DNA MAKES THE CUT: CLEAVAGE OF RNA BY A DNA ENZYME

Sushanth Bhaskarabhatla, Nicole DeCredico, Mona Gossmann, Diana Huang, Abhinav Jain, Vayu Kishore, Thomas Kuriakose, Cara Reynolds, Frank Romano, Victoria Stefanelli, Lulu Tsao, Vineet Tyagi

Advisor: Dr. Amber Flynn Charlebois
Assistant: Tim Howes

ABSTRACT

A DNA enzyme isolated via *in vitro* selection can be used to cleave essentially any RNA substrate in the presence of a divalent metal cation, preferably Mg^{2+}, provided that the substrate has a purine-pyrymidine junction. Practical applications exist, as some viral genomes are made of RNA and thus contain potential target sequences. H5N1 (avian flu) is one such virus, and a segment of its nucleoprotein gene sequence was successfully cleaved in this research. The results of this reaction, visualized through polyacrylamide gel electrophoresis and cresyl violet staining, have implications in drug therapy for this emerging infectious disease. In addition, it was verified that Mg^{2+} is not the only cation able to initiate the cleavage reaction. Ca^{2+} was utilized with the same sequences, yielding enzymatic activity similar to that of the Mg^{2+} reaction.

INTRODUCTION

DNA Enzymes

Before the isolation of DNA enzymes, storing genetic information was thought to be the sole function of DNA. Its counterpart, RNA, has long been considered the only nucleic acid capable of catalytic function. Researchers have used *in vitro* selection to isolate a specific DNA molecule that can catalyze the cleavage of RNA [1]. This process involves separating out catalytically active DNA sequences from an initial pool. Numerous rounds are carried out, with sequences from the previous round acting as templates for primer extension reactions. The DNA enzyme used in this research is referred to as 10-23 because it was the 23rd clone obtained after the 10th round of amplification. Comprised of 35 nucleotides, the enzyme contains a catalytic domain framed by two substrate-recognition domains. These binding arms possess the necessary energy to hold the RNA within the active site using Watson-Crick base pairing. Cleavage occurs on the 3' side of a purine that is followed by a pyrimidine, forming a 2',3' cyclic phosphate and a 5' hydroxyl group as products (Fig. 1). By changing the substrate-recognition domains, the DNA enzyme can be modified to cleave almost any RNA strand that includes a purine-pyrimadine junction. The length (8bp) of each substrate-recognition domain allows for a high degree of substrate specificity. While Mg^{2+} is generally utilized for the catalysis of the cleavage reaction, the reaction can occur in the presence of other divalent metal cations [2]. The sequence of the RNA substrate can also be altered in an attempt to generalize the function of the DNA enzyme. There is no variation permissible in the DNA catalytic domain — a single mutation can critically impair or even negate its catalytic ability [1].

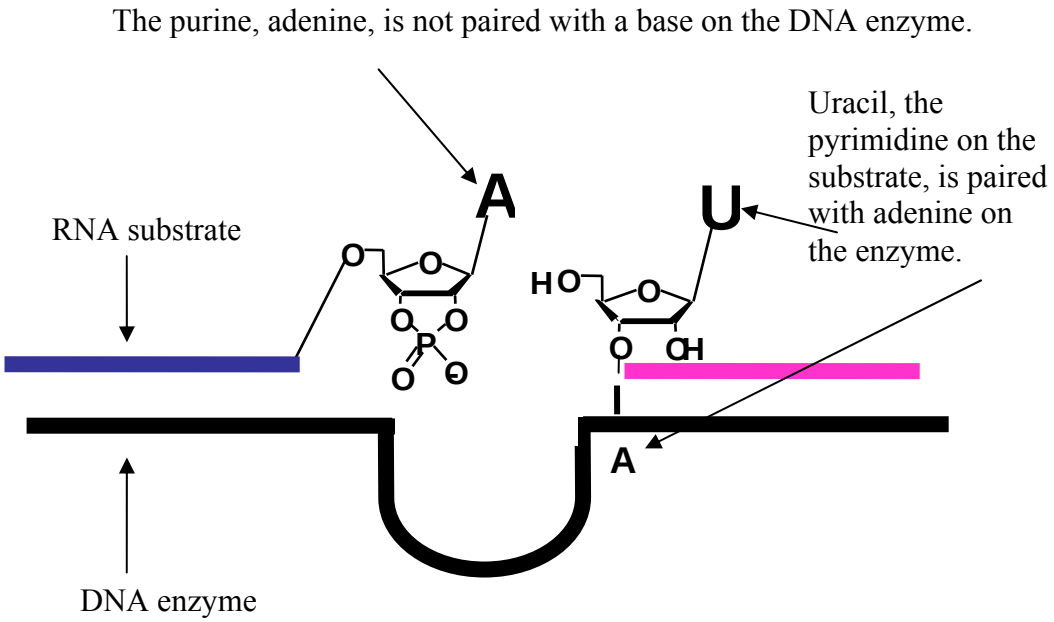

Fig. 1: Molecular structure of adenine and uracil after DNA enzyme cleaves the RNA substrate.

Nucleic acid catalysis is not an uncommon phenomenon — numerous reactions inside of living organisms are catalyzed by RNA enzymes. DNA enzymes can only be synthesized in the laboratory and are not found in nature, but greater stability and cost effectiveness make them superior candidates for kinetic applications. DNA enzymes are slightly less versatile, although more reliable, than RNA enzymes because they lack a 2'-hydroxyl group [3]. This reduction in possible hydrogen-bonding sites does not significantly impair the 10-23 enzyme's function.

Gel Electrophoresis

Polyacrylamide gel electrophoresis is used to separate cleaved nucleic acids based on their lengths and charges. The polyacrylamide gel used in this research is polymerized from monoacrylamide, which hardens to form a clear, flexible solid when TEMED and ammonium persulfate are added. Polyacrylamide was chosen over agarose because it creates a finer mesh, which is more applicable for smaller sequences such as the 11-mer and 9-mer used. In addition, agarose is not effective for single-stranded nucleic acids.

During the actual electrophoresis process, a current is passed through the gel to create an anode and a cathode (Fig. 2). Nucleic acids are negatively charged due to their phosphate groups and therefore travel from the cathode toward the anode [4]. The length of the nucleic acid influences how far it travels through the gel. Smaller fragments travel farther because they move more easily through the cross-linked polymer. Large pieces of nucleic acid are retarded on the upper portion of the gel.

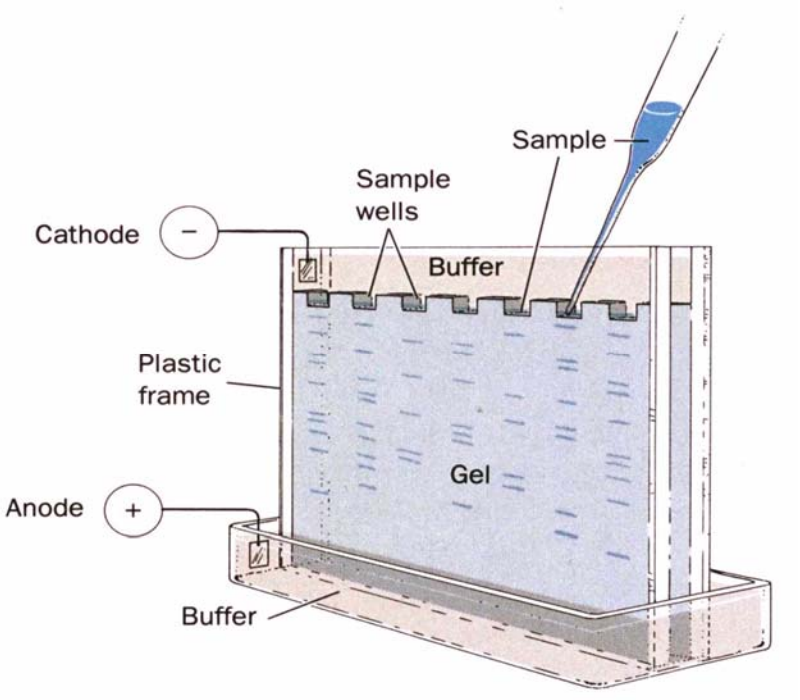

Fig. 2: Diagram of gel electrophoresis showing the anode and the cathode [4].

Once the nucleic acid molecules have migrated approximately halfway down the gel and the polyacrylamide is removed, it must be stained to show the position of the nucleic acid. Cresyl violet acetate (Fig. 3) was employed in this study because it assumes a blue color when bound to DNA and a purple color when bound to RNA. The reasons for this color difference are unknown.

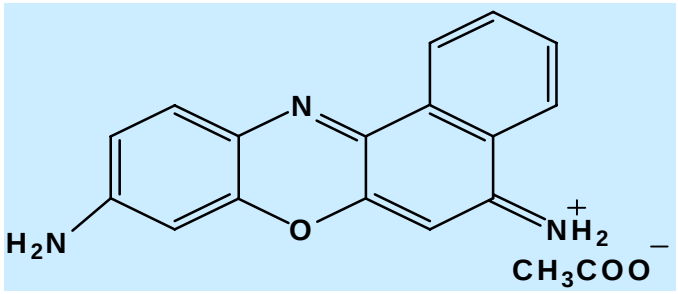

Fig. 3: The chemical structure of cresyl violet acetate

Past Research

In 1997, Santoro and Joyce isolated an RNA-cleaving DNA enzyme through *in vitro* selection [1]. The 10-23 DNA enzyme is a 35-base sequence, comprised of two binding arms that surround a 17-base active site (Fig. 4).

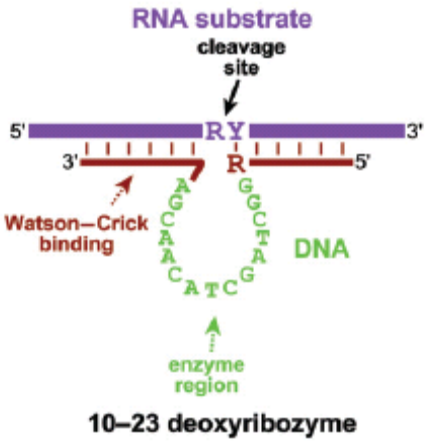

Fig. 4: The 10-23 enzyme developed in the Santoro-Joyce experiment [3].

The 10-23 enzyme was activated by Mg^{2+} and had a catalytic efficiency of 10^9 M^{-1} min^{-1}, higher than that of any other known nucleic acid enzyme. It cleaved the RNA under multiple turnover conditions; thus, the RNA concentrations were higher than those of the DNA, allowing cleavage of multiple strands of RNA. Formed by two arms and a "ring of nucleotides", the enzyme interacted with the substrate through Watson-Crick base pairing.

The original reactions were carried out in 2mM $MgCl_2$, 150 mM NaCl, and 50 mM Tris HCl (pH 7.5) at 37 °C. Later research on the 10-23 enzyme was published by Santoro and Joyce in 1998, when they studied the enzyme under a variety of conditions, including changed pH, divalent metal cofactor, and concentration of divalent metal cation [2]. The divalent metal ions Pb^{2+}, Zn^{2+}, Cd^{2+}, Mn^{2+}, Mg^{2+}, Ca^{2+}, Sr^{2+}, Ba^{2+}, and Co^{2+} were tested. The catalytic efficiency for Mg^{2+}, Mn^{2+}, and Ca^{2+} was determined. Mn^{2+} exhibited the highest level of activity of all metal cations tested in EPPS buffer solution, but not in the presence of Tris. Pb^{2+}, Mg^{2+}, and Ca^{2+} led to higher levels of activity than the remaining metals. The pH was also analyzed in conjunction with buffered solutions; reactions buffered by bis-tris propane had the highest pH range of 6.4-9.7. Since the DNA enzyme binds to RNA through Watson-Crick base pairing, the probability of substrate cleavage was far greater than the probability of enzyme-substrate dissociation. In addition, Santoro and Joyce discovered that the rate of cleavage by the 10-23 enzyme was completely independent of the RNA sequence, save for the two substrate nucleotides at the cleavage site. Substrate nucleotides in the form 5'-RU-3' (where R represents A or G) were cleaved most rapidly.

A more recent study for a practical application of the enzyme was conducted at the Laboratory of Virology at India's National Institute of Immunology [5]. The group tested the

effectiveness of various nucleic acid catalysts in cleaving HIV-1 viral genes. Their experimentation yielded the overall positive result that the 10-23 enzyme cleaves a segment of HIV RNA. The discovery that the 10-23 enzyme cleaves RNA viral sequences was a major impetus driving our decision to test the effects of the DNA enzyme on a viral gene sequence.

Avian Flu (H5N1) Nucleoprotein

H5N1, more commonly known as bird flu or avian influenza, is an RNA virus carried in the intestines of wild birds. Its symptoms include cough, fever, muscle aches, sore throat, pneumonia, severe respiratory distress, and eye infection. Although cases of humans contracting the virus from other humans are rare, the disease can be easily contracted from birds. It has a 60% mortality rate in humans and could develop into a pandemic [6].

Since H5N1 is an RNA virus, a DNA enzyme should be capable of cleaving the pathogen's RNA, rendering it incapable of transcribing and translating nucleoproteins (Fig. 5) that are essential for the replication of the virus [7]. If the DNA enzyme can successfully cleave the viral RNA, this research may have future applications for the treatment of H5N1.

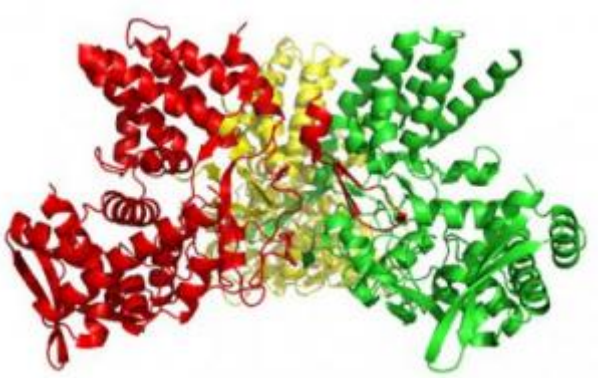

Fig. 5: Atomic structure of influenza A virus nucleoprotein [9].

MATERIALS AND METHODS

In the first reaction of this research, Santoro and Joyce's work was reproduced: the 10-23 enzyme was combined with the 20-mer RNA sequence in the presence of Mg^{2+}. In the second experiment, Ca^{2+} was used as the metal catalyst for the reaction of the same sequences . The variable in the third reaction was the sequence and substrate sequence. A 21-mer H5N1 RNA sequence was found in the GenBank database of the National Center for Biotechnology Information: 3'-UAGUCGAGGUAGUGGUAACAG-5' [8]. The sequence of the DNA enzyme was determined by preserving the catalytic domain from the 10-23 enzyme and specifying the substrate-recognition domains through complementary base pairing, excluding the adenine at the cleavage site. The resulting sequence was 5'ATCAGTCCAGGCTAGCTACAACGACACCATTGTC-3'. All reactions were carried out with a 4:1 ratio of RNA substrate to DNA enzyme. After the addition of the divalent metal catalyst, six time points were chosen to show the progress of the reaction. Polyacrylamide gel

electrophoresis provided evidence of RNA cleavage through the position and intensity of purple bands of dye.

Determination of DNA and RNA Concentrations

The DNA and RNA were ordered from Integrated DNA Technologies in pellet form and re-suspended in nanopure H_2O. The final volume of the reaction was 500 pmol of DNA and 2 nmol of RNA in 12 µL. Concentrations were verified through ultraviolet spectrophotometry at 260 nm, the maximum wavelength (λ_{max}) of nucleic acid. Beer's Law was used to calculate the concentrations: $A = \varepsilon bC$, where A is the absorbance, ε is the molar absorpivity, *b* is the pathway length, and C is the concentration. The measured absorbances and derived concentrations are listed in Table 1.

Reactions	Nucleic Acid	Absorbance	Concentration (µM)
Mg^{2+} and Ca^{2+}	D015 (35-mer enzyme)	0.4707	417
	R004 (20-mer)	0.7875	1,107
	R009 (11-mer)	0.1627	889
Bird Flu	DNA GS1 (36-mer)	0.3691	296.5
	RNA GS1 (21-mer)	0.2377	285.6

Table 1: Absorbances and concentrations of DNA enzymes, RNA substrates, and 11-mer standard.

Reaction of DNA enzyme with RNA substrate

For the Mg^{2+} and Ca^{2+} reactions, six time intervals ranging from 1 minute to 30 minutes were selected as quench points. Quench tubes contained 8 µL of 80% stop solution (50 mL prepared from 5 mL 10X TBE, 5 mL 0.5M EDTA, pH 8.0, 40 mL formamide, 12.5 g xylene cyanol FF). Xylene cyanol was the blue dye used to make the sample easier to see when loading the gel.

In the reaction tube, DNA (1.2 µL, 417 µM) and RNA (1.8 µL, 1,107 µM) were mixed in 2.4 µL of 5X reaction buffer (200 mM Tris, 750 mM NaCl for a final reaction concentration of 40 mM Tris, 150 mM NaCl). dH_2O was added to reach a 9.6-µL reaction volume. The sample was vortexed, centrifuged, and placed in a 95°C bath for 2 minutes to denature the nucleic acid. It was placed on ice for 5 minutes, allowing the nucleic acids to re-anneal, and then vortexed to evenly distribute the reactants throughout the buffer solution. The reaction was incubated at 37°C for 2 minutes to equilibrate the sample. $MgCl_2$ (2.4 µL of 300 mM, with a final reaction concentration of 60 mM) was added, and the sample was vortexed and centrifuged. Two µL were removed with a pipette and transferred to the first quench tube (as time = 0). Keeping the reaction incubated, 2 µL were transferred to tubes 2-6 at each time point. Each tube was centrifuged again to ensure mixing with the 80% stop solution.

For the experimental bird flu sequence, the procedure was repeated using different volumes of DNA (1.7 µL, 296.5 µM) and RNA (7 µL, 285.6 µM). 0.72 µL of 1.0 M $MgCl_2$ catalyzed the reaction. The last time point was set at 24 hours to increase the chance of some

catalytic activity occurring, even if the rate was exceedingly slow. When testing Ca^{2+} as an alternative divalent metal catalyst, 2.4 μL of 0.625 M $CaCl_2$ were added instead of $MgCl_2$. Time points were similar to those for the Mg^{2+} reaction.

Gel Electrophoresis

Each gel was prepared from 3.36 grams of urea, 12% polyacrylamide, 7 μL TEMED, 35 μL APS, 1X TBE, and dH_2O. The electrophoresis buffer was 1X TBE (89 mM Tris, 89 mM boric acid, 2 mM EDTA, pH 8.3). The 20-mer (un-cut RNA) and 11-mer (cut RNA) standards were loaded on opposite ends of the gel for verification of results. The six wells between the standards were loaded with reaction samples from tubes 1-6. Gels were run at 180 V for 35 to 45 minutes.

Staining and Destaining

The gels were stained in 0.02% cresyl violet for 30 minutes, and were then destained by being soaked in 5% acetic acid for 10 minutes. The acid was drained and replenished when judged to have absorbed enough dye, and this process was repeated twice. The results were recorded by digital photography.

RESULTS

Results of Mg^{2+}Reaction

The first set of reactions replicated the research formerly conducted by Santoro and Joyce in their 1997 study [1], establishing a standard reaction whose variables could be manipulated later. These reactions were carried out by combining 10-23 DNA enzyme and RNA substrate, both of which were identical to those referenced in the Santoro/Joyce paper [1], in the presence of Mg^{2+} cation, and then quenching the sample with stop solution at various time points.

Following polyacrylamide gel electrophoresis, cresyl violet staining, and ammonium acetate destaining, the gel obtained showed three rows of DNA and RNA bands, six columns representing different time points, and a 20-mer RNA standard (Fig. 6). The topmost bands, which appeared blue, were the 35-mer DNA enzyme, and remained constant in intensity because the enzyme was not consumed in the reaction. The bands in the second row were composed of 20-mer RNA substrate, which matched the standard on the left. The products of the cleavage reaction were located in the third row of bands, though the 11-mer standard was absent because it ran off the side of the gel. For all gels, the 9-mer sequences merged with the 11-mer band and were not visible as a distinct row. Increasing thickness and darkness of the product bands indicated an accumulation of product over time, proving that the Santoro and Joyce experiment was successfully replicated in our lab.

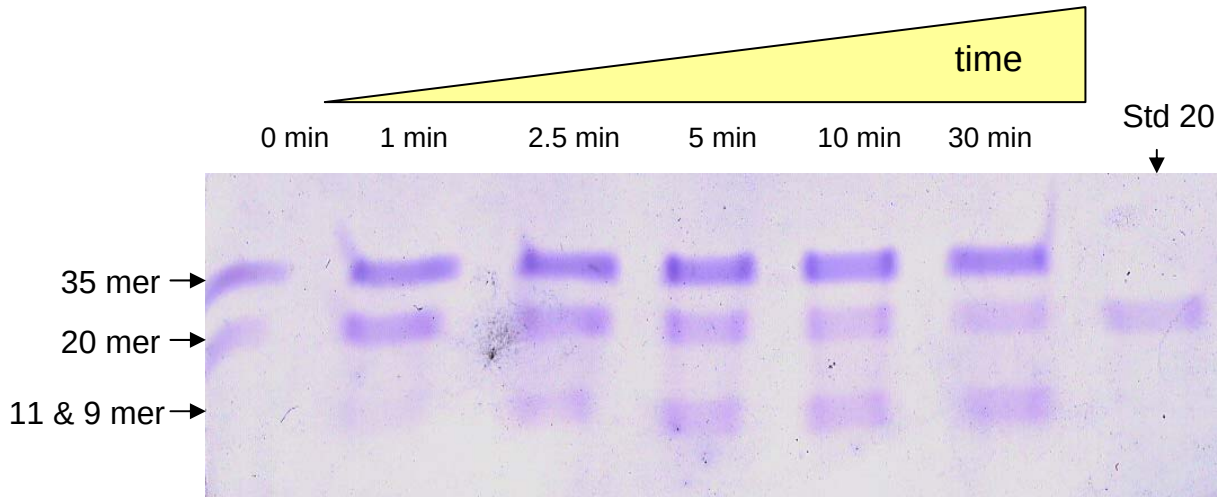

Fig. 6: Results of polyacrylamide gel electrophoresis of the Mg^{2+} reaction.

Results of Ca^{2+} Reaction

When Ca^{2+} was substituted as the divalent metal cation, cleavage also occurred. However, nucleic acid bands did not separate as distinctly on the gel and the 11-mer bands appeared to merge with the 20-mer bands (Fig. 7). The 20-mer standard was the rightmost band on the gel, and a small portion of 11-mer standard was visible on the left edge. The product band was most visible at the 24-minute time point. Aspects of the gel suggested that the reaction should be performed again to obtain more definitive results. DNA and product bands for the fifth time point were missing, though the 20-mer RNA was faintly visible. The sixth time point provided the most visible product band but also the largest and darkest substrate band. The 20-mer bands should actually decrease in intensity over time, a trend that appeared in the first four columns but not the last two.

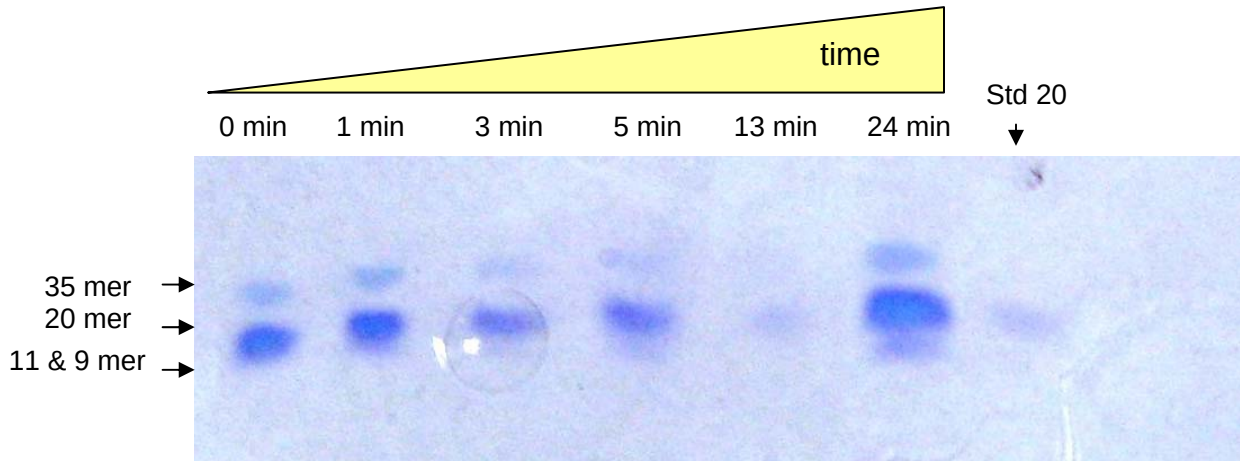

Fig. 7: Results of polyacrylamide gel electrophoresis of Ca^{2+}-mediated reaction.

Results of Avian Flu Reaction

When the reaction was run with experimental H5N1 RNA, cleavage occurred progressively over the time points. Two 11-mer standards were visible by the bands representing the 0-minute and 24-hour time points, while the 20-mer standard was a crooked line on the right edge of the gel (Fig. 8). As with the Ca^{2+} reaction gels, decreased intensity of the 21-mer was not evident at the fifth time point. The 24-hour time point indicated that the reaction ran to completion because there was no substrate remaining.

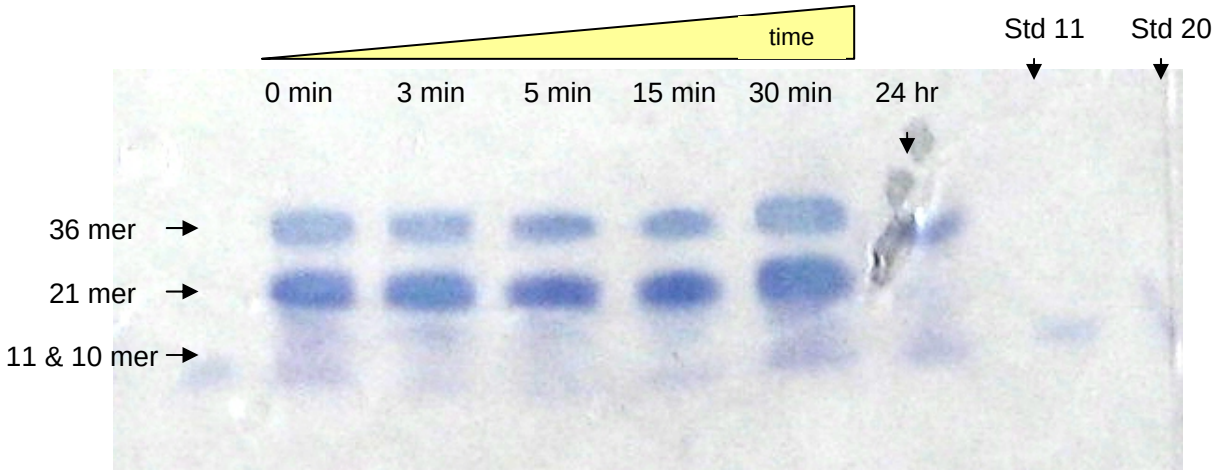

Fig. 8: Result of H5N1 cleavage experiment polyacrylamide gel electrophoresis.

DISCUSSION

Our experiment has validated the results of previous experiments by Santoro and Joyce [1]. The compatibility of the 10-23 DNA enzyme with divalent metal ion catalysis was supported by both the Mg^{2+} and Ca^{2+} cations successfully cleaving the RNA substrate. Moreover, the cleavage of H5N1 bird flu RNA demonstrated that the 10-23 enzyme could be generalized to many RNA sequences with the required bases. We were unable to quantitatively measure data in our laboratory, as we did not have access to radioactive materials for tracking the rate of cleavage. While the cresyl violet dye used in this experiment was safer and less expensive, alternative methods are more precise and sensitive to DNA and RNA, requiring only 1000 times as much nucleic acid. Although this is a considerable advantage in large research laboratories, cresyl violet is better suited to a classroom environment. With isotope tagging, we could have used an imaging device such as the Molecular Dynamics Phosphorimager. This would clarify whether the severe reduction in H5N1 RNA substrate at the 24-hour time point was due to catalysis or gradual degradation in the buffer solution. The loss of substrate was not accompanied by significant product increase.

If we had analyzed kinetics, we would also have compared the reaction rates to find the optimal concentrations of divalent metal ions. Characteristics of the substrate, such as length, symmetry, and base variation, should be further examined to determine conditions for highest catalytic efficiency. Santoro and Joyce, in their 1998 paper, postulated that optimal length of the target sequence depends on its purine and pyrimidine content [4]. The bird flu sequence, comprised of 10 pyrimidines and 11 purines, did not allow us to investigate this variable. With quantitative data, we would also be able to determine the differences between the cleavage rate for bird flu RNA and the standard RNA reaction.

Significant advances are necessary to engineer a vector by which the DNA enzyme can be applied *in vivo* rather than *in vitro*. As of now, all reactions have been conducted under laboratory conditions. Consequently, limitations specific to *in vivo* research, such as pH, temperature, and targeting of viral RNA, have not been studied to determine whether DNA enzymes can inactivate RNA in the body. Possible limitations of DNA enzymes include their ability to access RNA sequences in a folded conformation. A DNA enzyme must locate its target sequence, and after entering the cell, avoid degradation by deoxyribonuclease or other enzymes.

By performing the reaction with additional variations, measuring reaction rates, and using more precise labeling techniques, an improved DNA enzyme could be designed from the 10-23 enzyme. Overall, DNA enzymes present a multitude of opportunities for future investigation and potential therapeutic function.

CONCLUSION

The 10-23 enzyme cleaved two RNA substrates, the Santoro-Joyce sequence and a segment of the H5N1 nucleoprotein sequence. It also cleaved the former in the presence of Ca^{2+}, exhibiting activity levels similar to those of the Mg^{2+}-catalyzed reaction. The reactions were quenched after various intervals and run through gel electrophoresis, with different wells showing cleavage at each time point. The intensity of the purple bands reflected the extent to which RNA was cleaved, while the blue bands of DNA remained constant throughout. Sources of error during the reaction could have affected the substrate and product bands on the gel. The first time point reported was not at 0 seconds. The reaction had to be vortexed and spun down before it could be placed into a stop solution, causing t=0 to be inaccurate. Other time points may have been distorted by the time required to mix the metal catalysts into solution and transfer the reaction to the quench tube. Another source of error may have occurred in the creation of the polyacrylamide gels. If the chemical components of the gel had not been evenly mixed, the resulting mesh-like structure may have been inconsistent, causing an inaccurate placement of bands. Furthermore, as levels of buffer solution were not strictly monitored, this may have resulted in a crooked appearance of bands on the electorphorhesis. Overall results from the 15 gels run were positive, though some gels exhibited no reaction and others were inconclusive.

Previous experiments by Santoro and Joyce, as well as Unwalla et al., used DNA enzymes to interrupt HIV-1 gene expression [1, 2, 5]. Our reactions with H5N1 sequence sought to test broader applications for inactivation of target RNAs by DNA enzymes. If the 10-23 enzyme can cleave any RNA sequence with a purine-pyrimidine junction, it should theoretically

be able to eliminate the functionality of multiple RNA sequences. This includes those of retroviruses, as well as the mRNA transcripts of harmful DNA sequences found in bacteria, protozoans, or mutated somatic cells. Santoro and Joyce also isolated another DNA enzyme, the 8-17, that successfully cleaved RNA under simulated physiological conditions. The junction required was 3'-GA-5' instead of 3'-YR-5' (Y = U or C, R = A or G). This difference near the cleavage site provides greater flexibility for the RNA substrate.

Results derived from the reaction with Ca^{2+} confirmed Santoro and Joyce's findings that multiple metal cations can catalyze the reaction. Nevertheless, gels with Mg^{2+} reactions showed clearer visual trends and the expected changes in intensity, with 20-mer substrate being cleaved into 11-mer and 9-mer products. Due to limited experimentation time, reactions with Mn^{2+} were not performed.

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