Analysis of "Suppression of RNA Recognition by Toll-like Receptors: The Impact of Nucleoside Modification and the Evolutionary Origin of RNA **(Kariko et al.)**"

Scientific Poster on Analysis of Research Knowledge

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Overview

In this report, the team sought to determine whether naturally occurring nucleoside modifications modulate the immunostimulatory potential of RNA and the role TLRs might play in this process. Previously, it was discovered that : 1. nucleoside modified RNA activates immune cell the most. 2. RNA is more immunogenic in Bacteria just like DNA. 3. Human immunoregulatory can discriminate between eukaryotic and Bacterial mRNA. The team approached these problems with the idea that the nucleoside modification might be the reason of these. They executed 4 experiments and found out: 1. Naturally Occurring RNAs Are Not Equally Potent Activators of DCs. 2.In Vitro-Transcribed RNA Stimulates Human TLR3, TLR7 and TLR8 but most of Nucleoside RNAs are not stimulatory. 3. Modified Nucleosides Reduce the Capacity of RNA to Induce Cytokine Secretion and Activation Marker Expression by DC. And TLRs are activated differently by RNA (m6A ,s2U modifications : not activate TLR3/ and those with m5C, m5U, s2U, m6A, Ψ : not activate TLR7 or TLR8) 4. Suppression of RNA-Mediated Immune Stimulation Is Proportional to the Number of Modified Nucleosides Present in RNA. This discover might be utilized in serious necrosis problems or in vivo biotechnologies which need minimum of immune response.

Methods

MDDC generation
In this paper, GM-CSF-IL4 generated MDDC and GM-CSF-INF-a generated MDDC is used. After in-vitro cell generation, those cells were seeded into 96-well plates and exposed to R-848 or RNA with prior complexing to lipofection.

In T cells, binding of IL-4 to its receptor induces proliferation and differentiation into Th2 cells. Granulocyte-macrophage colony-stimulating factor(GM-CSF) stimulates stem cells to produce granulocytes (a type of white blood cell: neutrophils, eosinophils, and basophils) and monocytes. (GM-CSF) and interleukin-4 (IL-4) trigger monocytic differentiation into DCs so for the in vitro DC differentiation, monocyte is usually cultured in GM-CSF and IL-4. Interestingly in this paper, IFN-alpha is also used as IL-4 during differentiation. The main function of the IFN-alpha is to alert the organism in case of viral infection by detection of abnormal double stranded DNA, but also to inhibit virus multiplication by action on the translation in infected cells. However the only difference was how long the monocytes should be cultured (IFN-a: 3 days, IL-4: 5 days). Using IFN-alpha, they could get the result faster.

Lipofection
Lipofection is a lipid-based transfection technology which belongs to biochemical methods including also polymers, DEAE dextran and calcium phosphate. Lipofection principle is to associate nucleic acids with cationic lipid formulation. The lipid-based reagents used for lipofection are generally composed of synthetic cationic lipids that are often mixed with helper lipids such as DOPE(1,2-dioleoyl-phosphatidyl-ethanolamine) or cholesterol. These lipids mixture assembles in liposomes or micelles with an overall positive charge at physiological pH and are able to form complexes (lipoplexes) with negatively charged nucleic acids through electrostatics interactions. The resulting molecular complexes, known as lipoplexes, are then taken up by the cells. In this paper, levels of substances are analyzed by ELISA and FACS analysis. Also, HPLC and blotting was used to check whether the substances are well generated according to the plan.

Cytokine ELISA & Blotting
In this paper, for most of the experiments, cytokines secreted by DCs are detected by indirect Enzyme-Linked ImmunoSorbent Assay (ELISA) . Also, western blot and northern blot were used to check whether the RNA modification or generation has gone well as the desired form.

FACS analysis
FACS Fluorescence-activated cell sorting (FACS) is a specialized type of flow cytometry. It provides a method for sorting a heterogeneous mixture of biological cells into two or more containers, one cell at a time, based upon the specific light scattering and fluorescent characteristics of each cell. Flow Cytometry is a technique used to detect and measure physical and chemical characteristics (ell size, cell granularity, total DNA, new synthesized, DNA gene expression, surface receptor, intra cellular proteins, transient signal.) of a population of cells or particles. In this process, a sample containing cells or particles is suspended in a fluid and injected into the flow cytometer instruments.
FACS works as following: (1)The cell suspension is entrained in the center of a narrow, rapidly flowing stream of liquid. The flow large separation between cells relative to their diameter. Ultrasonic nozzle vibrator forces the stream of cells to break into individual droplets which mostly contains one cell per droplets. (2)An electrical charging ring is placed just at the point where the stream breaks into droplets. A charge is placed on the ring based on the immediately prior fluorescence intensity(more cell which has fluorescently-tagged antibody, higher the intensity) measurement before the break of stream, and the opposite charge is trapped on the droplet as it breaks from the stream. The uncharged droplet pass through while the charged droplets will be fall through an electrostatic deflection system that diverts droplets into containers based upon their charge. (3)Droplets containing a single cell are assigned a positive or negative charge, based on whether the cell has a fluorescently-tagged antibody. Droplets containing a single cell are then detected by an electric field and diverted into separate collection tubes according to their charge allowing for easy separation of the cells marked with the fluorescent antibody.
In this paper, the team measured CD83 and HLA-DR attaining of modified or unmodified DCs using fluorescent tag.

HPLC assay
High-performance liquid chromatography(HPLC) is a technique based on the simple fact that individual compounds behave differently in water. HPLC separates and purifies compounds according to their polarity, or their tendency to like or dislike water. HPLC commonly consists of: Mobile phase, Stationary phase which is also called HPLC column, made up with carbon chains, sample detector containing flow cell connected with computer data station. The detected data is commonly analyzed by Chromatogram.

Elution works as following: As the gradient kicks in, the solvent concentration increases while the water concentration decreases. This makes the mobile phase more and more apolar. Compounds contained in the sample will stick to the carbon chains in the column, with the most apolar compounds sticking the strongest, and the most polar compounds sticking weakly. The compounds bind to the column and are flushed out at different times, depending on whether they are more likely to stick to the column or the mobile phase as it is pumped through. The time that each compound elutes (or flushes out) from the column is known as that compound's retention time (R_t).
The detector contains a flow cell that detects each separated compound band against a background of mobile phase. An appropriate detector has the ability to sense the presence of a compound and send its corresponding electrical signal to a computer data station.
Chromatogram is a series of peaks rising from a baseline drawn on a time axis. Each peak represents the detector response for a different compound. the chromatogram begins when the sample was first injected and starts as a straight line set near the bottom of the screen. This is called the baseline; it represents pure mobile phase passing through the flow cell over time. As the analyte compound passes through the flow cell, a stronger signal is sent to the computer. The line curves, first upward, and then downward, in proportion to the concentration of the compound in the sample band. This creates a peak in the chromatogram. After the compound passes completely out of the detector cell, the signal level returns to the baseline; the flow cell now has, once again, only pure mobile phase in it.

Hence, HPLC(RP-HPLC) works according to following process: (1)Pumps the mobile phase, usually the mixture of water and organic solvent, into the HPLC column. (2)Along the pathway of (1), inject the sample so that the sample and mobile phase move through the HPLC column. (3)Elution of compounds in the sample along a concentration gradient(commonly: the percentage of water in the mobile phase decreases over time, while the percentage of the apolar solvent increases simultaneously) in HPLC column. (4)Detector's flow cell deteects the compound and it is shown as electrical data by chromatogram. (One peak represents one compound)

In this paper, nucleotides were detected by using a photodiode array at 245 nm.

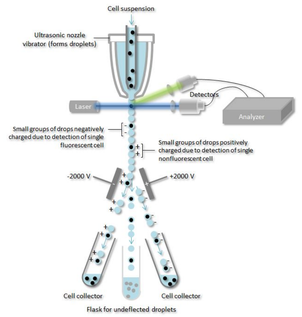
1.Naturally Occurring RNAs Are Not Equally Potent Activators of DCs

To investigate the immunostimulatory potential of different cellular RNA subtypes, and to identify the likely RNA components from necrotic cells activating DCs, team used the following method:

1. Isolate RNA from mammalian, E-coli sources: (1) different subcellular compartments which are: cytoplasm, nucleus, and mitochondria. (2) total RNA, tRNA, and polyA-tail-selected mRNA. 2. Complex them in lipofectin and add to MDDC generated with GM-CSF and IL-4. with or without the RNase digestion. 3.TNF-a from DCs measured by ELISA

(RNA samples were digested with Benzonase, capable of cleaving both ssRNA and dsRNA. RNA signaling was abolished in MDDCs so verifying that RNA is the active component that triggers of RNA with different primary sequences ranging in TNF-α secretion.)

The control: lipofection for negative control and the poly, R-848, in vitro transcribed RNA 1886 for positive control. As you can see in Figure 1, mammalian tRNA did not induce any detectable level of TNF-α. In Vitro-Transcribed RNA stimulated human TLR3. Mitochondrial mt RNA was the most inducing. This is due to mtRNA 's properties: most potent, hardly modified, most similar with bacterial RNA. Conversely, tRNA which is most modified did not induced the TNF-α. In the case of E-coli, tRNA induced the TNF-a although it is modified. Total RNA sample both induced TNF-α but, E-coli 's induced more than mammalian 's. This result disputes that not all naturally occuring RNAs are equal. Also it shows that nucleotide modification and activation of TNF is inversed.



Method. FACS analysis

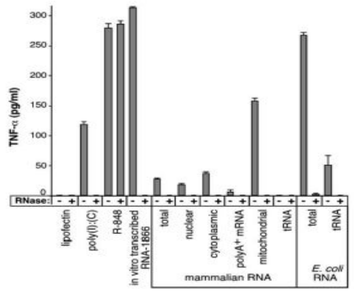


Figure 1. Production of TNF-α by MDDCs Transfected with Natural RNA

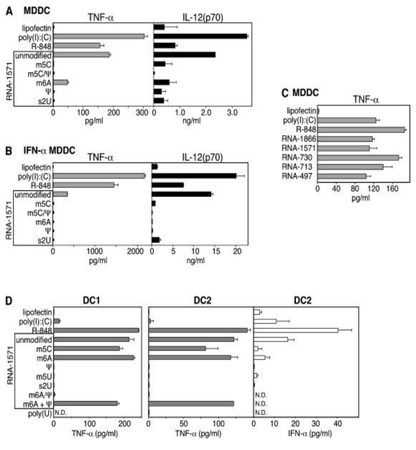
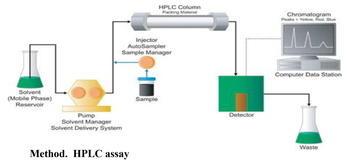


Figure 3. Cytokine Production by RNA-Transfected DCs

Method. HPLC assay



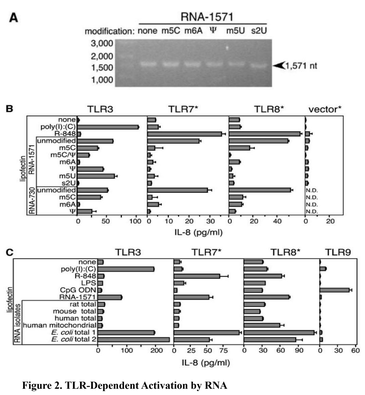
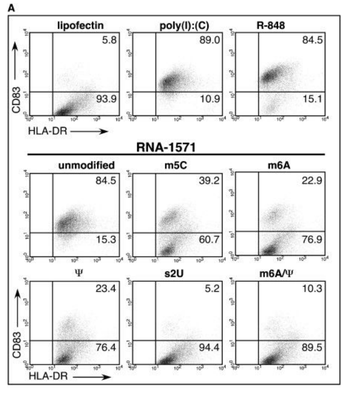


Figure 2. TLR-Dependent Activation by RNA



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	TNF-α+	CD80	CD86
	pg/ml	mean fluorescence	
lipofectin	0	7.6	55.3
poly(I):(C)	45.6	59.4	257.4
R848	48.3	55.2	235.4
RNA-1866			
unmodified	26.7	52.7	246.4
m5C	0	16.4	108.6
m6A	0	12.4	78.4
Ψ	0	12.0	87.5
s2U	0	8.0	62.7
m6A/Ψ	0	8.6	68.4

Figure 4. Activation of DCs by RNA

2. In Vitro-Transcribed RNA Stimulates Human TLR3, TLR7 and TLR8 but most of Nucleoside RNAs are not stimulatory

In first experiment, set out RNA with selected modifications(one or two of four NTPs were substituted with modified NTP) Modified RNAs analyzed by denaturing gel electrophoresis. In vitro - transcribed RNA activates human TLR 7, chemically synthesized oligoribonucleotides stimulate murine TLR 7, human TLR 8

To find out how nucleotide is modificated can influence RNA –mediated activation of TLRs utilize human 293 cell lines

1) TLR3- transformed cells were treated with lipofectin - complexed RNA -> high levels of IL-8 secretion
2) RNA containing m6A, s2U modifications did not induce IL-8(detectable level)
3) other nucleoside modification such as m5C, m5U, Ψ or m5C/Ψ in RNA -> less or not activate TLR 3

(Figure 2B, 2C and not shown) RNA containing any of the nucleoside modifications did not stimulate cell and to rule out clonal artifacts were responsible for RNA -induced stimulation

-> RNA activates human TLR and nucleotide modification suppress the ability of RNA to stimulate TLRs RNA m6A, s2U modifications suppress the ability of RNA to stimulate TLR 3, but m6A, m5C, s2U, Ψ modification block stimulation of TLR 7, TLR 8

In second experiment, RNA isolated from natural source were tested

1) RNA from different species were transfected into 293 cells -> not induce substantial IL-8 secretion. but RNA from different two different bacterial cell -> induce robust IL-8 secretion (Figure 2C)
2) mitochondrial RNA from human stimulated human TLR8 but not TLR3 or TLR7 (Figure 2C)

Collectively, RNA that contains modified nucleosides and isolated from bacteria or mitochondria stimulate selected human TLRs, whereas total mammalian RNA abundant in nucleoside modification are non or minimally stimulatory

3. Modified Nucleosides Reduce the Capacity of RNA to Induce Cytokine Secretion and Activation Marker Expression by DCs

[Table 3.] Demonstrates that nucleoside modifications diminish the ability of RNA to induce TNF-α and IL-12 secretion. When other sets of RNA with the same base modifications but different primary sequence and lengths were tested or When the RNAs were modified by adding 5' cap structure or 3' end poly A-tail or by removing the 5' triphosphate moiety, the results were similar. RNAs of different length and sequence induced varying amounts of TNF-α from DCs.

However, when MDDCs from different donors were used. More variability can be found. In experiments, MDDCs responded to RNA treatment(Table 3.A). But, the presence of m6A reduced the RNA-mediated MDDC activation than m5C or Ψ did. Under those environment, the relative sensitivity of MDDCs to poly(I):(C)and R-848 treatments also differed. By using Northern analysis, find out the reason of this variability. Cellular uptake and stability of the transfected RNAs were not influenced by the nucleoside modification such as m5U, m5U, Ψ etc. (data not shown)

To determine whether primary blood DCs responded to RNA like generated DCs, Use primary monocytoid(DC1), and plasmacytoid(DC2, absence of TLR3). Transfection of vitro transcripts, both cell induced TNF-α.(Figure 3.D). transcripts which uridine was replaced with m5U, Ψ or s2U were not stimulatory of DC1 and DC2 but RNA containing m5C and m6A were inducers of cytokines.

And more experiments, Find out transcripts with m6A, Ψ double modification were a nonstimulatory, whereas the mixture of RNA with single type of modification was a cytokine inducer. This suggested that primary DCs have an additional RNA signaling entity that recognizes m5C, m6A modified RNA and whose signaling is inhibited by modification of U residues.

[Figure 4] revealed that modified nucleosides decrease the ability of RNAs to induce cell surface of expression of CD 80, CD 83, CD86 and MHC II

Collectively, Capacity of RNA to induce DCs to mature and secrete cytokines depends on subtype of DC and characteristics of nucleoside modification present in the RNA

4. Suppression of RNA-Mediated Immune Stimulation Is Proportional to the Number of Modified Nucleosides Present in RNA

Question: What is the minimal frequency of specific modified nucleosides sufficient to limit the immunostimulatory potential of RNA?

Method 1: Transcription in vitro of RNA in the presence of decreasing amounts of m6A, Ψ, m5C and increasing amounts of unmodified NTPs.
Expectation: Incorporating of modified nucleoside phosphates into RNA is proportional to the ratio contained in the transcription reaction.
Result [HPLC analysis]
1: In the presence of UTP:ΨTP in a same ratio, nearly equal amounts of incorporated UMP, ΨMP were released after digestion of transcribed RNA (Figure 5A)
2: An increasing amount of modified nucleosides proportionally inhibited the capacity of RNA to induce TNF-α (Figure 5B).
3: Inhibition of Cytokine secretion was detected in the presence of 0.2%-0.4% m6A, Ψ or m5C in RNA corresponding to about 3-6 modified nucleosides per RNA molecule (Figure 5B).
4: Test RNAs with modified nucleoside levels of 1.7%-3.2% - RNA maintained only half of its capacity to induce expression of TNF-α.
5: Similar experiments were performed on TLR expressing 293 cells. - A higher percent of modified nucleoside content was required to inhibit RNA-mediated signaling events (data not shown).

Method 2: Chemically synthesized 21-mer ORNs with phosphodiester internucleotide linkages and 5′ monophosphate and identical primary sequences but with modified nucleosides(m5C, Ψ, or 2′-O-methyl-U (Um)) (Figure 6A).
Results
1: Although short unmodified ORNs induce TNF-α secretion, the presence of a single nucleoside modification could abolish this effect (Figure 6B).
2: Control ORN induced 293-hTLR8 cells to secrete IL-8 whereas cells containing modified nucleosides did not.
3: When ORNs were tested in hTLR3 or hTLR7 expressing cell lines, no IL-8 secretion was found under any conditions (data not shown).
4: [Northern assay] Test the 21-mer chemically synthesized ORNs along with 31-mer in vitro transcripts for their ability to induce TNF-α mRNA in MDDCs. - ORNs containing a single modified nucleoside induced less TNF-α mRNA, ORN2-Um, the 2′-O-methylated ORN, was the least stimulatory (Figure 6C).

Conclusion & Our Thoughts

Conclusion
1. DNA and RNA stimulate the mammalian innate immune system through activation of Toll-Like Receptor. TLR3 can be activated by dsRNA, TLR9 can be activated by DNA Containing unmethylated CpG island(In eukaryotes, DNA CpG island is methylated), TLR 7 can be activated by ssRNA, TLR8 can be activated by RNA Oligonucleotides
→ Mammalian mRNA have modification. But Bacterial mRNA Contains no nucleoside modifications. It can be distinguished by immune cell

2. modified Nucleosides diminish the ability of RNA to induce IL-12. TNF- α. modifying adenine, uracil, cytosine suppresses the capacity of RNA to induce immunoactivity. RNA with modification did not activate TLR8, TLR7
1) RNA containing m6A or s2U modifications did not induce TLR3
2) RNA containing m6A, m5C, m5U, s2U, pseudo modification block stimulation of TLR7, TLR8
→ RNA containing naturally occurring modified nucleoside(m5C, m6A, m5U, pseudouridine or 2'-o-methyl-U) can eliminate or reduce activation of human dendritic cells

: Research about Nucleoside modifications in pathogen's RNA is a significant resource about treatment of cancer that makes abnormal genes

Our thoughts
1. Why RNA is used for identification of immune system and why DNA is not used?
Cell usually contain 5 to 10 times more RNA than DNA, RNA could be a rich molecular source for sampling by immune system.

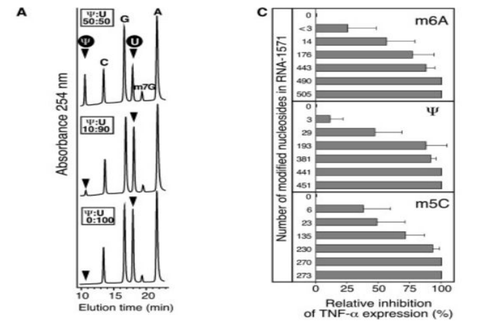
2. A study on the causes of inducing nucleoside modification. The immune potential of mammalian RNA might also explain why degradation during apoptosis is so critical. It does not induce immune response. Although less described, a well-orchestrated degradation of cellular RNA also occurs in apoptotic, but not necrotic.

3. If the cause that induces nucleoside modification is identified, it can be used for cancer treatment. (tumor necrosis, normal cell necrosis etc.)

4. Currently, one of the methods to treat autoimmune diseases like Systemic Lupus Erythematosus (SLE) is to use TNF-a blockers. However, using TNF-a blockers can cause cancer as a side effect. The point at which cancer does not occur while treating autoimmune diseases is different for each individual so it can be the next research,

5. If using in vivo biotechnologies (drug delivery using your own cells, organoids, etc.) needs to minimize the immune response, it can minimize the immune response.

6. Also found that the human immune response can discriminate between eukaryotic and bacterial mRNA → found in association with nucleoside mod: potent immune stimulation with bacterial but not with mammalian was due to difference in modified nucleoside content (amount of modified rRna) + Also find out what kind of mod is also important



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Modified nucleoside in reaction[a] (%)	m6A						Ψ						m5C					
	0	1	10	50	90	99	100	1	10	50	90	99	100	1	10	50	90	99	100
in RNA[b]																			
expected (%)	0	0.3	3.2	16	29	32	32	0.3	2.9	14	26	28	29	0.2	1.7	9	16	17	17
measured (%)	0	<0.2	0.9	11	28	31	32	0.2	1.9	12	24	28	29	0.4	1.4	9	15	17	17
measured (number)	0	<3	14	176	443	490	505	3	29	193	381	441	451	6	23	135	230	270	273

Figure 5. Analyzing RNA Containing Different Amounts of Modified Nucleosides

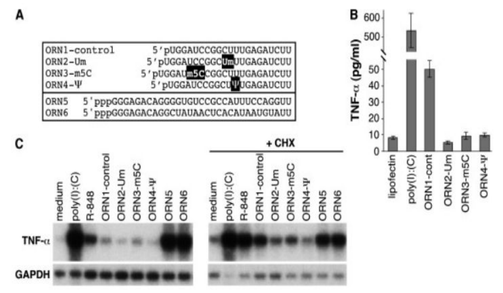

Figure 6. TNF-α Expression by RNA-Transfected DCs

Terminology

Innate immune system
Innate immunity provides the early line of defense against microbes. It consists of cellular and biochemical defense mechanisms that are in place even before infection and are poised to respond rapidly to infections.

Dendritic cell(DCs)
DCs that originate from Hematopoietic stem cell phagocytosis antigen and make antigen small fragment. Through this process DCs can securate CD80,CD86 to activate T cell . Antigen presenting cell and thus initiate all T-cell-dependent immune responses.

Toll-like receptors (TLRs)
The innate immune system is initiated by the recognition of pathogen-associated molecular patterns (PAMPs) and endogenous damage-associated molecular patterns (DAMPs-> molecular pattern of damaged tissue or cell) by pattern recognition receptors (PRRs). Among all the PRRs identified, the TLRs are the most ancient class, with the most extensive spectrum of pathogen recognition.

Monocyte-derived Dendritic cells (Mo-DC)
Mo-DC are a distinct DC subset, involved in inflammation and infection, they originate from monocytes upon stimulation in the circulation. Their activation and function may vary in autoimmune diseases.

Tumour Necrosis Factor alpha (TNF-α)
TNF-α, is an inflammatory cytokine produced by macrophages, monocytes during acute inflammation and is responsible for a diverse range of signalling events within cells, leading to necrosis or apoptosis. TNF-α-dependent maturation of local dendritic cells is critical for activating the adaptive immune response to virus infection. largely produced by Ly6c(+)CD11b(+) DCs. Induce necrosis.

Endosome acidification
endosome acidification induce endosome escape of virus virus. During the endosomal journey, acidification triggers a conformational change of the virus spike protein hemagglutinin (HA) that results in escape of the viral genome from the endosome into the cytoplasm.

Interleukin-2 (IL-2)
IL-2 is a 15-kDa cytokine predominantly secreted by activated T cells and represents a key player in the cell-mediated immune response in allograft rejection. IL-2 has an immunoregulatory role; it promotes the growth and development of peripheral immune cells in the initiation of the (defensive) immune response, and keeps them alive as effector cells.

Interferon
A group of signaling proteins made and released by host cells in response to the presence of several viruses. they trigger killer immune cells to fight those invaders. interfere with viruses and keep them from multiplying.

RNAP
RNAP is RNA polymerase or DNA-directed/dependent RNA polymerase. The enzyme that Synthesize RNA that is complementary DNA template. Using the enzyme helicase, RNAP locally opens the double-stranded DNA. One strand of the exposed nucleotides can be used as a template for the synthesis of RNA, a process called transcription. Transcription factor and its associated transcription mediator complex must be attached to a DNA binding site(promoter region) before RNAP can initiate the DNA unwinding at that position. RNAP not only initiates RNA transcription, it also guides the nucleotides into position, facilitates attachment and elongation, has intrinsic proofreading and replacement capabilities, and termination recognition capability.