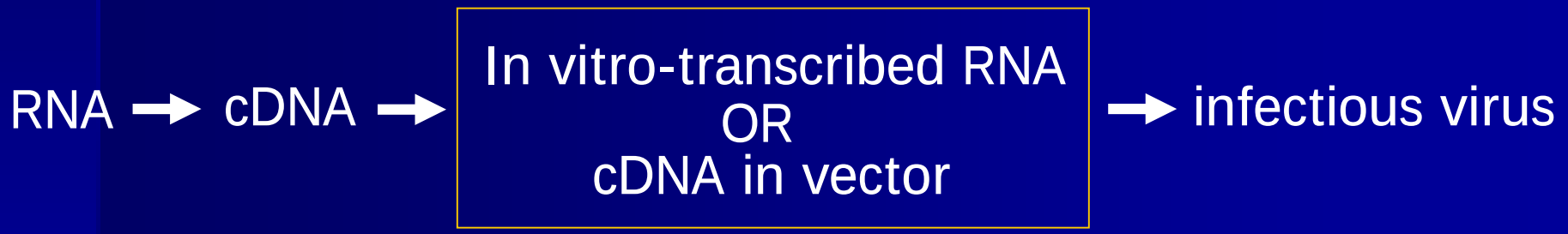
Reverse Genetics of RNA Viruses

Reverse Genetics (RG)

- The creation of a virus with a full-length copy of the viral genome
- The most powerful tool in modern virology

RG of RNA viruses

- Generation or recovery (rescue) of infectious virus from cloned cDNA



"Infectious Clone"

Nature of RNA viruses

- Polarity (+ sense or – sense)
- Size of the genome
- Segmented or not
- Site of replication (nucleus or cytoplasm)

Families of RNA Viruses

	Non-segmented	Segmented
+ve sense	Arteriviridae: 13-15 kb (PRRS) Caliciviridae: 7.4-7.7 kb (Hepatitis E) Coronaviridae: 27-32 kb (SARS) Flaviviridae: 9.5-12.5 kb (West Nile) Picornaviridae: 7.2-8.4 kb (FMD)	Birnaviridae: DS RNA: 6 kb (IBD) Reoviridae: DS RNA: 16-27 kb (Blue Tongue) Arenaviridae: ambisense: 10.6 kb (LCV)
-ve sense	Rhabdoviridae: 11-15 kb (Rabies) Paramyxoviridae: 15-16 kb (Newcastle Disease)	Bunyaviridae: 11-20 kb (Hanta) Orthomyxoviridae: 10-13.6 kb (Influenza)

Polarity

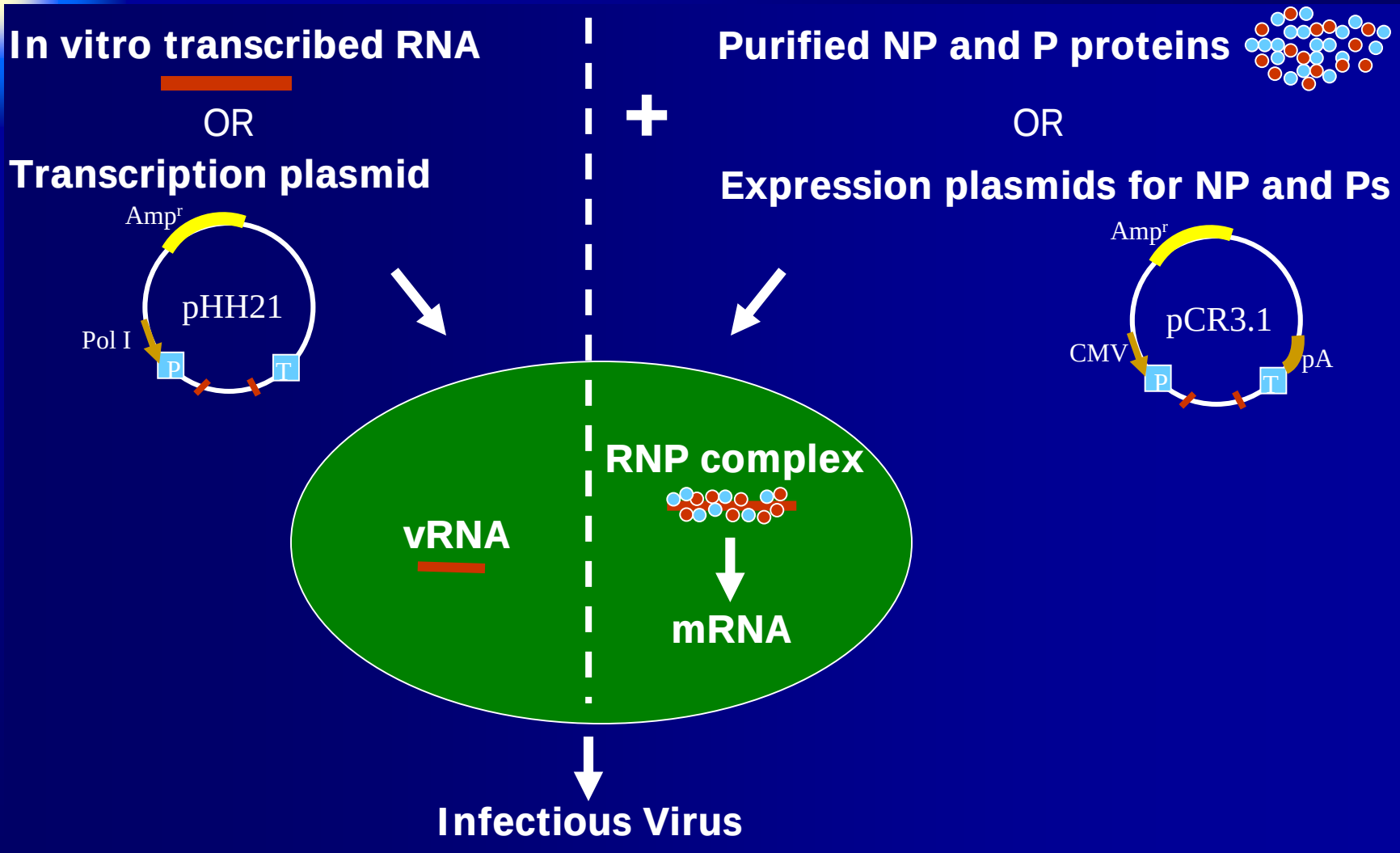
- Plus-stranded RNA viruses

  - deproteinated genomes of these viruses are able to utilize the host cell machinery to initiate their life cycle

- Negative-stranded RNA viruses

  - requires encapsidation with the viral nucleoprotein before it can serve as a functional template to initiate transcription/replication

Schematic Diagram of RG Systems



Construction of a full-length cDNA clone

- Long and tedious!
- Require the presence of the entire viral sequence
  - published sequence
  - or sequencing new isolate
- cDNA synthesis
  - require thermostable and high fidelity reverse transcriptase and DNA polymerase
  - require systematic assembly of large RNA genome
  - difficult to produce in vitro transcripts devoid of vector derived sequences
- Cloning
  - instability of full-length cDNA clones in bacteria
- Sequence verification

Plus-stranded RNA viruses

- Poliovirus infectious clone (1981)
  - Racaneillo and Baltimore, Science 214:916
  - cloned in bacterial plasmid pBR322

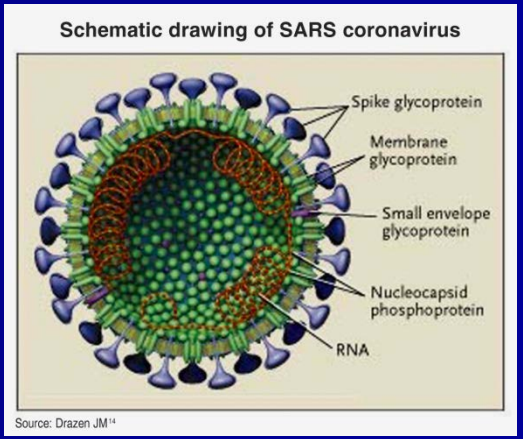
- Coronavirus
  - Almazan et al., 2000 (PNAS, 97:5516)
  - Yount et al., 2000 (J Virol, 74:10600)
  - Thiel et al., 2001 (J Gen Virol, 82:1273)

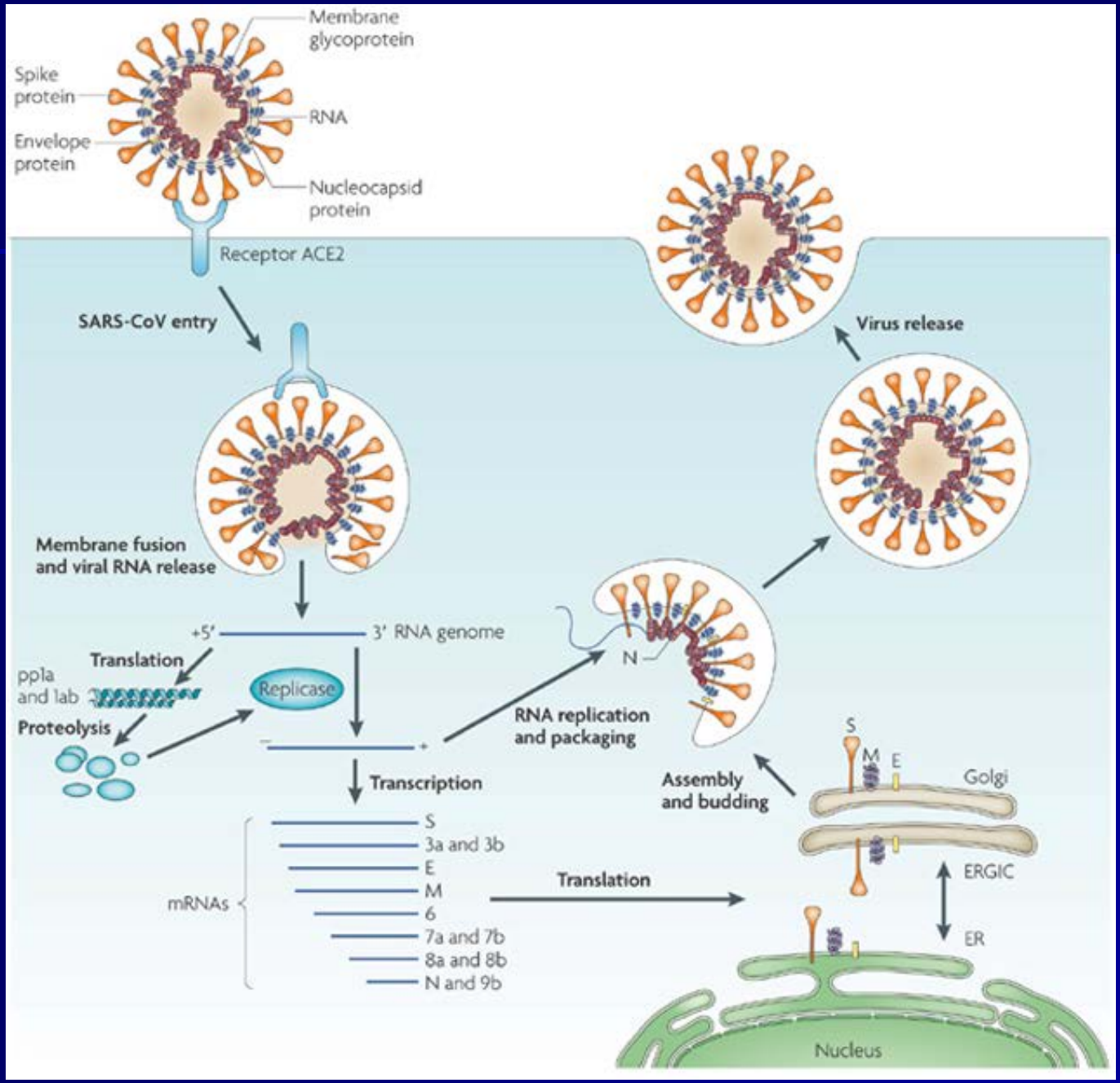
Reverse genetics with a full-length infectious cDNA of severe acute respiratory syndrome coronavirus

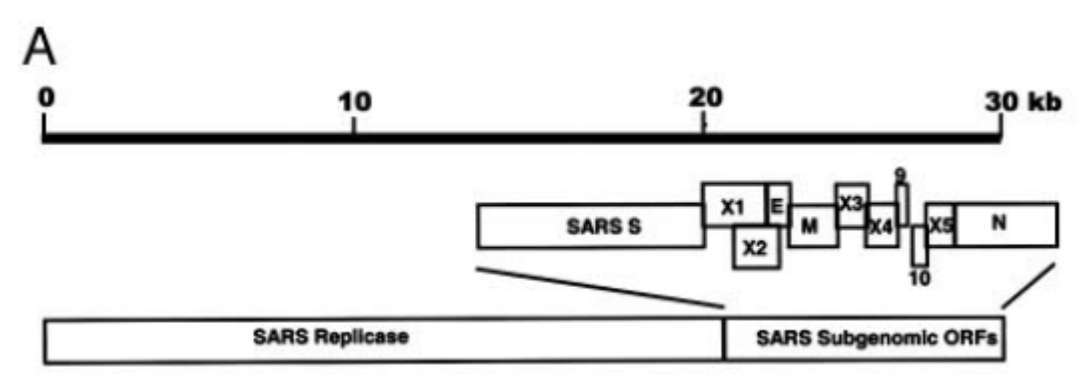
Boyd Yount*†, Kristopher M. Curtis*†, Elizabeth A. Fritz‡, Lisa E. Hensley‡, Peter B. Jahrling‡, Erik Prentice§, Mark R. Denison§, Thomas W. Geisbert‡, and Ralph S. Baric*¶‖

Severe acute respiratory syndrome, is the disease caused by SARS coronavirus

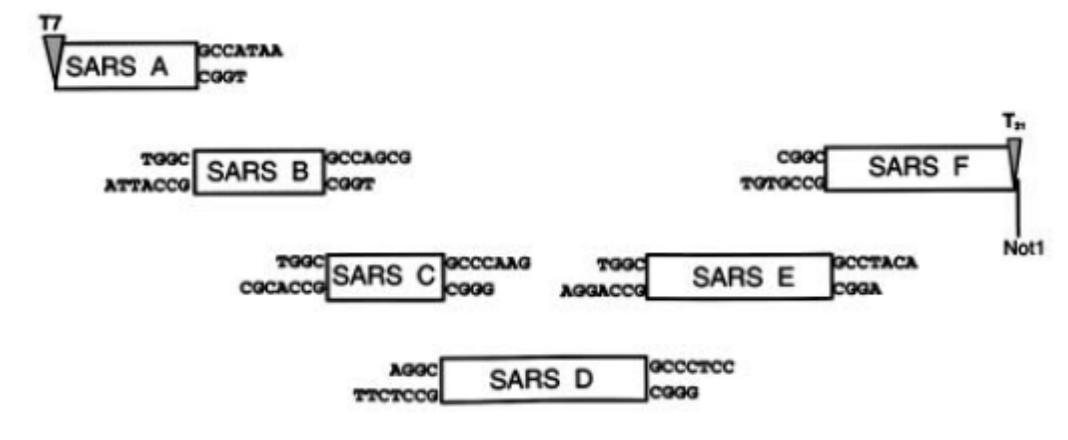
SARS coronavirus is a **positive** and **single stranded RNA** virus belonging to a family of enveloped coronaviruses. Its genome is about **29.7kb**, which is one of the largest among RNA viruses.




Membrane glycoprotein
Spike protein
RNA
Envelope protein
Nucleocapsid protein
Receptor ACE2
SARS-CoV entry
Virus release
Membrane fusion and viral RNA release
+5'
3' RNA genome
Translation
pp1a and lab
Replicase
Proteolysis
RNA replication and packaging
N
S
M
E
Golgi
Assembly and budding
Transcription
mRNAs
S
3a and 3b
E
M
6
7a and 7b
8a and 8b
N and 9b
Translation
ERGIC
ER
Nucleus



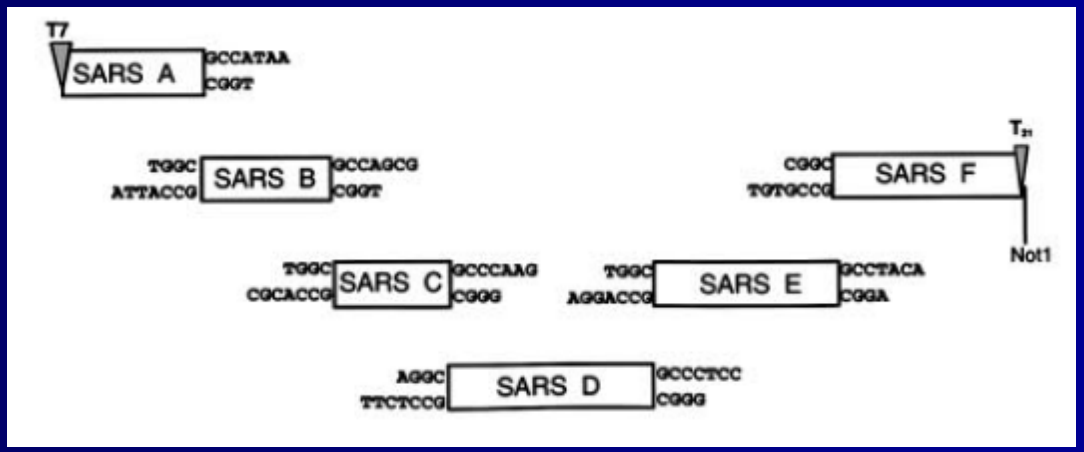
Genome structure



Aprroach used to cloned the entire genome

B **SARS Junctions**

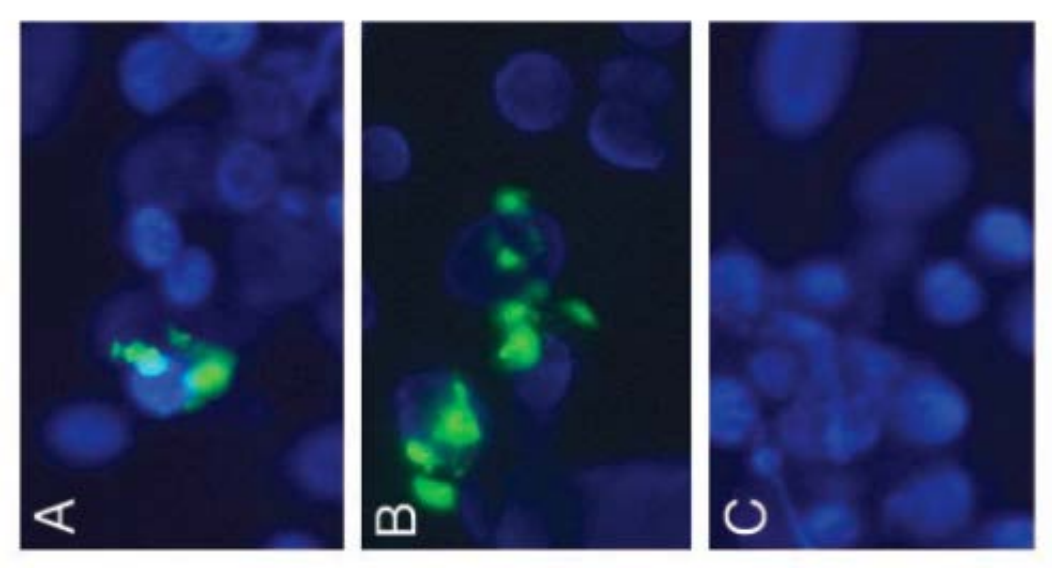
SARS A/B Junction	GCCATAATGGC CGGTATTACCG	4,419
SARS B/C Junction*	GCCAGCGTGGC CGGTCGCACCG	t 8,749
SARS C/D Junction	GCCCAAGAGGC CGGGTTCTCCG	g 12,113
SARS D/E Junction*	GCCCTCCTGGC CGGGAGGACCG	at g t 18,966
SARS E/F Junction*	GCCTACACGGC CGGATGTGCCG	t 24,088



Cloned cDNA genome → In vitro transcription

↓

Cell transfection



Negative-stranded RNA viruses

- Difficulties

  - precise 5’ and 3’ ends are required for replication and packaging of the genomic RNA

  - the viral RNA polymerase is essential for transcribing both mRNA and complementary, positive-sense antigenomic template RNA

  - both genomic and antigenomic RNAs exist as viral ribonucleoprotein (RNP) complexes

- In 1994 (Schnell et al., EMBO, 13:4195-4203)

  - the rescue of the first NS RNA virus, rhabdovirus rabies virus, starting entirely from cDNA

Rescue of non-segmented negative-stranded viruses

Transctiption plasmid for genomic RNA

+

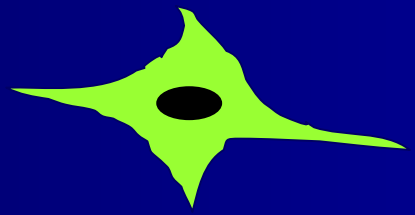
T7 Polymerase Expression System
- Vaccinia virus
- or Cell lines

+

Expression plasmids For NP, P, etc.

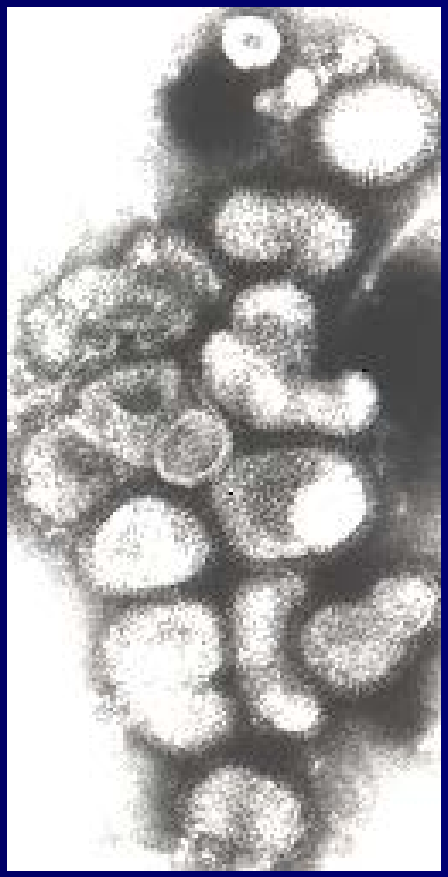
OR

Helper virus



Infectious virus

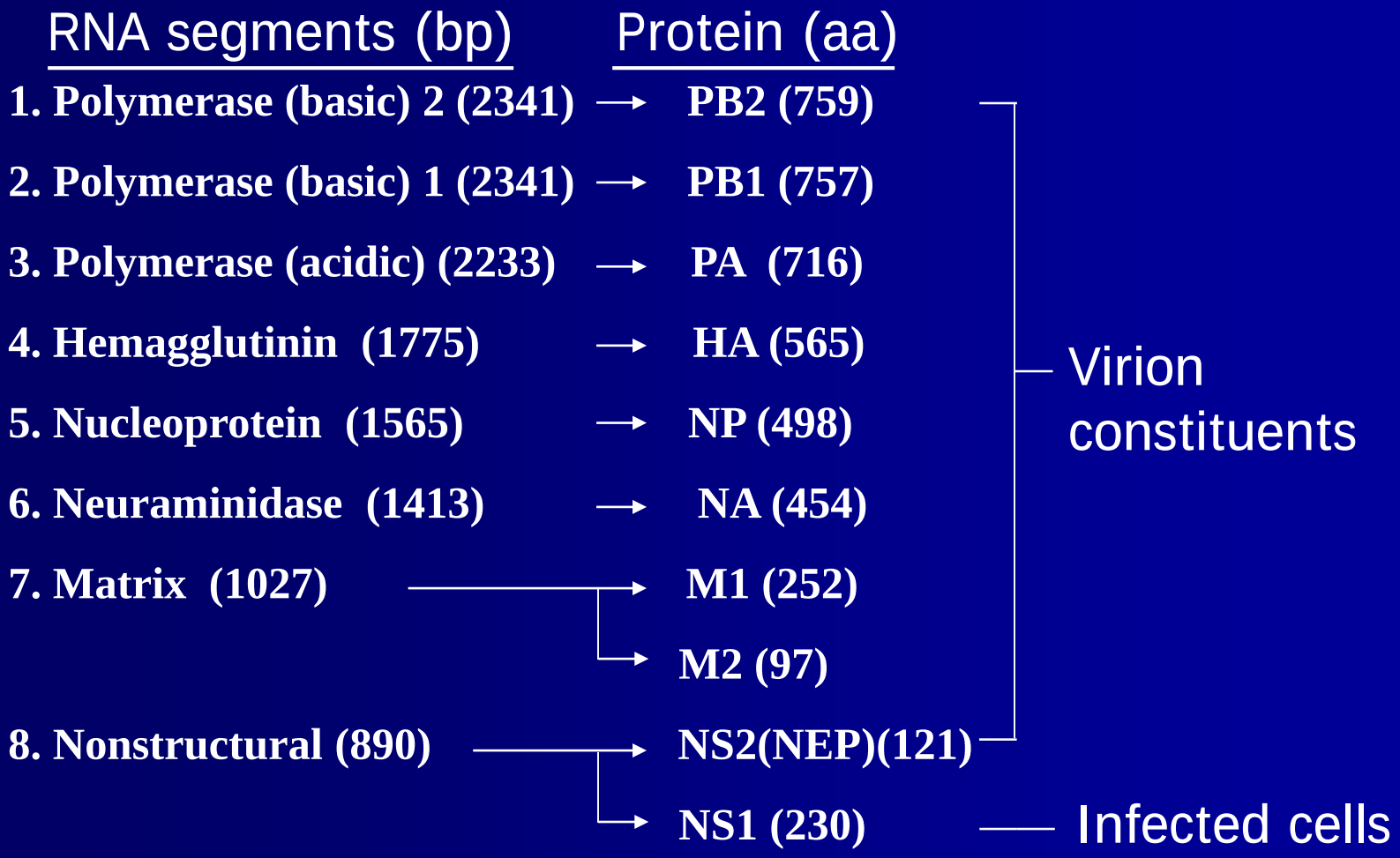
Rescue of Influenza Virus



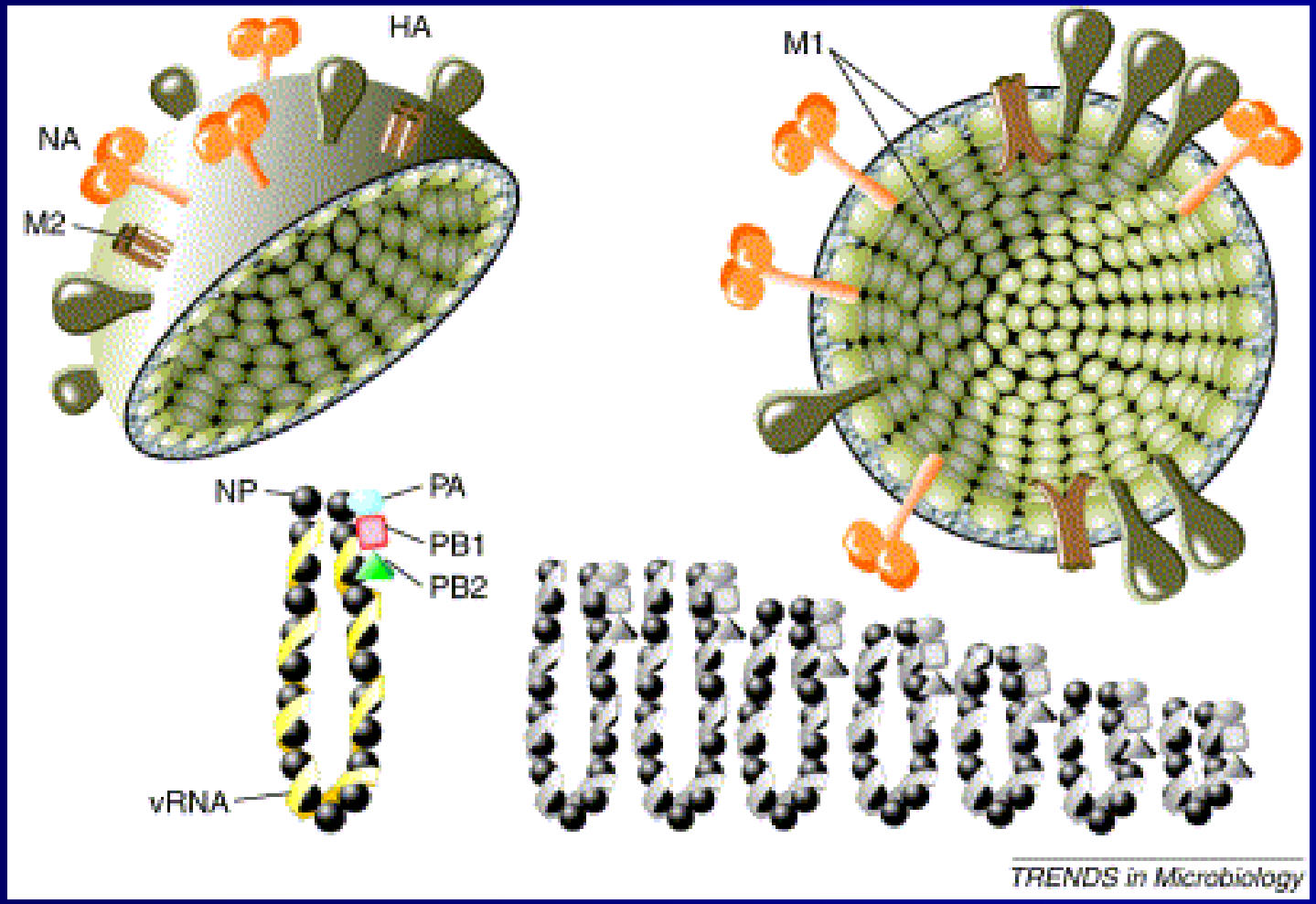
Chang-Won Lee, 1996

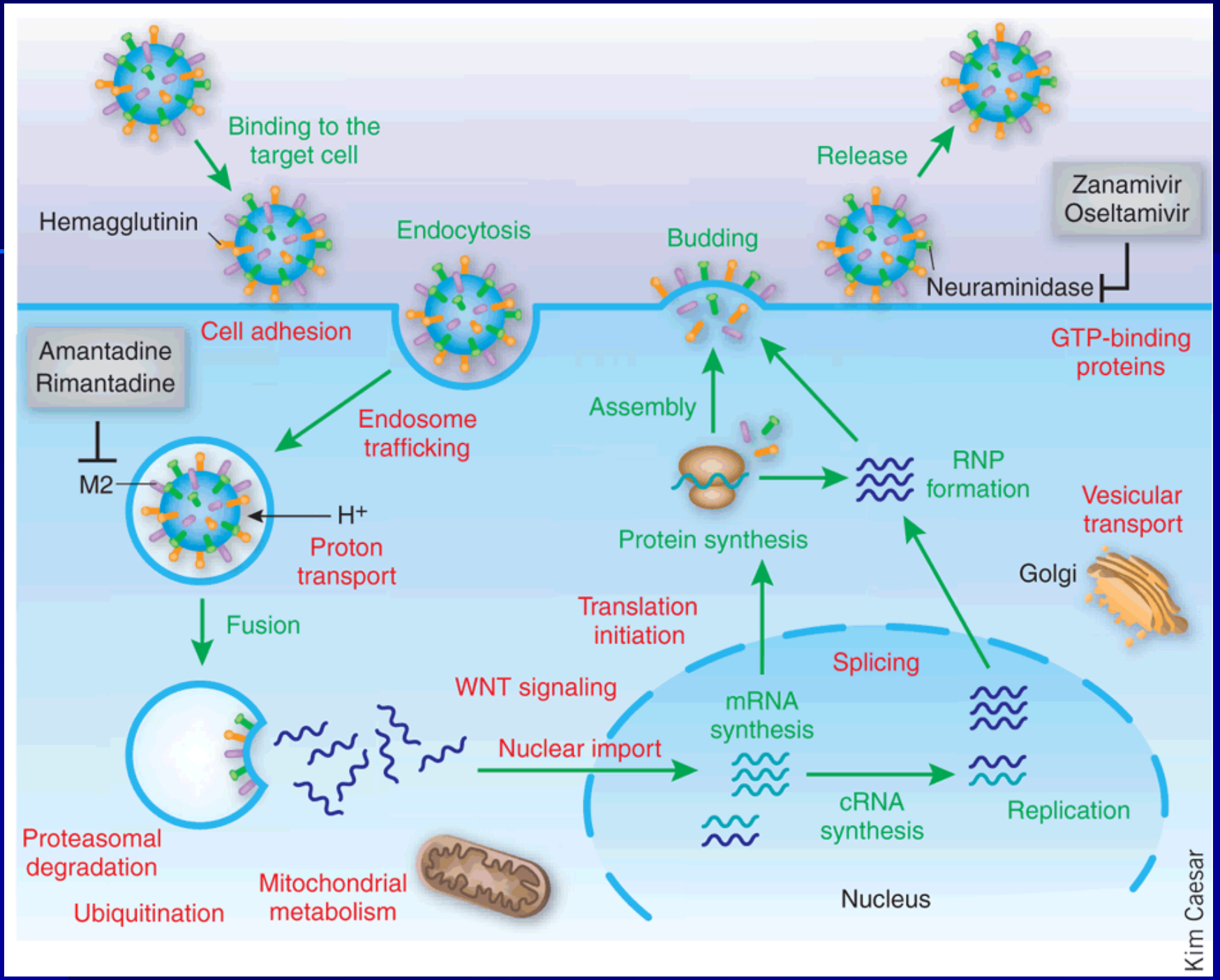
- Family : *Orthomyxoviridae*
- Genera
  - influenza A virus
  - influenza B virus
  - influenza C virus
  - thogotovirus
- Segmented RNA genome
- Negative polarity
- Replicates in the nucleus of infected cells

Genomes


RNA segments (bp)
Protein (aa)
1. Polymerase (basic) 2 (2341)
PB2 (759)
2. Polymerase (basic) 1 (2341)
PB1 (757)
3. Polymerase (acidic) (2233)
PA (716)
4. Hemagglutinin (1775)
HA (565)
5. Nucleoprotein (1565)
NP (498)
6. Neuraminidase (1413)
NA (454)
7. Matrix (1027)
M1 (252)
M2 (97)
8. Nonstructural (890)
NS2(NEP)(121)
NS1 (230)
Virion constituents
Infected cells

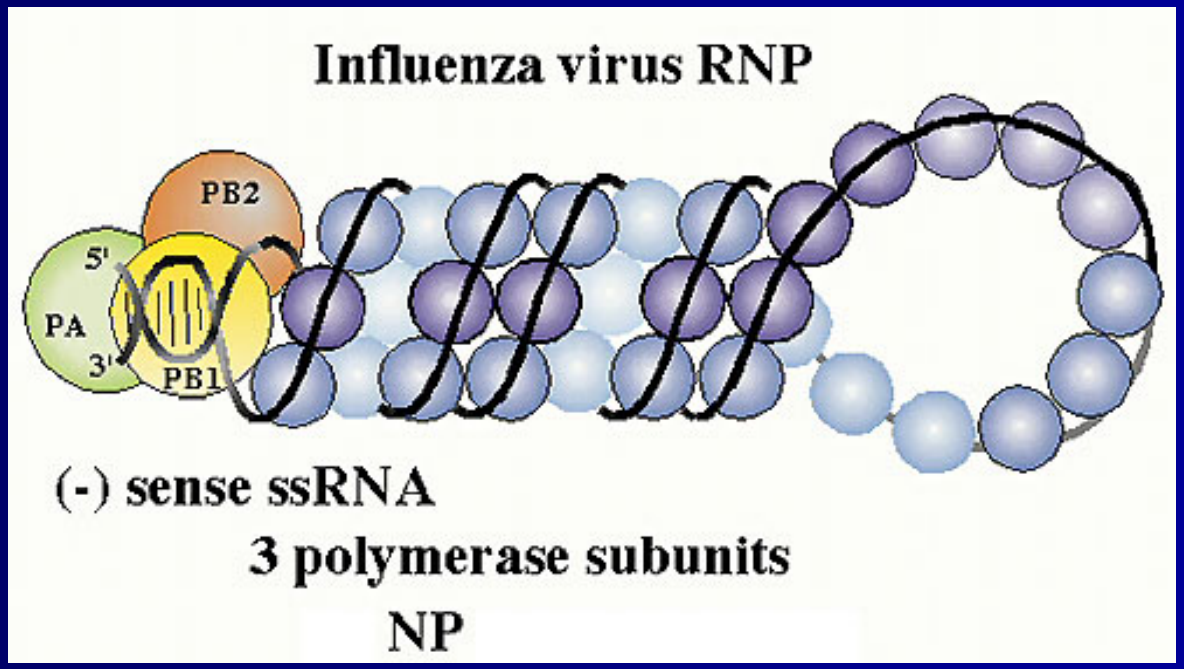
Structure of influenza virus



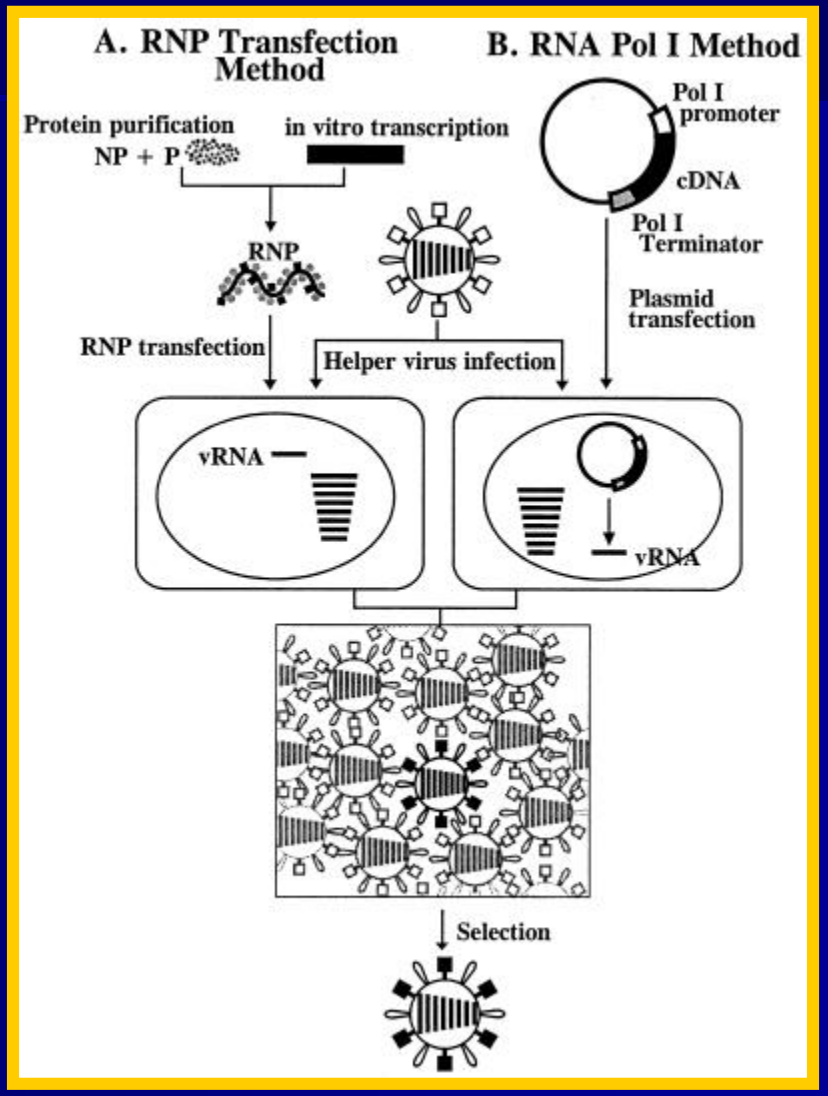

Binding to the target cell
Hemagglutinin
Endocytosis
Budding
Release
Zanamivir
Oseltamivir
Neuraminidase
Cell adhesion
GTP-binding proteins
Amantadine
Rimantadine
Endosome trafficking
M2
H+
Proton transport
Assembly
RNP formation
Protein synthesis
Vesicular transport
Golgi
Translation initiation
Fusion
Splicing
WNT signaling
mRNA synthesis
Nuclear import
cRNA synthesis
Replication
Proteasomal degradation
Ubiquitination
Mitochondrial metabolism
Nucleus
Kim Caesar

Key to generation of influenza virus

- vRNA encapsidated by NP must be transcribed into mRNA by the viral polymerase complex
- The vRNP complex is the minimal functional unit



Helper virus-based method



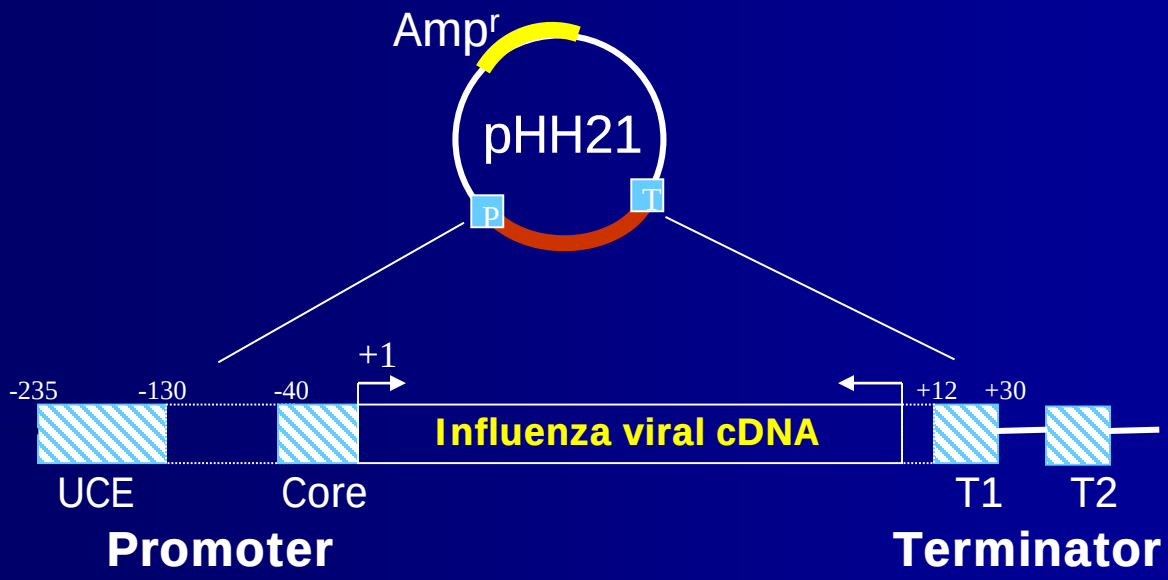
BUT

vast background of wild-type virus

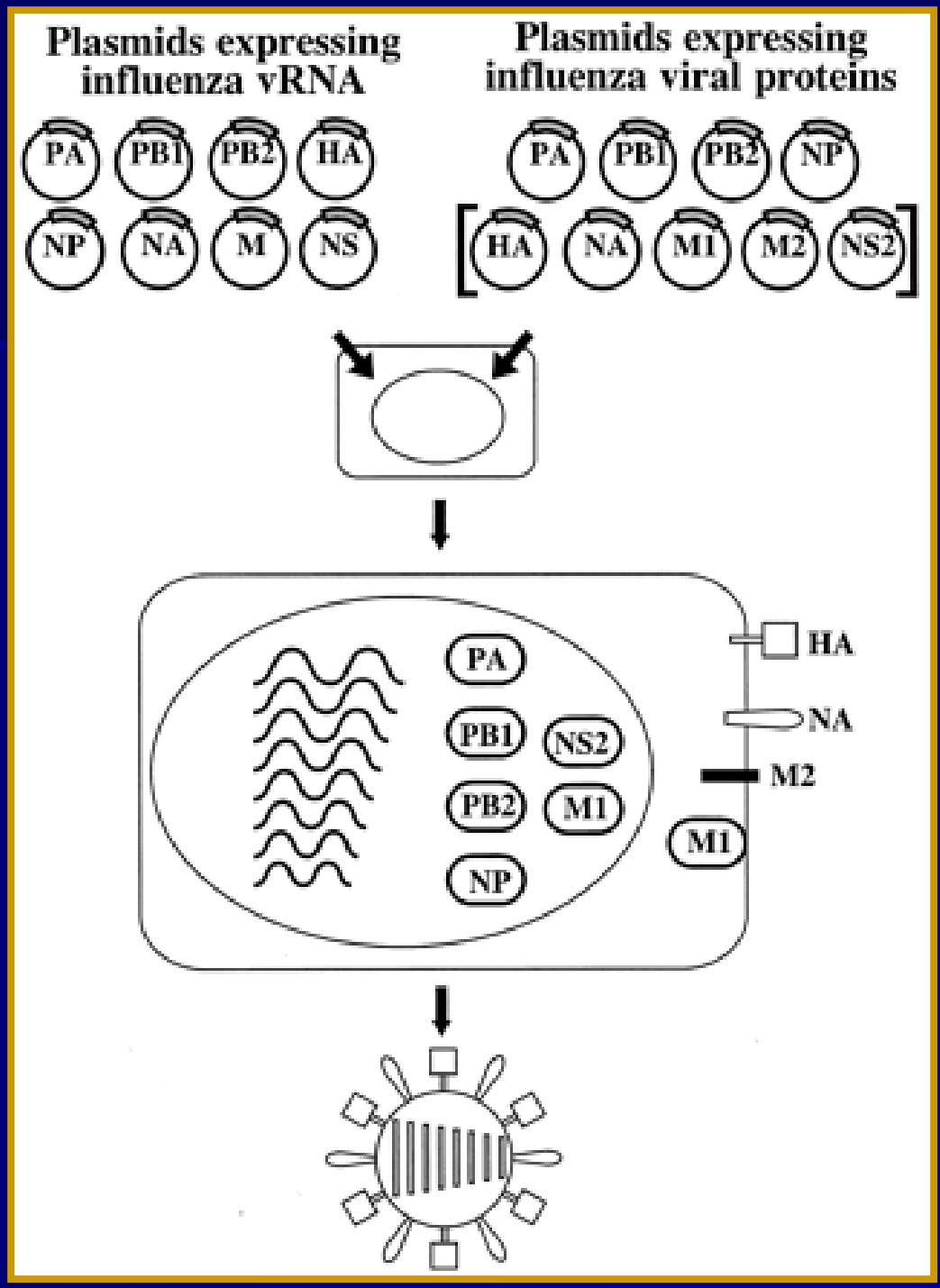
RNA polymerase I

- A nucleolar enzyme, which transcribes ribosomal RNA
  - In growing cells, rRNA accounts for 80% of the total RNA

- A Replacement of the rDNA template with a cDNA encoding an influenza viral gene did not impair the precise initiation and termination of transcription (Neumann *et al.*, 1994)

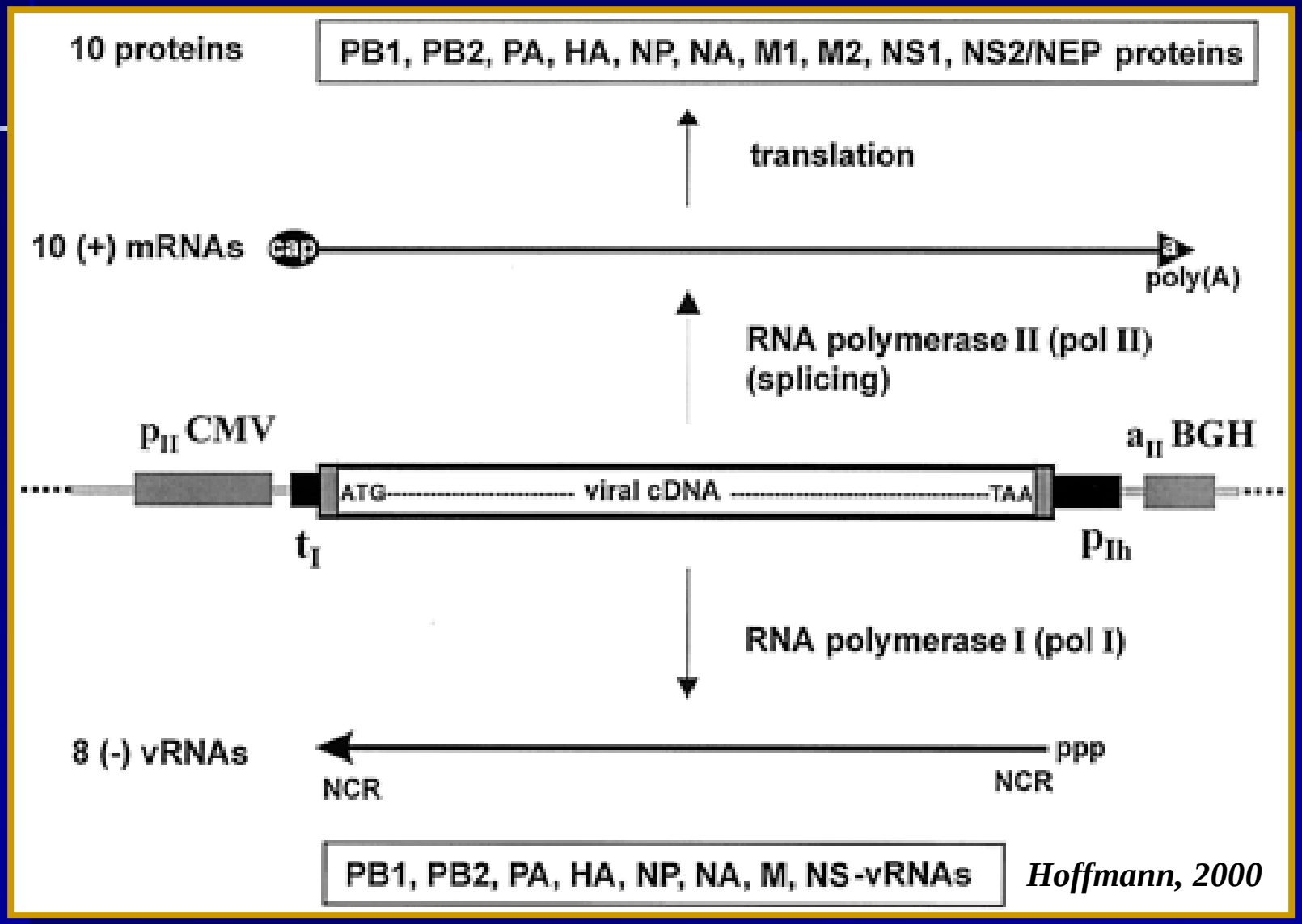


Plasmid-Based Reverse Genetics

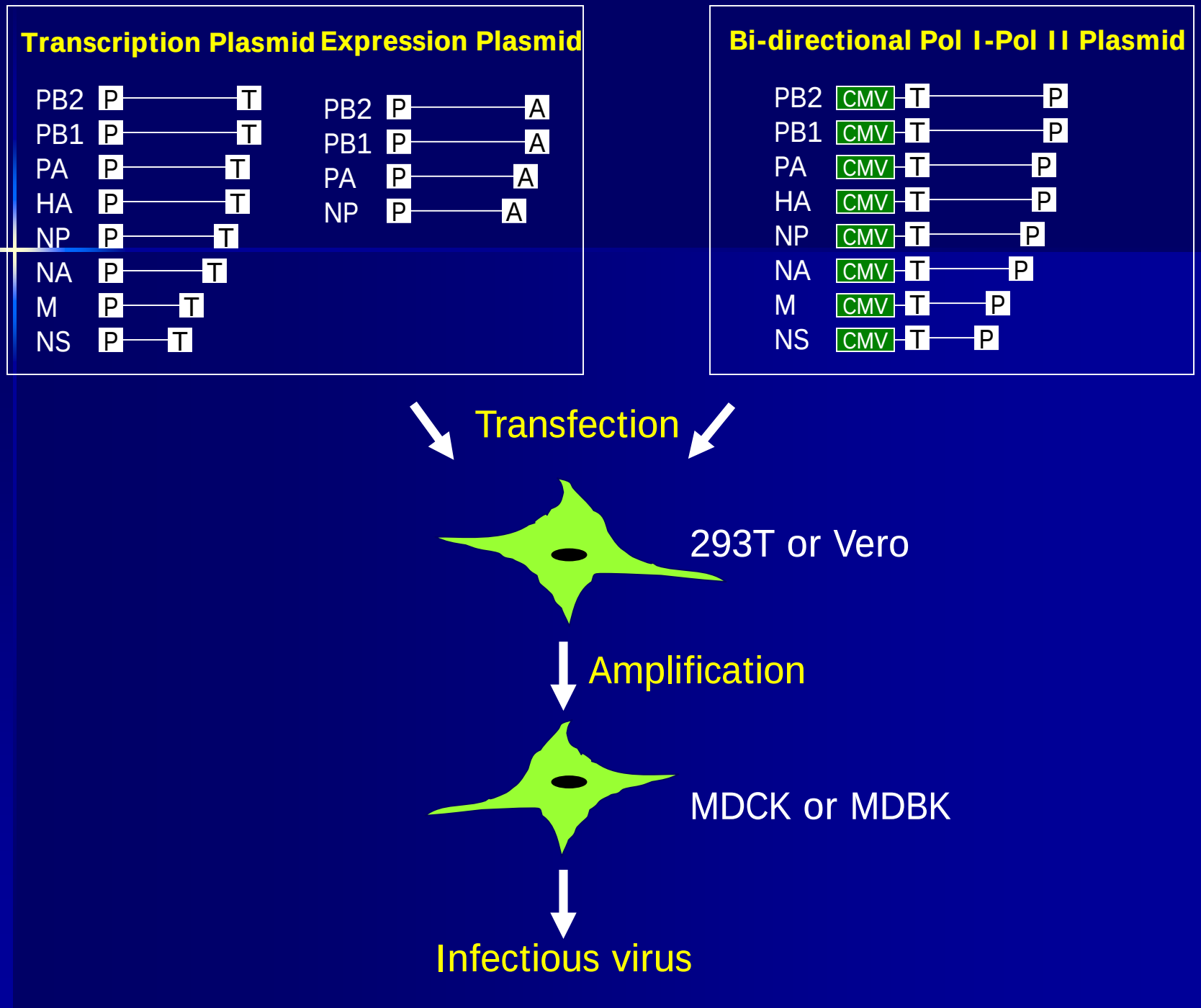


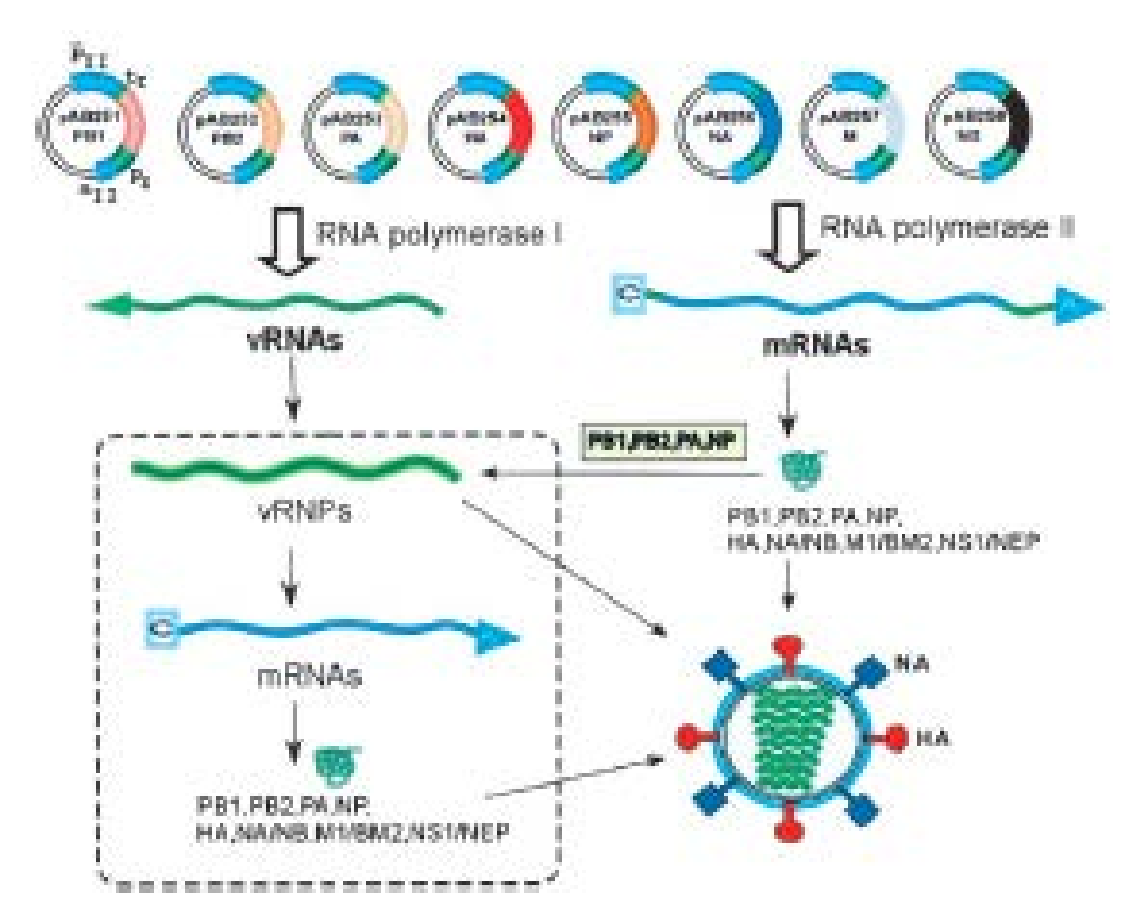
Neumann et al. PNAS 96:9345-9350, 1999

Bidirectional pol I - pol II transcription system



Hoffmann et al. PNAS, 97:6108-6113, 2000


Transcription Plasmid
PB2 P T
PB1 P T
PA P T
HA P T
NP P T
NA P T
M P T
NS P T
Expression Plasmid
PB2 P A
PB1 P A
PA P A
NP P A
Bi-directional Pol I-Pol II Plasmid
PB2 CMV T P
PB1 CMV T P
PA CMV T P
HA CMV T P
NP CMV T P
NA CMV T P
M CMV T P
NS CMV T P
Transfection
293T or Vero
Amplification
MDCK or MDBK
Infectious virus


RNA polymerase I
RNA polymerase II
vRNAs
mRNAs
PB1,PB2,PA,NP
vRNPs
PB1,PB2,PA,NP,
HA,NA/NB,M1/BM2,NS1/NEP
mRNAs
PB1,PB2,PA,NP,
HA,NA/NB,M1/BM2,NS1/NEP
NA
HA

Unidirectional RNA polymerase I–polymerase II transcription system for the generation of influenza A virus from eight plasmids

Erich Hoffmann[1] and Robert G. Webster[1,2]

Bidirectional

(+) v cDNA

↓

(-) vRNA

Unidirectional

(+) v cDNA

↓

(+) cRNA

↓

(+) cRNP

↓

(-) vRNA

(-) vRNP