cell. (E), (F), and (G) display magnifications of the nucleoli in the cell in (A), (B), and (C) respectively.

Similarly to the results in Figure 13A and 13E, SRP72-GFP fusion molecules showed prominent nucleolar signal and also nucleoplasmic and cytoplasmic signal (Figure 14A and 14D). SRP RNA probes again showed prominent nucleolar and cytoplasmic signal and some nucleoplasmic signal (Figure 14H and 14N). There was onlyautofluorescence detected in cells that were transfected with control plasmids (Figure 14G and 14J) and cells that were not transfected (Figure 14M and 14P). There was also only autofluorescence detected in cells that went through FISH using SRP RNA anti-sense scrambled probes (Figure 14B and 14K) and cells that went through FISH using control probes (Figure 14E and 14Q).

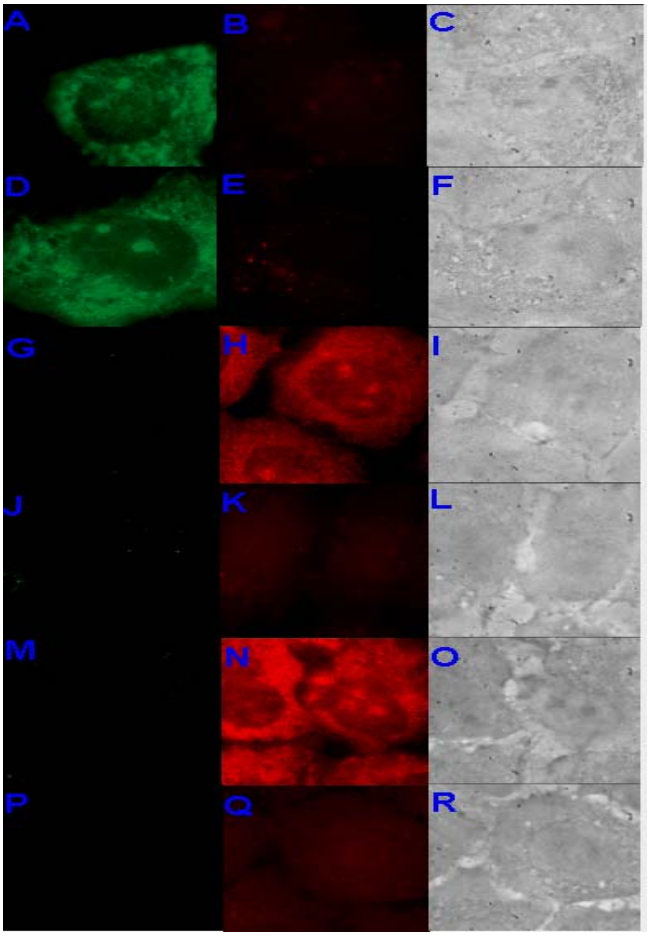


Figure 14. Controls from the SRP72 and SRP RNA colocalization study in NRK cells. Each row in the figure displays an example of an NRK cell on a slide that was either not transfected or transfected with a SRP72-GFP plasmid or control mixture and then had FISH performed using different types of probes. (A) and (D) display green fluorescence from SRP72-GFP plasmid transfected cells, (G) and (J) display green fluorescence from control mixture transfected cells, and (M) and (P) display green fluorescence from non-transfected cells. (H) and (N) display red fluorescence from FISH using SRP RNA probes, (B) and (K) display red fluorescence from FISH using SRP RNA anti-sense scrambled probes, and (E) and (Q) display red fluorescence from FISH using a control mixture. (C), (F), (I), (L), (Q), and (R) are the phase images of the control NRK cells used.

Telomerase was the next cellular component studied, and the first objective was to determine the localization of the telomerase RNA (hTR) in mammalian cells. The HeLa cell line was chosen to study the localization hTR because these cells have known hTR in them, and the GM00847 cell line was chosen as a control cell line because they are known to not contain hTR. To localize hTR, either hTR LNA or hTR PNA probes were used during the FISH procedure. Images were captured using a fluorescence

microscope and a 100X objective lens. Only one plane of the cell was captured to show the localization of hTR.

When the TH1 PNA was used during the FISH procedure, there was mostly signal in the nucleoli and cytoplasm and some in the nucleoplasm in both HeLa and GM00847 cells. Since the latter cells do not contain hTR, these results suggest that this probe had non-specific binding to other cellular components (Figure 15A and 15C). When TH4 PNA was used during the FISH procedure, there was mostlyautofluorescence and maybe some non-specific binding detected (Figure 15E and 15G). A positive control, SRP-2 PNA, was used to show specific binding to SRP RNA, which revealedsignal in the nucleoli, cytoplasm, and nucleoplasm (Figure 15I and 15K). This means that the FISH method was working correctly. There was onlyautofluorescence detected in cells that had yeast PNA and control probes used during the FISH procedure (Figure 15M, 15O, 15Q, and 15S).

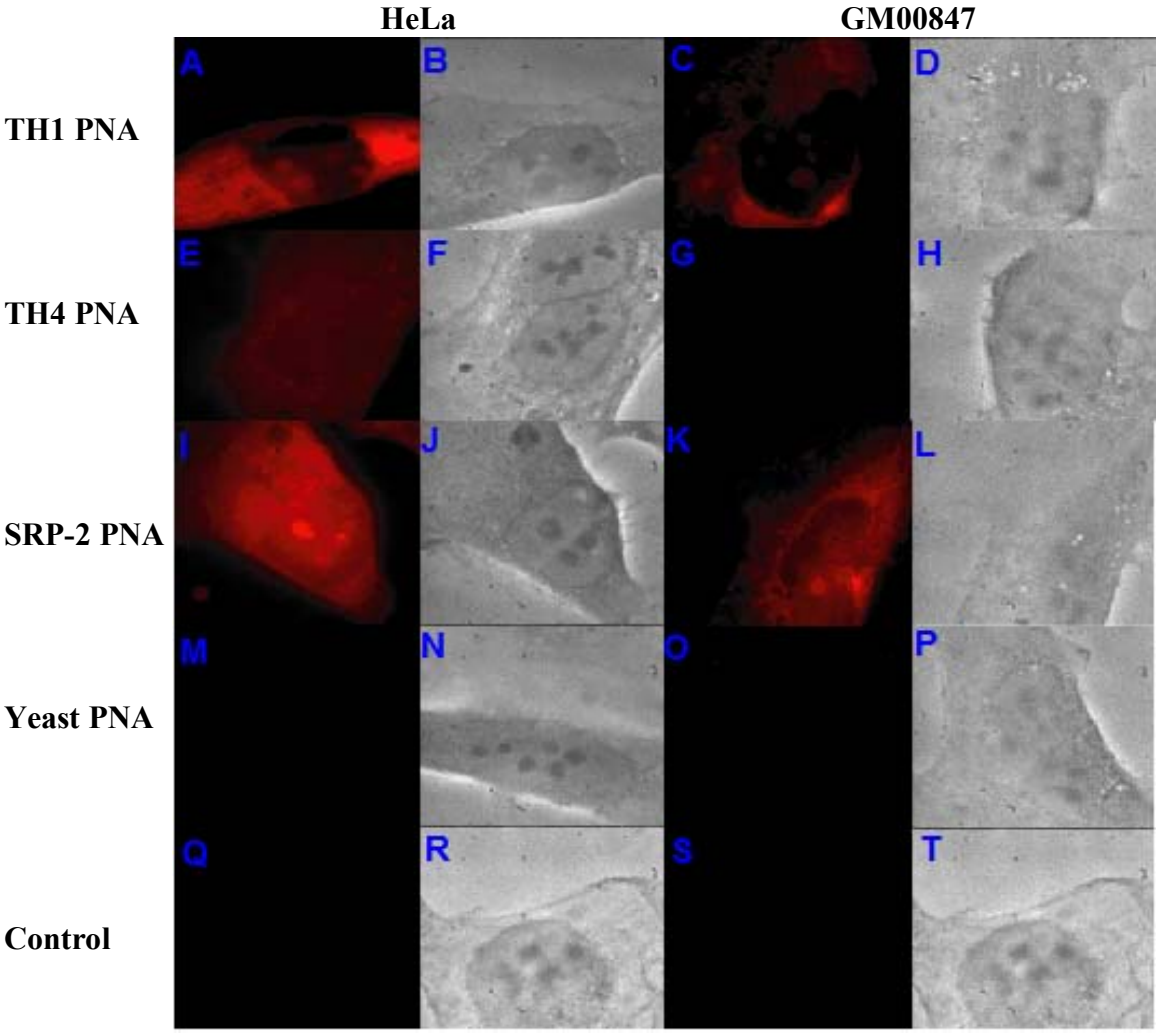


Figure 15. Results and controls from the telomerase RNA (hTR) localization in HeLa and GM00847 cells using TH1 PNA, TH4 PNA, SRP-2 PNA, yeast PNA, and control probes. This figure displays examples of the middle plane of HeLa and GM00847 cells on a slide that have their intensity scaled the same and have gone through FISH using telomerase hTR (TH1 and TH4 PNA) probes and control probes. (A) and (C) display red fluorescence from FISH using TH1 PNA probes in HeLa and GM00847 cells respectively. (E) and (G) display red fluorescence from FISH using TH4 PNA probes in HeLa and GM00847 cells respectively. (I) and (K) display red fluorescence from FISH using SRP-2 PNA probes in HeLa and GM00847 cells respectively. (M) and (O) display red fluorescence from FISH using yeast PNA probes in HeLa and GM00847 cells respectively. (Q) and (S) display red fluorescence from FISH using control probes in HeLa and GM00847 cells respectively. (B), (F), (J), (N), and (R) are phase images of the HeLa cells used, while (D), (H), (L), (P), and (T) are phase images of the GM00847 cells used.

Since the PNA probes didn't produce results that showed localization of hTR in HeLa and not in GM00847 cells, it was thought that a LNA probe to hTR would be beneficial to try. When TH1 LNA was used during the FISH procedure, there was mostly signal in the nucleoli and cytoplasm and some in the nucleoplasm in both HeLa and

GM00847 cells, suggesting that this probe, like its PNA counterpart, also had non-specific binding to other cellular components (Figure 16A and 16C). There was so much signal that when all the images were scaled to the same intensity, there was no signal showing in any of the positive or negative controls, further suggesting that there had to have been non-specific binding to other cellular components (Figure 16E, 16G, 16I, 16K, 16M, and 16O).

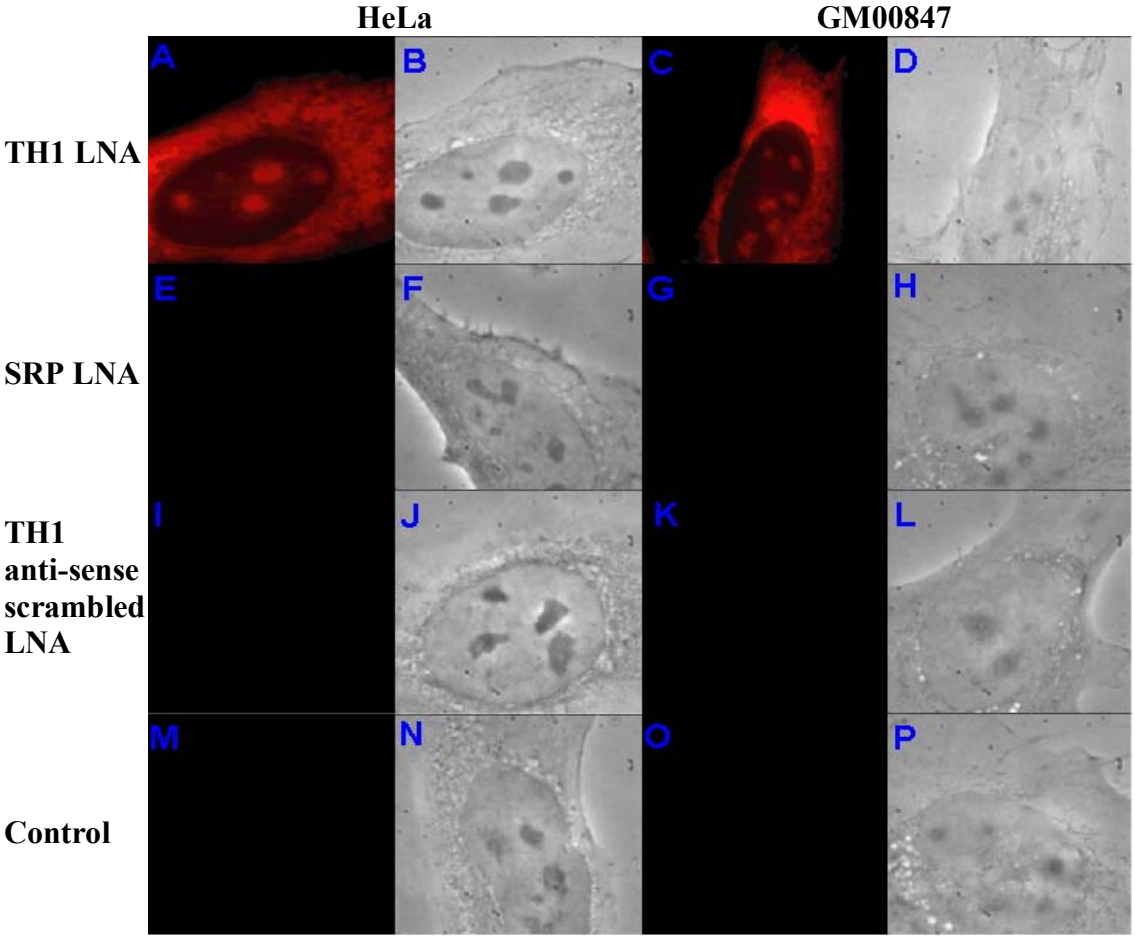


Figure 16. Results and controls from the telomerase RNA (hTR) localization in HeLa and GM00847 cells using TH1 LNA, SRP LNA, TH1 anti-sensescrambled LNA, and control probes. This figure displays examples of the middle plane of HeLa and GM00847 cells on a slide that have their intensity scaled the same and have gone through FISH using a telomerase hTR (TH1 LNA) probe and control probes. (A) and (C) display red fluorescence from FISH using TH1 LNA probes in HeLa and GM00847 cells respectively. (E) and (G) display red fluorescence from FISH using SRP LNA probes in HeLa and GM00847 cells respectively. (I) and (K) display red fluorescence from FISH using TH1 anti-sense scrambled LNA probes in HeLa and GM00847 cells respectively. (M) and (O) display red fluorescence from FISH using control probes in HeLa and GM00847 cells respectively. (B), (F), (J), and (N) are phase images of the HeLa cells used, while (D), (H), (L), and (P) are phase images of the GM00847 cells used.

Since the previous PNA and LNA probes didn't produce results that showed localization of hTR in HeLa and not in GM00847 cells, another LNA probe called the "TERNS" probe was used during FISH. The probe displayed prominent nucleolar signal and also nucleoplasmic and cytoplasmic signals in HeLa cells and less of those signals in GM00847 cells (Figure 17A and 17C). When scrambled probes were used during FISH, there was prominent nucleolar signal and also nucleoplasmic and cytoplasmic signal in both HeLa and GM00847 cells (Figure 17E and 17G). When anti-sensed probes and control probes were used during FISH, only autofluorescence was detected (Figure 17I, 17K, 17M and 17O).

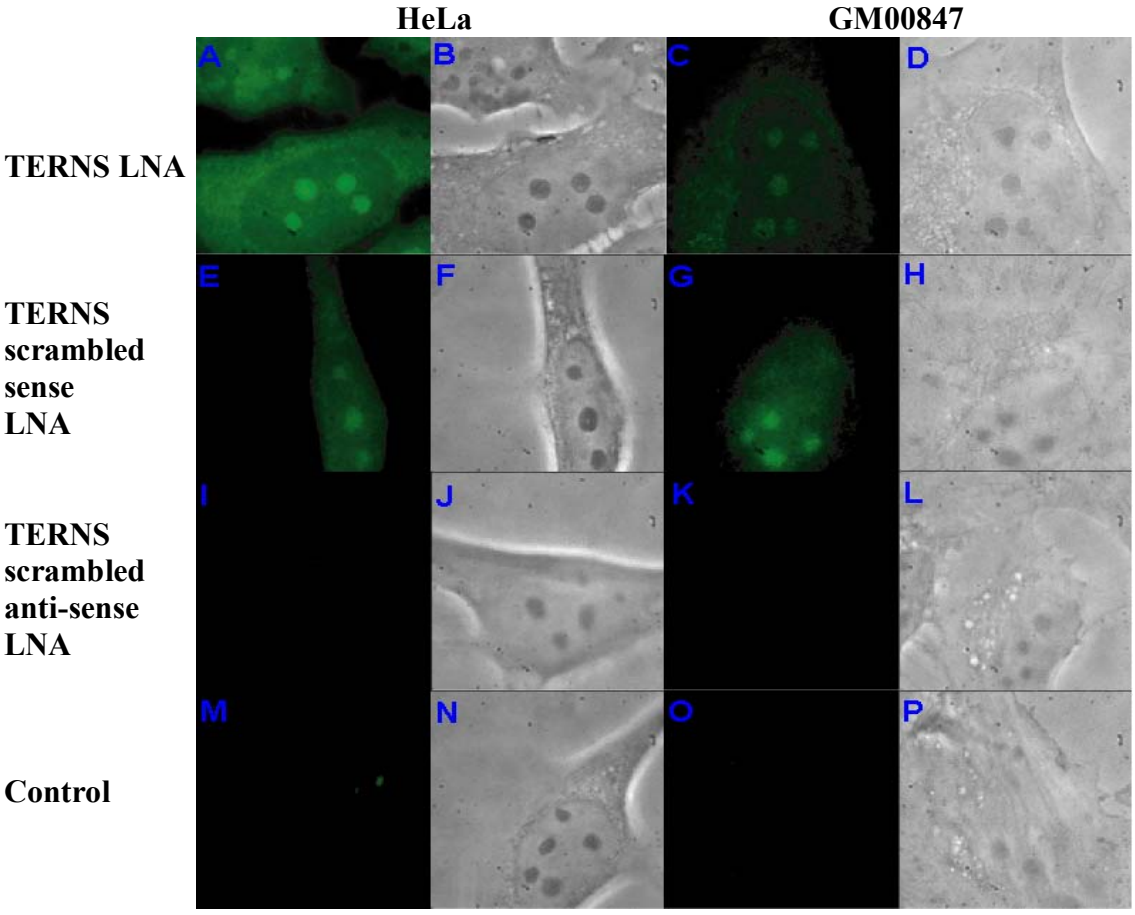


Figure 17. Results and controls from the telomerase RNA (hTR) localization in HeLa and GM00847 cells using TERNS LNA, TERNS sensescrambled LNA, TERNS anti-sense scrambled LNA, and control probes. This figure displays examples of the middle plane of HeLa and GM00847 cells on a slide that have their intensity scaled the same and have gone through FISH using a telomerase hTR (TERNS LNA) probe and control probes. (A) and (C) display green fluorescence from FISH using TERNS LNA probes in HeLa and GM00847 cells respectively. (E) and (G) display green fluorescence from FISH using the TERNS sense scrambled LNA probes in HeLa and GM00847 cells respectively. (I) and (K) display green fluorescence from FISH using the TERNS antisense scrambled LNA probes in HeLa and GM00847 cells respectively. (M) and (O) display green fluorescence from FISH using control probes in HeLa and GM00847 cells respectively. (B), (F), (J), and (N) are phase images of the HeLa cells used, while (D), (H), (L), and (P) are phase images of the GM00847 cells used.

Telomerase-associated proteins were the last cellular components studied in this project. The HeLa and U2OS cell lines were chosen to study the localization of  and TRF-2 because they are known to have those components in them, and the VA13 and GM00847 cell lines were chosen as controls because they are known to not have the those components in them.  To localize  and TRF-2, antibodies specific for those proteins and a fluorescently labeled donkey  anti-rabbit (DAR) secondary antibody were used

during the immunostaining procedure. Images were captured using a fluorescence microscope and a 100X objective lens. Only one plane of the cell was captured to show the localization of hTERT.

The first of two telomerase-associated proteins studied was TRF-2, and it was thought to localize to the nucleolus. When theTRF-2 antibody was used during the immnunostaining procedure, there was mostly signal detected in the nucleoplasm in both HeLa and U2OS cells (Figure 18A and 18C). When just the secondary antibodies were applied to the cells, onlyautofluorescence was detected in both of the cell types (Figure 18E and 18G). Since there was no clear signal detected in the nucleolus, dilutions were made to the primary antibody to see in lowering the concentration will affect the pattern of the signal detected.

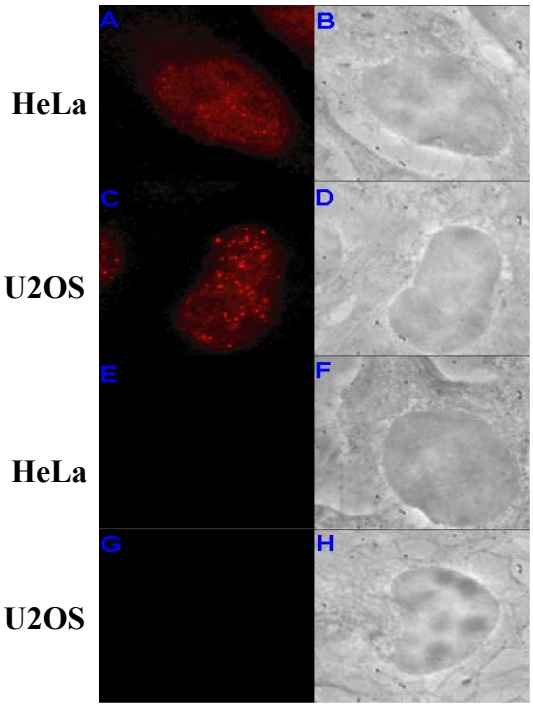


Figure 18. Results and controls from the TRF-2 ocalization in HeLa and U2OS cells using a primary TRF-2 antibody and a fluorescently labeled donkey anti-rabbit (DAR) secondary antibody. This figure displays examples of the middle plane of HeLa and U2OS cells on a slide that have their intensity scaled the same. (A) and (C) display red fluorescence from immunostaining using TRF-2 (1:100 dilution) and DAR (1:200 dilution) antibodies in HeLa and U2OS cells respectively. (E) and (G) display red fluorescence from immunostaining using just DAR (1:200 dilution) antibodies in HeLa and U2OS cells respectively. (B) and (F) are phase images of the HeLa cells used, while (D) and (H) are phase images of the U2OS cells used.

Dilutions (1:100, 1:250, and 1:500) were made for TRF-2 antibodies and then used during the immnunostaining procedure on just HeLa cells. There was mostly signal, which corresponded well to the dilutions, in the nucleoplasm in the cells (Figure 19A, 19C, and 19E). When just the secondary antibody was applied to these cells, only autofluorescence was detected (Figure 19G). Along with the results from Figure 18, these results demonstrate that the nucleoplasm is a region in the cell where TRF-2 might localize.

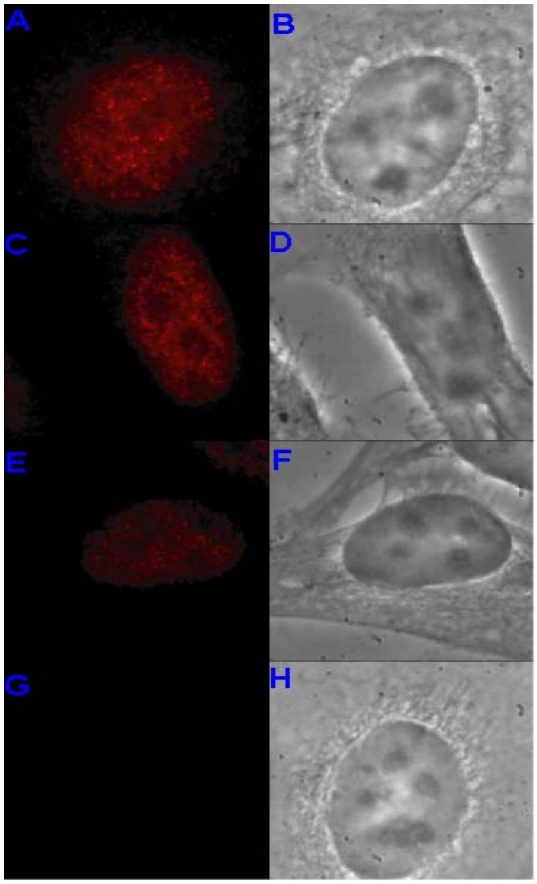


Figure 19. Results and controls from theTRF-2 localization in HeLa cells using a primary TRF-2 antibody (TRF-2) and a fluorescently labeled donkey anti-rabbit (DAR) secondary antibody. This figure displays examples of the middle plane of HeLa cells on a slide that have their intensity scaled the same. (A) displays red fluorescence from immunostaining using TRF-2 (1:100 dilution) and DAR (1:200 dilution) antibodies in a HeLa cell. (C) displays red fluorescence from immunostaining using TRF-2 (1:250 dilution) and DAR (1:200 dilution) antibodies in a HeLa cell. (E) displays red fluorescence from immunostaining using TRF-2 (1:500 dilution) and DAR (1:200 dilution) antibodies in a HeLa cell. (G) displays red fluorescence from immunostaining using just DAR (1:200 dilution) antibodies in a HeLa cell. (B), (D), (F), and (H) are phase images of the HeLa cells used.

When the antibody (hTERT) was used during the immnunostaining procedure, it was thought the results would indicate localization in the nucleolus. When different dilutions (1:250, 1:500, and 1:750) were used during the immunostaining procedure, there was mostly signal detected in the nucleoplasm in HeLa cells (Figure 20A, 20C, and 20E). When just the secondary antibody was applied to these cells, only autofluorescence was detected in the cells (Figure 20G). Like the TRF-2 results, since there was no clear signal detected in the nucleolus, dilutions were made to the primary antibody to see in lowering the concentration will affect the pattern of the signal detected. Two negative control cell lines were also used to see if the antibody is binding to a different cellular component.

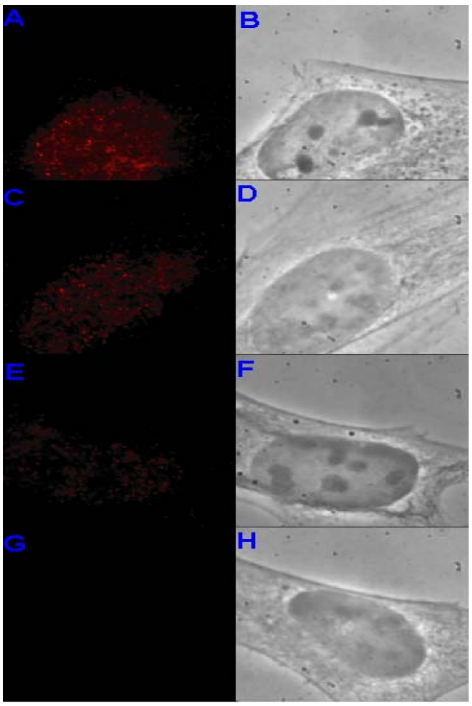


Figure 20. Results and controls from the hTERT localization in HeLa cells using a primary telomerase hTERT antibody and a fluorescently labeled donkey anti-rabbit (DAR) secondary antibody. This figure displays examples of the middle plane of HeLa cells on a slide that have their intensity scaled the same. (A) displays red fluorescence from immunostaining using hTERT (1:250 dilution) and DAR (1:200 dilution) antibodies in a HeLa cell. (C) displays red fluorescence from immunostaining using hTERT (1:500 dilution) and DAR (1:200 dilution) antibodies in a HeLa cell. (E) displays red fluorescence from immunostaining using hTERT (1:750 dilution) and DAR (1:200 dilution) antibodies in a HeLa cell. (G) displays red fluorescence from immunostaining using just DAR (1:200 dilution) antibodies in a HeLa cell. (B), (D), (F), and (H) are phase images of the HeLa cells used.

Dilutions (1:750 and 1:1500) of the hTERT antibody were used during the immnunostaining procedure on HeLa, Va13, and GM00847 cells, and there was mostly signal in the nucleoplasm that was barely aboveautofluorescence in all of the cells (Figure 21A, 21C, 21E, 21G, 21I, and 21K). When just the secondary antibody was applied to these cells, onlyautofluorescence was detected (Figure 21M, 21O, and 21Q). These results indicate that there was there was no clear signal detected in the nucleolus and that the primary antibody could either be binding weakly to hTERT in the nucleoplasm or that it is binding to another cellular protein.

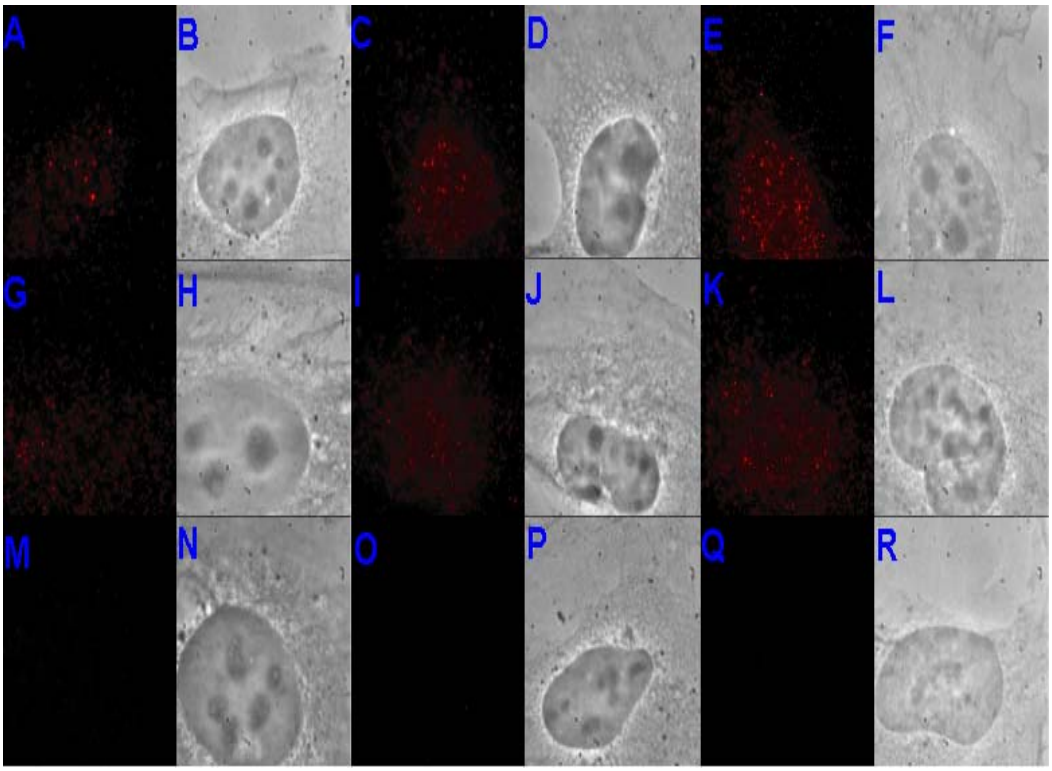


Figure 21. Results and controls from the hTERT localization in HeLa, VA13, and GM00847 cells using a primary telomerase hTERT antibody and a fluorescently labeled donkey anti-rabbit (DAR) secondary antibody. This figure displays examples of the middle plane of HeLa, VA13, and GM00847 cells on a slide that have their intensity scaled the same. (A), (C), and (E) display red fluorescence from immunostaining using hTERT (1:750 dilution) and DAR (1:200 dilution) antibodies in HeLa, VA13, and GM00847 cells respectively. (G), (I), and (K) display red fluorescence from immunostaining using hTERT (1:1500 dilution) and DAR (1:200 dilution) antibodies in HeLa, VA13, and GM00847 cells respectively. (M), (O), and (Q) display red fluorescence from immunostaining using just DAR (1:200 dilution) antibodies in HeLa, VA13, and GM00847 cells respectively. (B), (H), and (N) are phase images of the HeLa cells used. (D), (J), and (P) are phase images of the VA13 cells used. (F), (L), and (R) are phase images of the GM00847 cells used.

## Discussion

The results from the SRP study demonstrate that there is colocalization in the nucleolus between the SRP RNA and three protein subunits. By using fluorescence and confocal microscopy, three-dimensional images were created to show the overlap of the SRP proteins and the SRP RNA. This finding is consistent with the idea the SRP complex is partially assembled in the nucleolus and then exported through the nucleoplasm and into the cytoplasm where SRP function is located. Further studies need to be done on SRP protein and SRP RNA colocalization to more fully understand where SRP subunits travel in the cell.

To further study the results produced in this report on SRP, one would first want to examine the effect of exogenous SRP-GFP fusion proteins in certain cells. There is a possibility the SRP-GFP fusion proteins create an environment where the cell would want to localize them to nucleoli. However, in at least frog oocytes, Dr. Pederson's lab previously showed that endogenous SRP19 protein (i.e. not a GFP fusion protein) is localized in the nucleoli (5). There is also the possibility that the SRP RNA probe is cross reacting with other cellular RNAs that localize in the nucleolus, but this is less likely due to the controls producing consistent images and the overlap between SRP-GFP proteins and the SRP RNA probe. Though these results are very promising, more studies need to be performed to further solidify the nucleolus being the colocalization site between certain SRP proteins and SRP RNA.

As for telomerase, it was first thought the telomerase hTR and hTERT would colocalize in the nucleolus, and that TRF-2 would localize in the nucleolus. After FISH procedures were performed on HeLa and GM00847 cells using various hTR specific

probes, it was demonstrated that there was signal in the nucleolus both in HeLa cells and in the telomerase deficient cell line GM00847. These findings suggest that either the probe was hybridizing to a different cellular component or that there is some hTR in the GM00847 cells. The latter possibility seems unlikely because the cells were obtained from a well regarded national cell repository and used directly. The results from the FISH using the TERNS probe could lead to another conclusion. There is a possibility that the signal in HeLa cells is hTR hybridization and the signal in GM00847 cells is just background compared to HeLa.

The results for the two telomerase-associated proteins, TRF-2 and hTERT, were inconclusive There was no nucleolar localization like expected, but instead there was nucleoplasmic localization with concentrated signals that looked like speckles. One way to interpret these results is to say that TRF-2 and could possibly localize to specific structures in the nucleoplasm. Another way to interpret these results is to say that the antibodies weren't specific enough. In retrospect, a positive immunostaining control should have been included in my experimental design, which would involve using an antibody that did give a positive reaction for some particular nuclear protein to assure that the immunostaining protocol was working in my hands.

To conclude, the results from the SRP study demonstrate that there is colocalization in the nucleolus between the SRP RNA and the three SRP protein subunits studied. The results from the study on telomerase demonstrate that further modifications need to be made to the techniques used because of the difficulty of localizing this small RNA and its associated proteins in the cell. The results obtained will be refined to further study the localization of these molecules and other molecules in the cell. Particular

molecules that are of current interest in molecular biology are miRNAs, and their localization patterns are starting to be examined. The techniques used in this report could be modified and applied to further study miRNAs and other RNAs and proteins whose presence and function are just starting to be acknowledged.

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