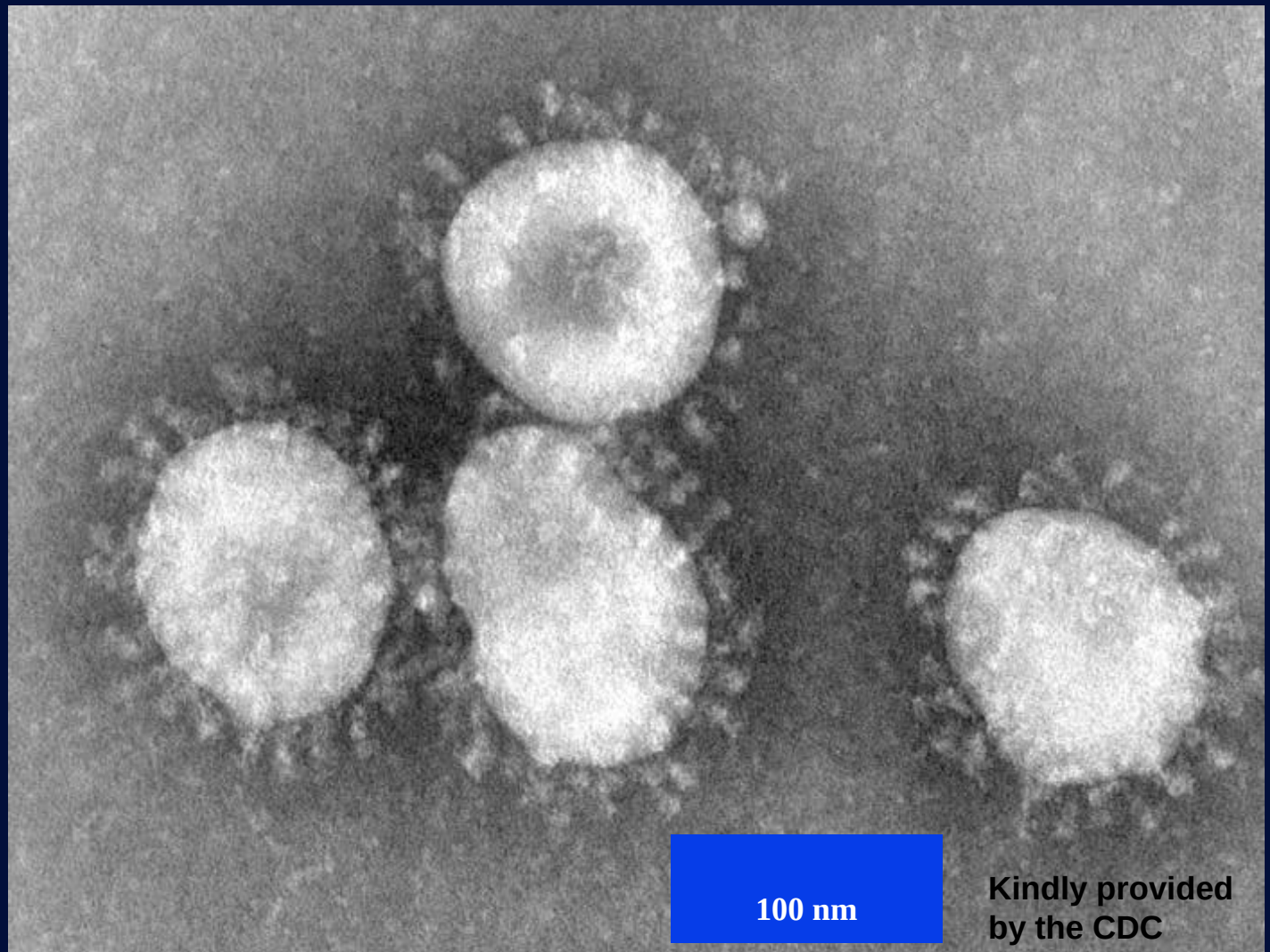
SARS-CoV: Reverse Genetics and Pathogenesis

Boyd Yount, Kris Curtis, Amy Sims



NIDOVIRALES ORDER

Coronaviridae

-Group 1

TGEV

HCV229E

FIP

HuCV-NL

-Group 2

MHV

BCV

HCV-OC43

SARS-CoV

-Group 3

IBV

Molecular Clones available for TGEV, HCV229E, MHV, SARS-CoV and IBV

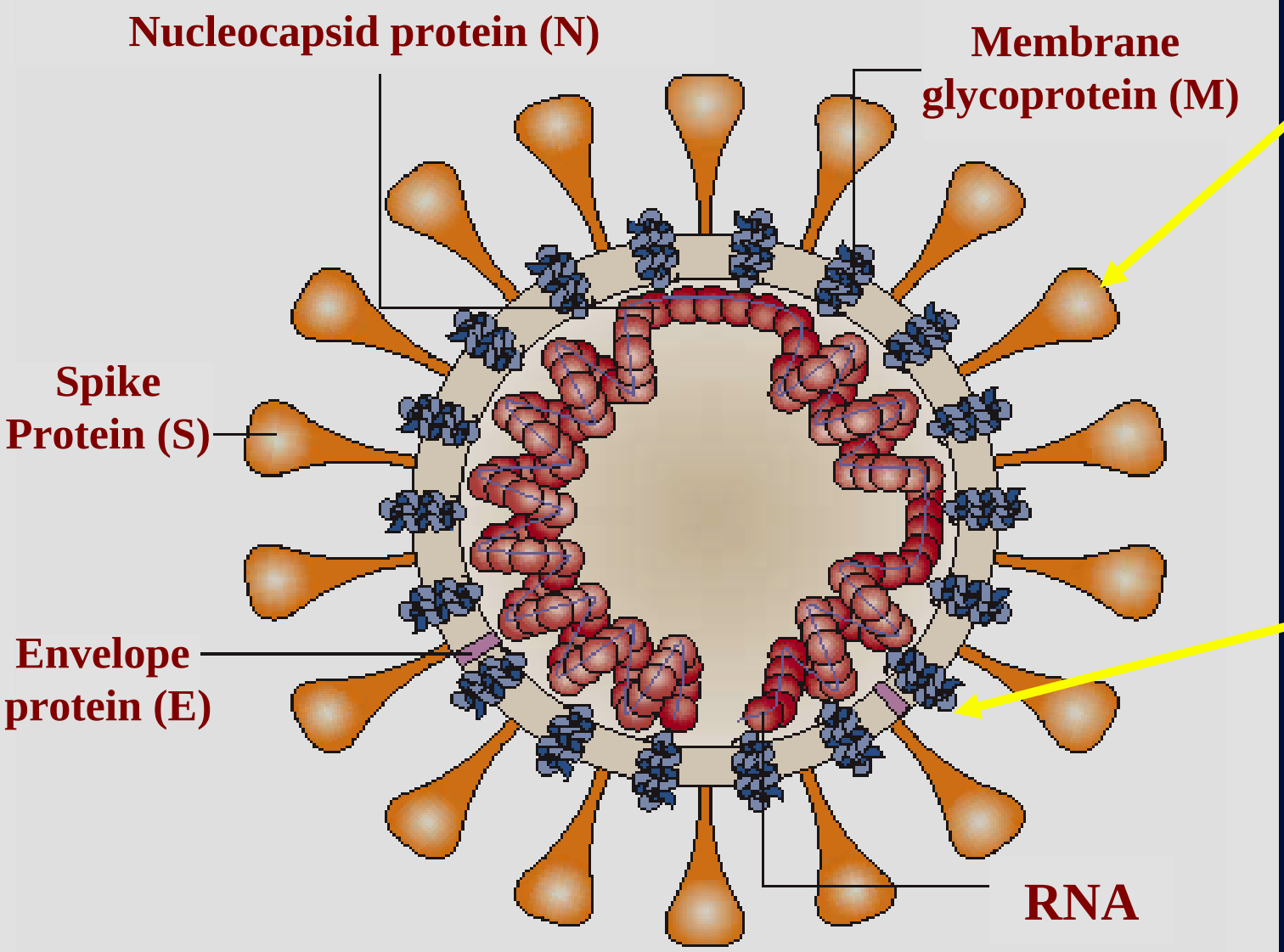
SARS-CoV
CASE MORTALITY RATES

- Overall Mortality Rate: 10-12%; ~20% require intensive care and/or mechanical ventilation
  - <1% Age 24 or younger
  - 6% Age 25-44
  - 15% Age 45-64
  - >50% Age 65 or older
- Future of the SARS Outbreak?
  - Three laboratory acquired infections in 2003-2004 (Taiwan, Singapore and China)
  - Four SARS-CoV infections reported in China in Dec 2003-04; source: likely zoonosis transmission
  - ~1-2% of serum samples collected in Hong Kong and in Guangdong province in 2001-2002 were seropositive to SARS-CoV
  - SARS-CoV likely introduced multiple times into human population in Southeast Asia

OBJECTIVES

- Coronavirus Life Cycle and Replication
- Coronavirus Molecular Genetics Techniques
  - Targeted Recombination
  - Coronavirus reverse genetics
- SARS CoV Reverse Genetics
  - Pathogenesis, replication and vaccine development
  - Replication Competent Propagation defective viruses
- Safety Measures
  - Facilities
  - Personnel
  - Community
- Summary

SARS-CoV Schematic

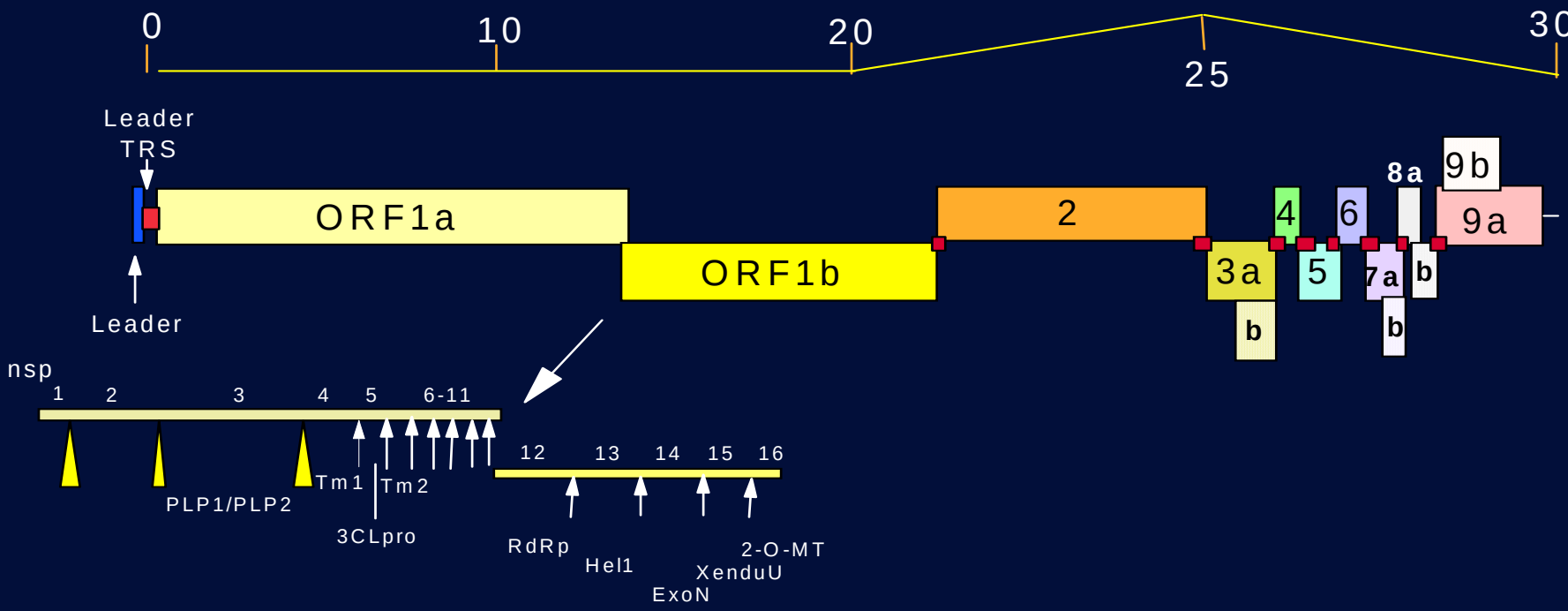


ss, linear, positive polarity RNA genome of ~29 Kb in length

S glycoprotein interacts with human ACE2 molecule for entry into cells in culture; Major determinant of host range; neutralizing epitopes

E and M drive assembly of mature particles into an intermediate compartment between golgi and rER

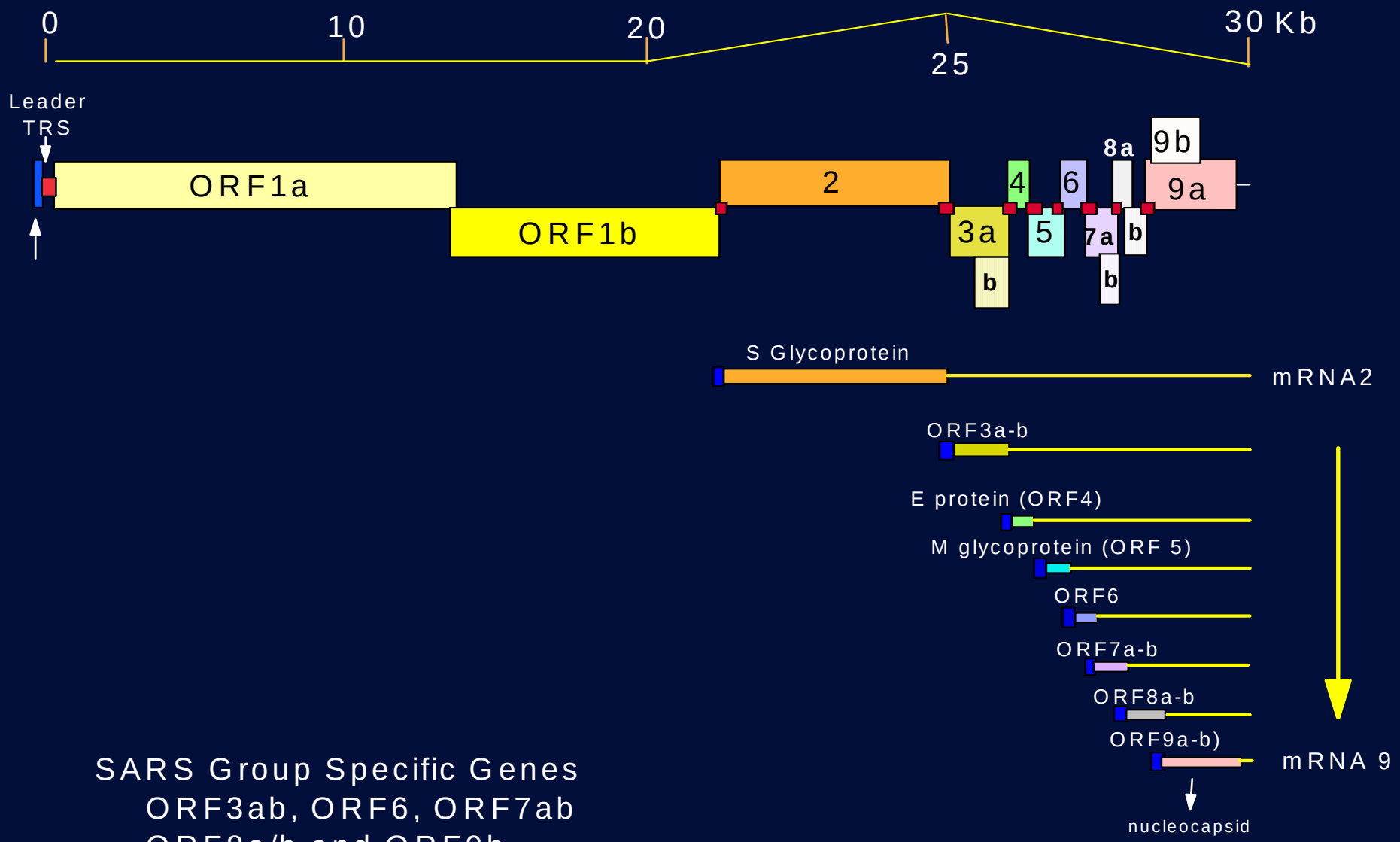
Readily inactivated by 65^{o}C, 70-100% alcohol, or phenolic based disinfectants



SARS Group Specific Genes
ORF3ab, ORF6, ORF7ab
ORF8a/b and ORF9b

- Transcription Regulatory Sequence (TRS)
  CS-ACGAAC

ORF8a/b 29 bp insertion in Civit CoV produces a single ORF;



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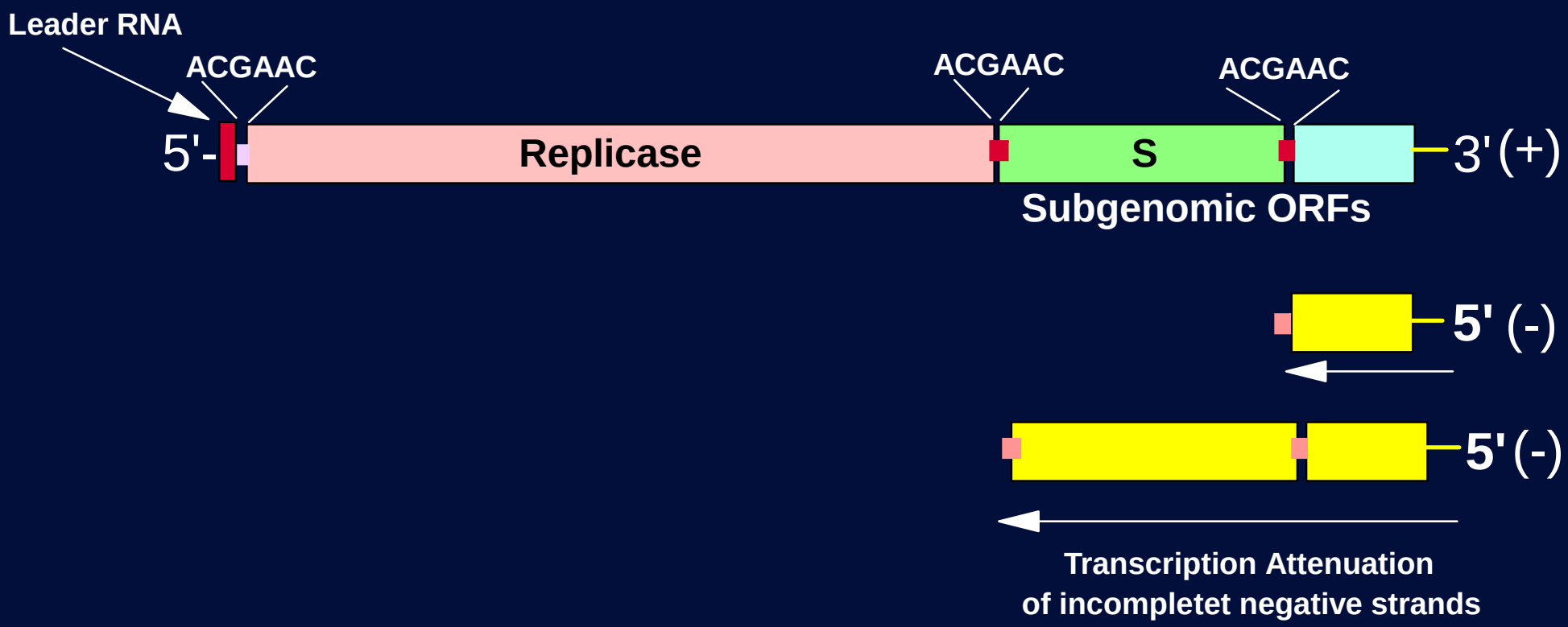
Transcription Attenuation



Sawicki and Sawicki 1990, Sethna and Brian 1989-90;
Schaad et al., 1993; van Marle G. et al., 1999, Baric et al., 2000

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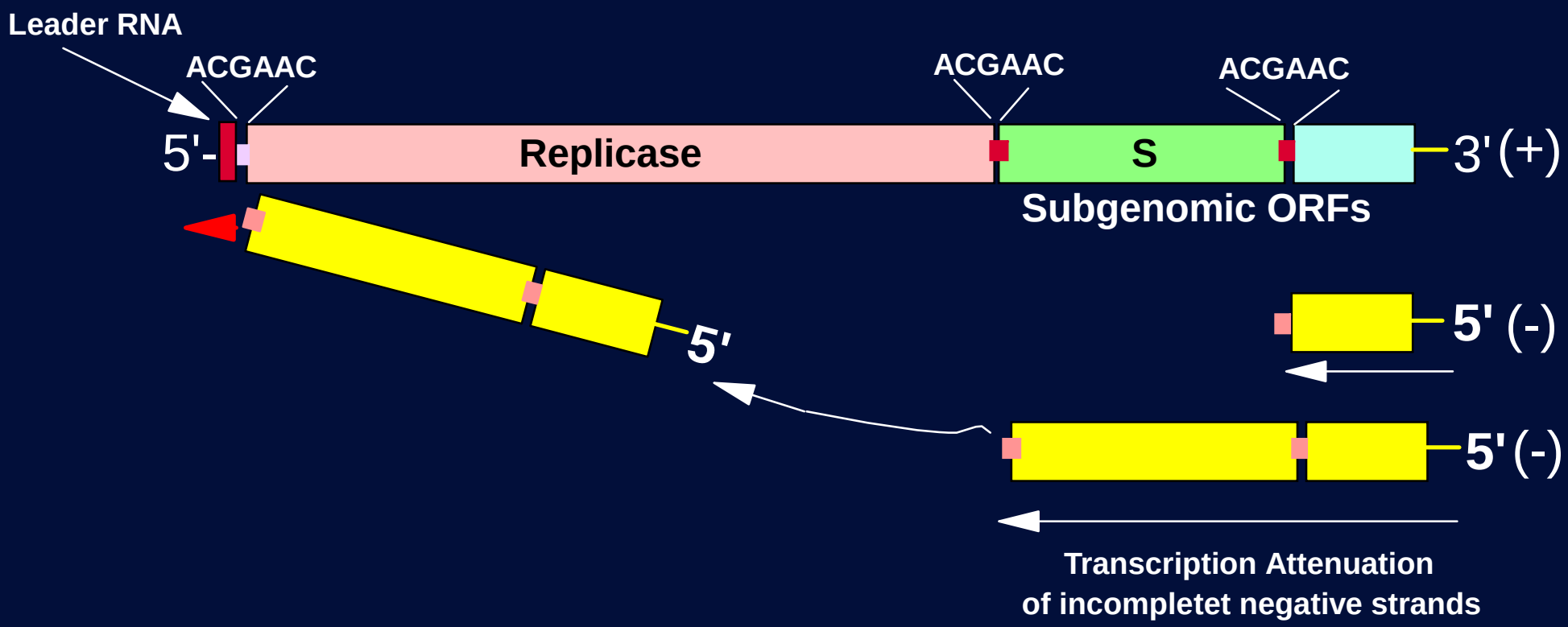

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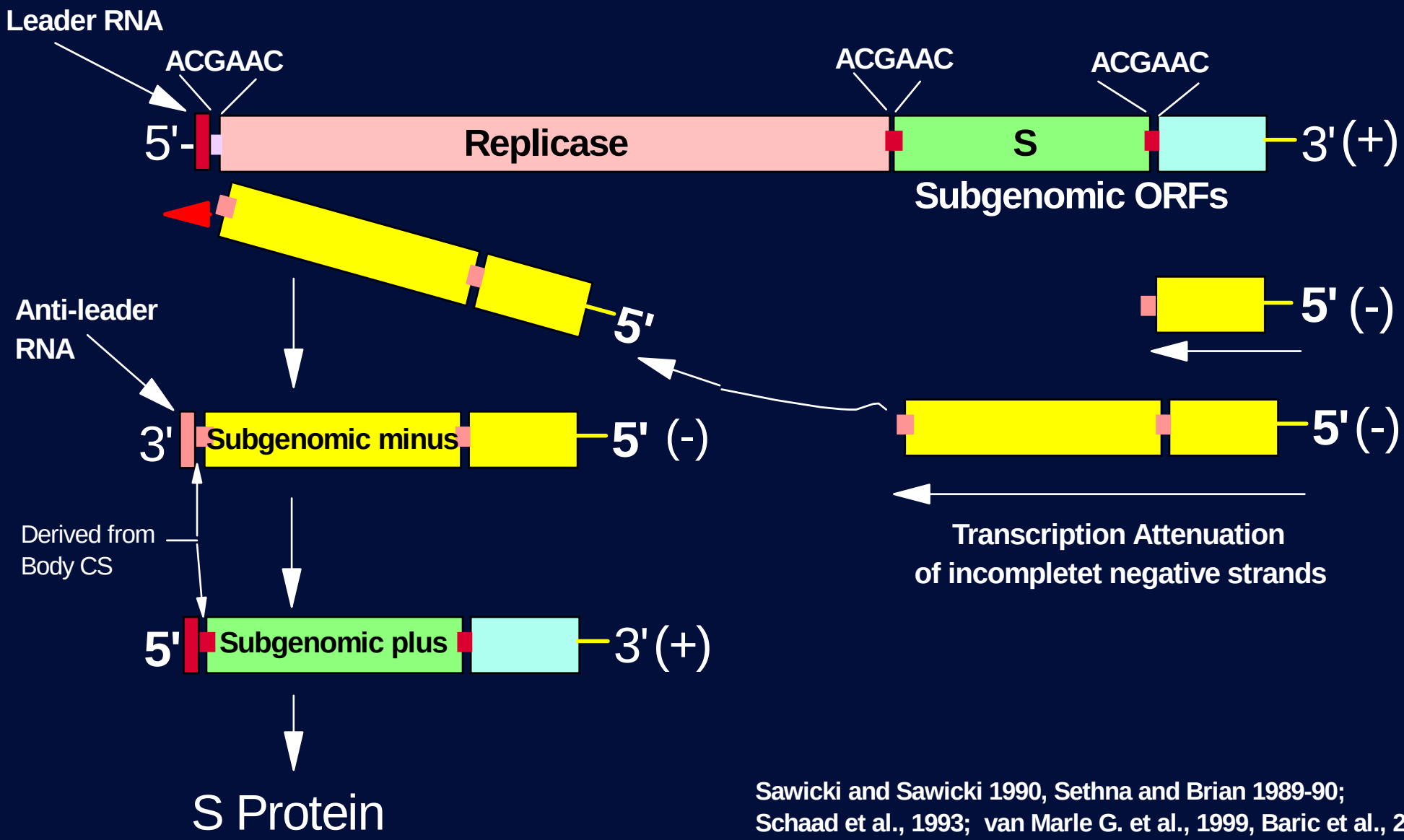

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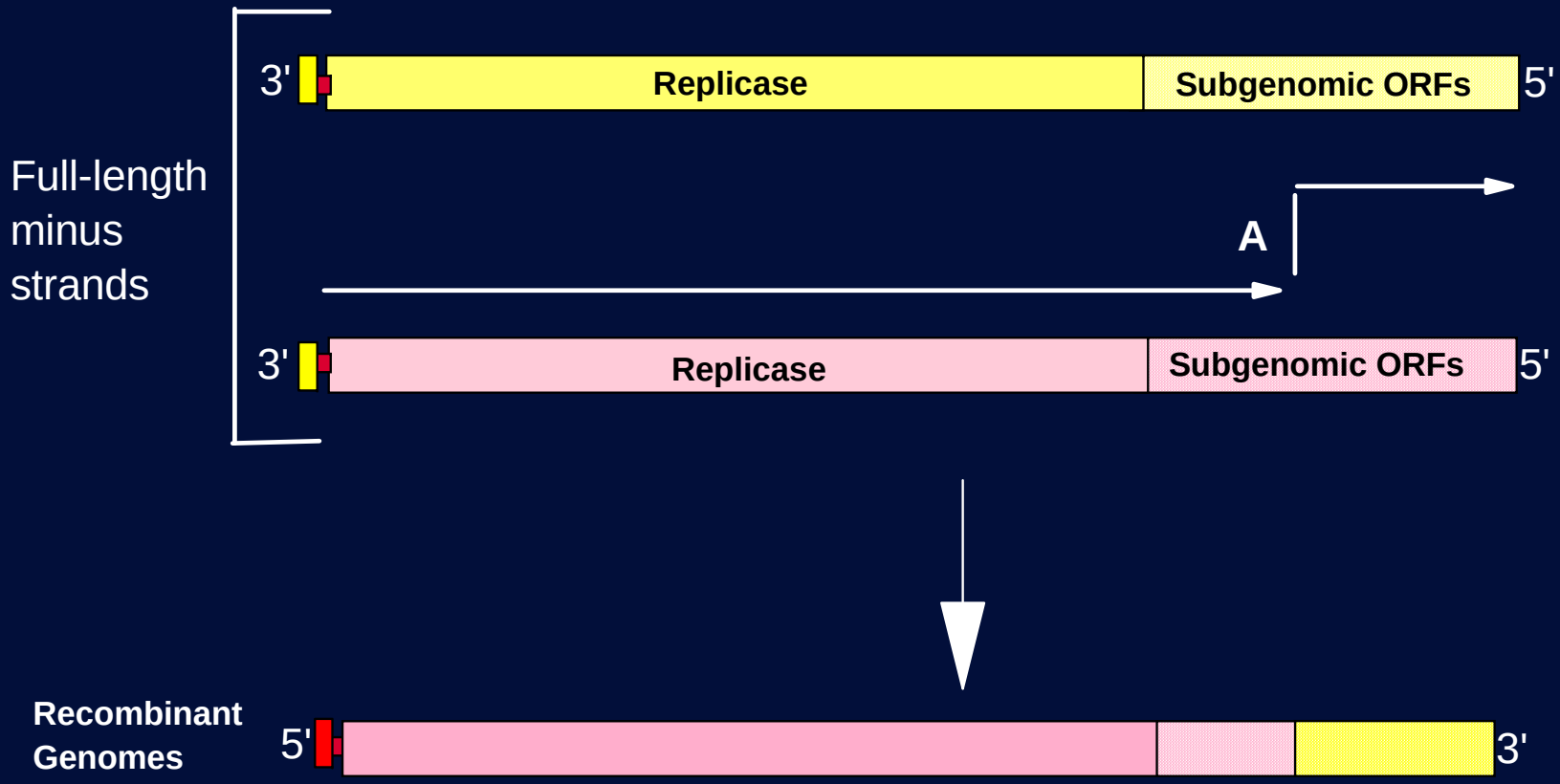
Coronavirus RNA Recombination

(Positive or Negative RNA Synthesis- ~20%)



Koetzner et al., 1992 J. Virol. 66, 1841-1848; Fu and Baric, 1993 Virology

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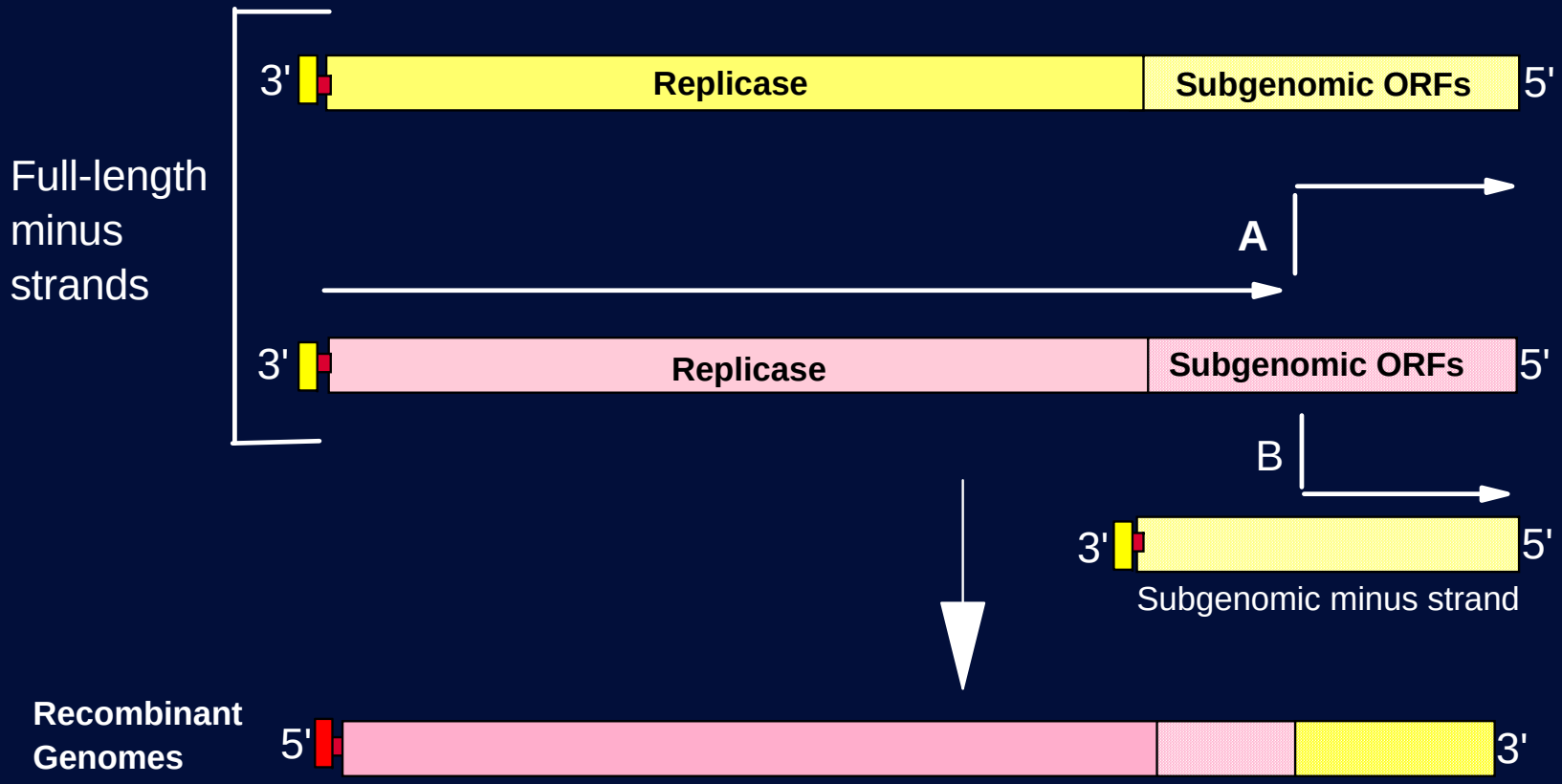


Koetzner et al., 1992 J. Virol. 66, 1841-1848; Fu and Baric, 1993 Virology

Coronavirus Genetics
Targeted RNA Recombination

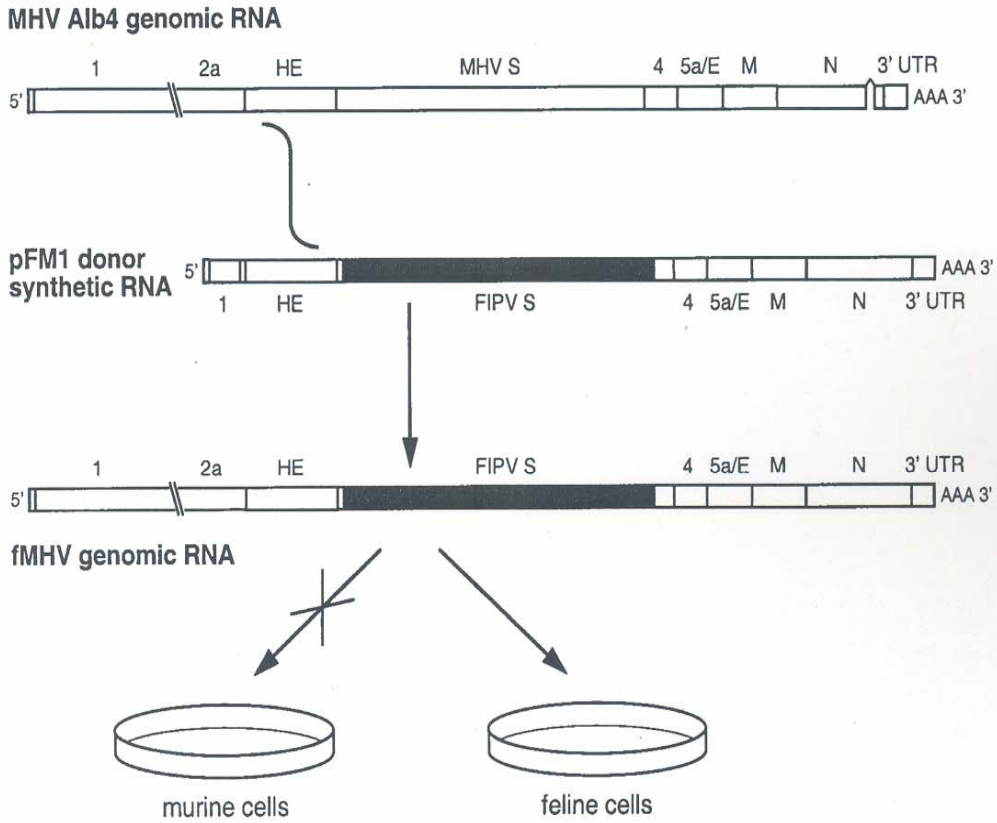
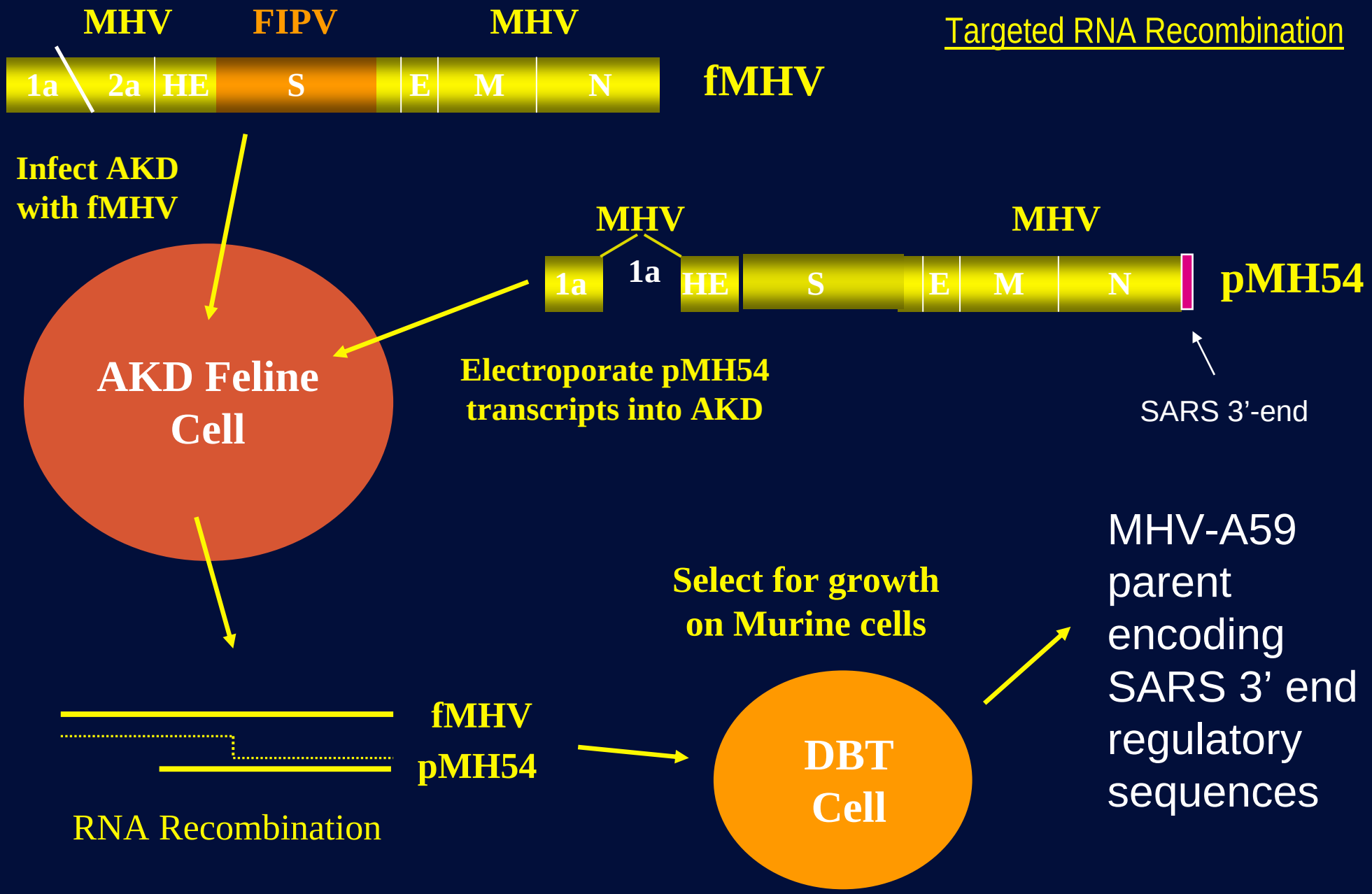
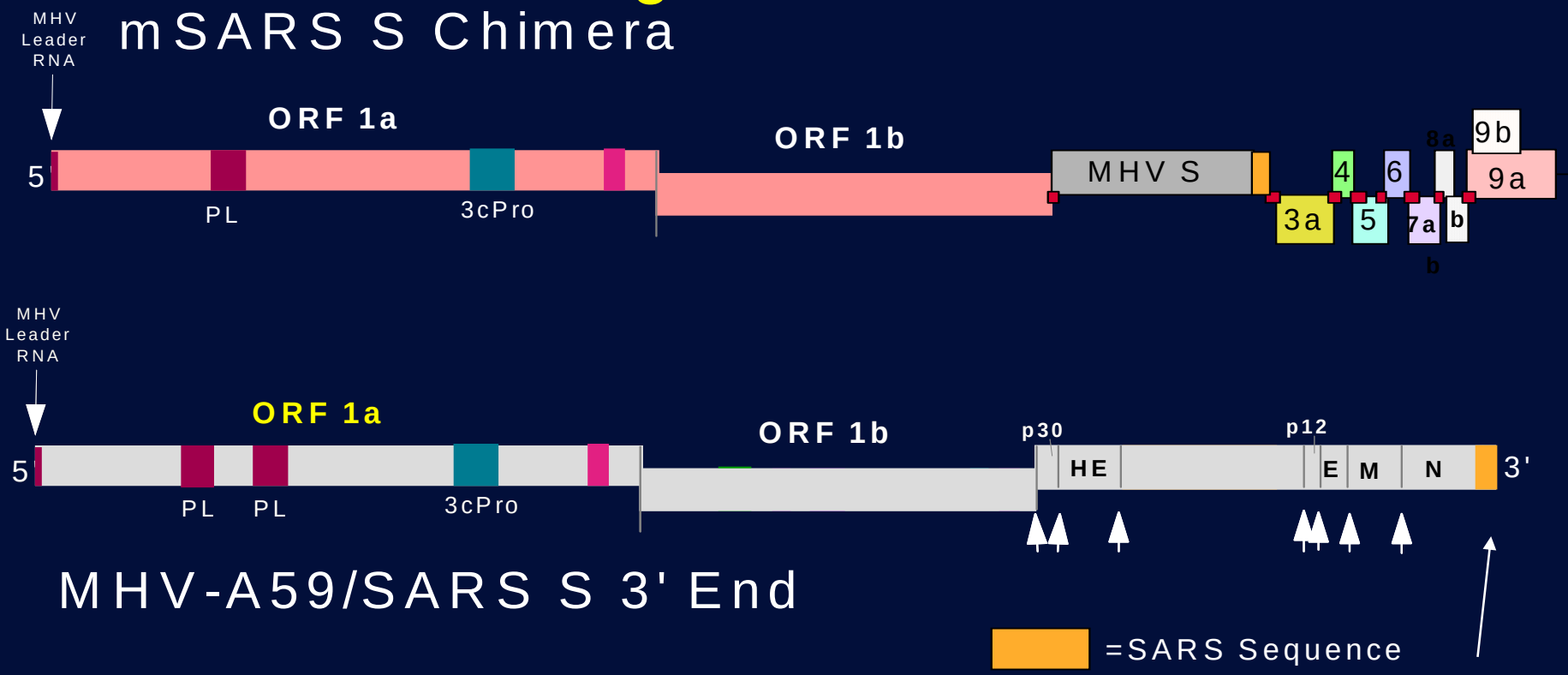


FIG. 2. Scheme for construction of fMHV by targeted recombination between the MHV N gene deletion mutant, Alb4 (27), and donor RNA transcribed from the plasmid pFM1. The deletion in the Alb4 N gene is shown as a discontinuity. A single crossover event anywhere within the HE gene fragment of the donor RNA should generate a recombinant, fMHV, containing both the ectodomain-encoding region of the FIPV S gene (shaded) and the wild-type MHV N gene. The recombinant should simultaneously lose the ability to infect murine cells and gain the ability to infect feline cells.

Kuo et al., 2000 J. Virol. 74, 1393-1406

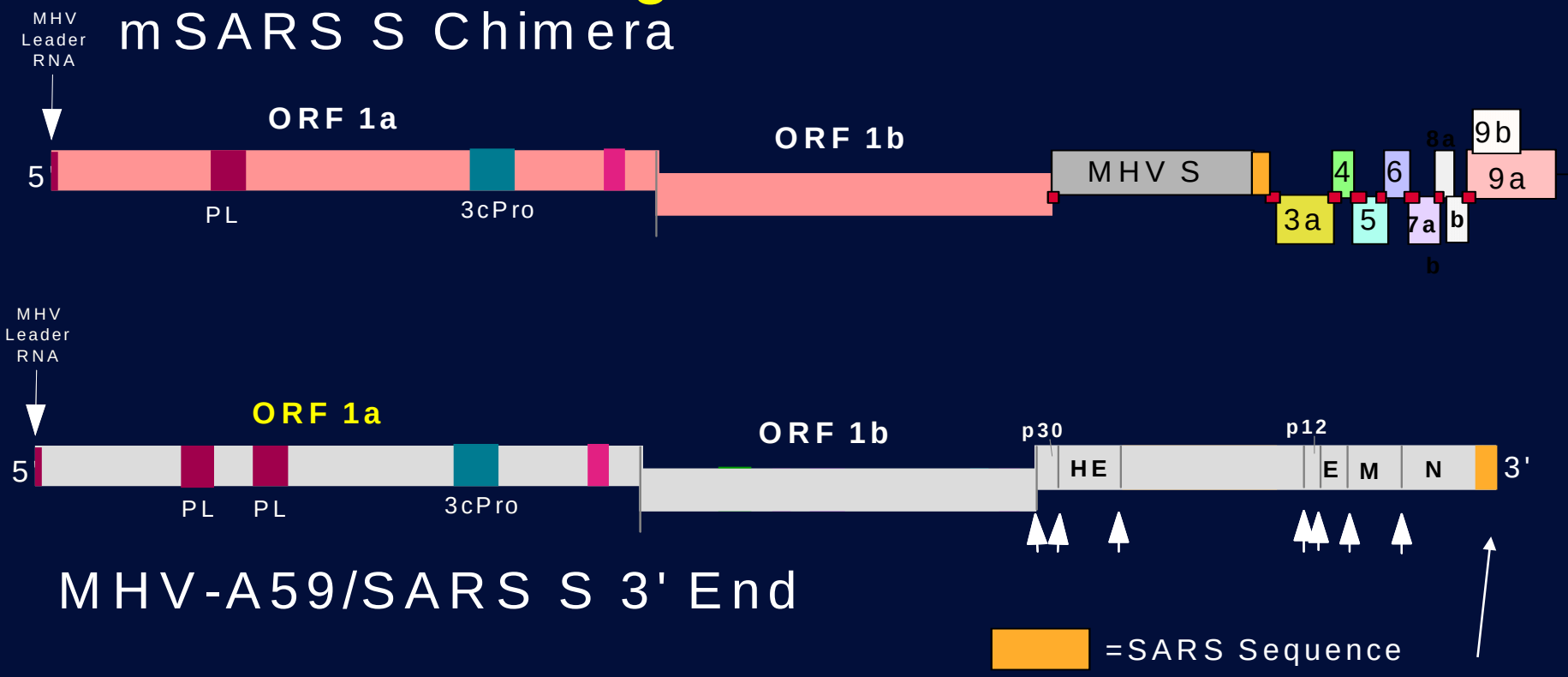

MHV
FIPV
MHV
1a
2a
HE
S
E
M
N
fMHV
Targeted RNA Recombination
Infect AKD with fMHV
AKD Feline Cell
MHV
MHV
1a
1a
HE
S
E
M
N
pMHV54
Electroporate pMH54 transcripts into AKD
SARS 3'-end
fMHV
pMH54
RNA Recombination
Select for growth on Murine cells
DBT Cell
MHV-A59 parent encoding SARS 3' end regulatory sequences

SARS Targeted Recombination



Goebel et al., 2004 J. Virology 78, 7846-7851

SARS Targeted Recombination



Goebel et al., 2004 J. Virology 78, 7846-7851

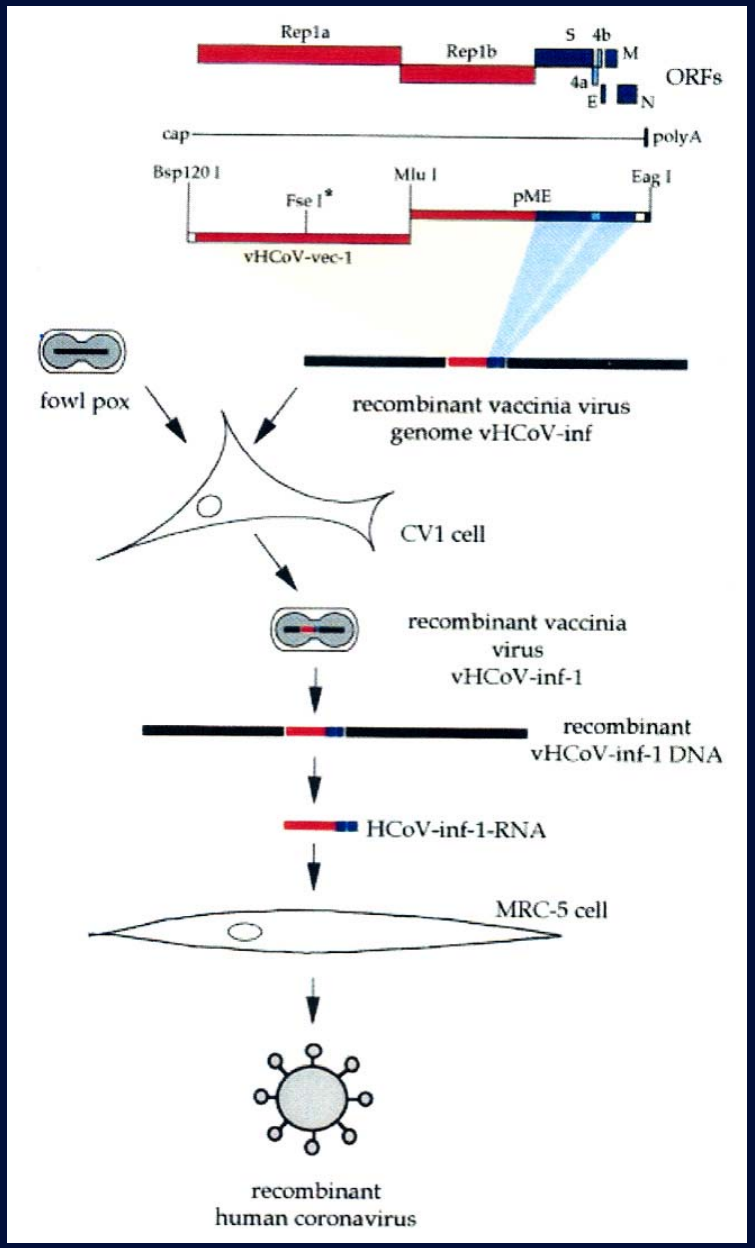
- Concerns:
  - Humanized coronavirus encoding SARS S glycoprotein/SARS genetic backbone targeted to murine cells and rodents
  - Weakly pathogenic coronaviruses encoding SARS sequences or virulence alleles
  - Recombinant SARS-CoV encoding genes from other viruses or cells

Coronavirus Infectious Clone

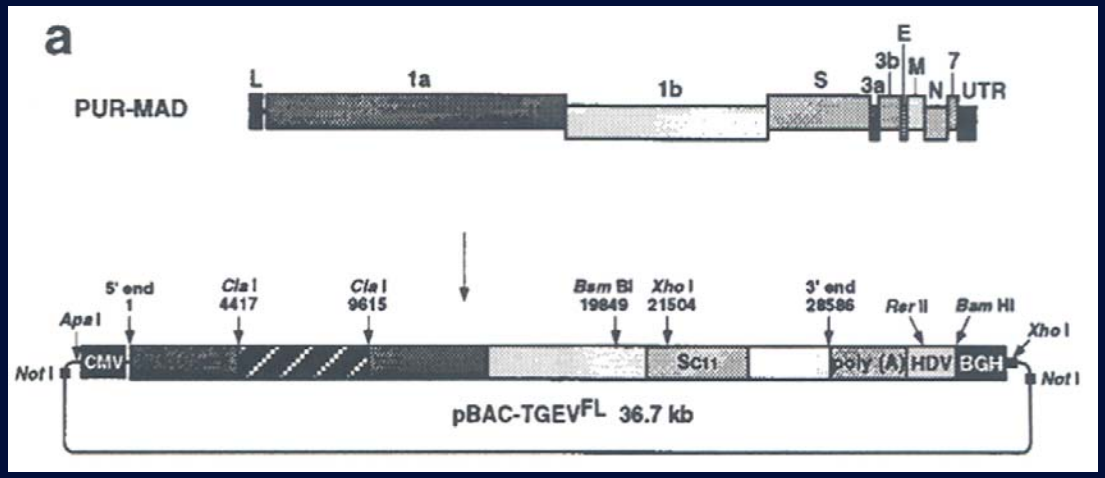
- Large Size of the Viral Genome
- Stable Cloning Vectors
- Regions of Chromosomal Toxicity
- Synthesizing Infectious Transcripts
- Ease of Manipulation
  - –the availability of rare cutting restriction sites for reverse genetic applications

- **Solutions:** Clone genome into BAC vectors, Vaccinia Vectors, or use Systematic Assembly of component clones

HCV 229E, IBV*



TGEV*



DNA launch using host RNA polymerase

**Full length genomes in self-replicating vectors (vaccinia or BAC), easily renewable resource

Thiel V et al., 2001. J. Gen. Virol 82, 1273-1281

Almazan, F. et al., 2000. PNAS USA 97, 5516-5521

Systematic Assembly of Adjoining cDNA Clones
(MHV, TGEV, SARS-CoV)
Class IIS Restriction Endonucleases unique interconnecting junctions

Bgl I 5′….GCCN**NNN**▼NGGC….3′

3′….CGGN▲**NNN**NCCG….5′

Recursive Assembly Strategy: Bgl1 = 2^{64} fragments; Sfi1 (8 cutter with 3 nucleotide variable overhang) = 2^{64} fragments of ~64Kb.

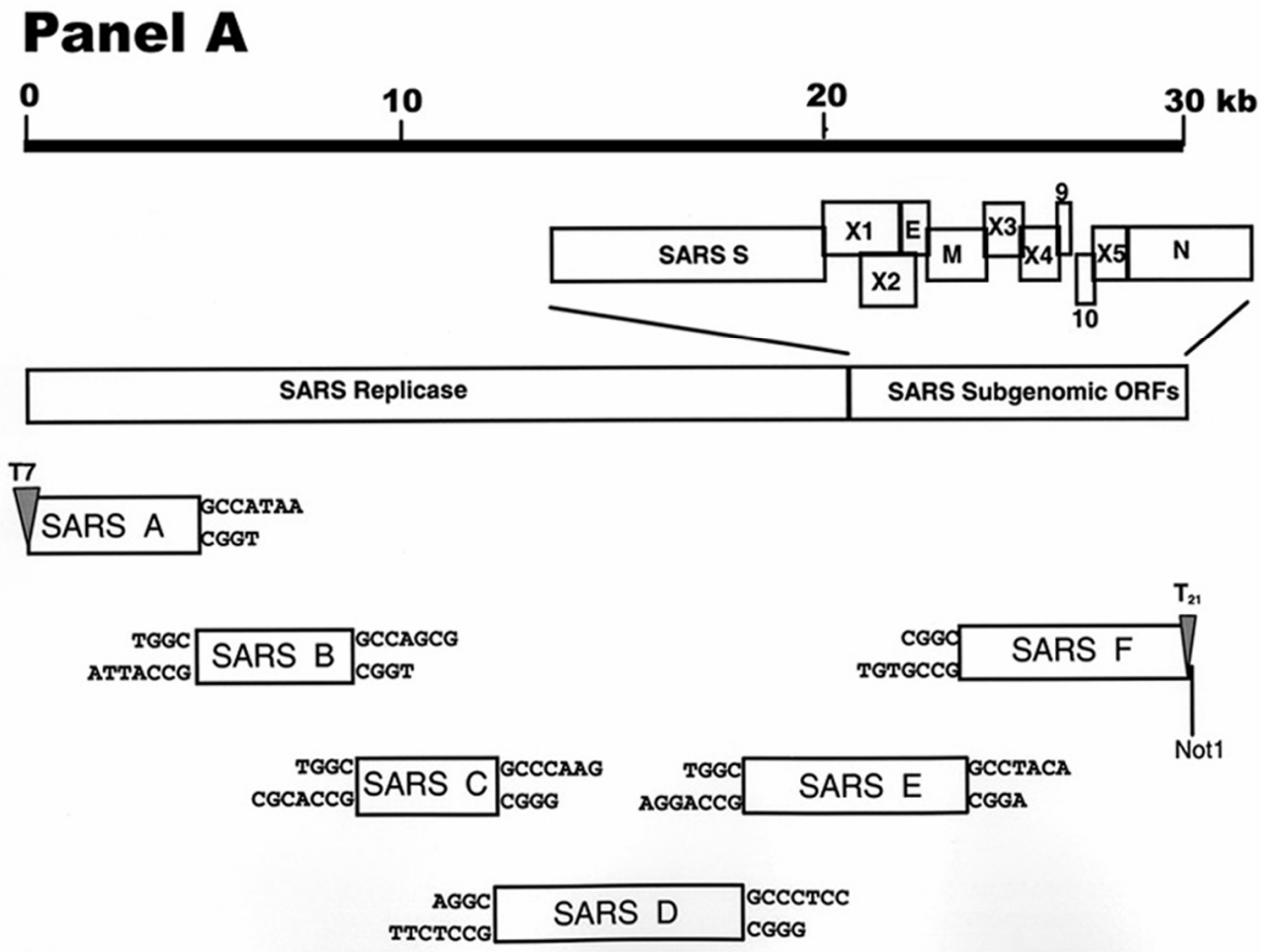
*allows for directional assembly of recombinant or synthetic DNAs into full length, million base pair microbial genomes

-synthetic cDNAs of ~5-8 Kb in length are readily available commercially

Yount et al., J.Virol 74, 10600-10611 (2000)

GCCNNNNNGGC- Bgl1 Site

SARS ASSEMBLY STRATEGY

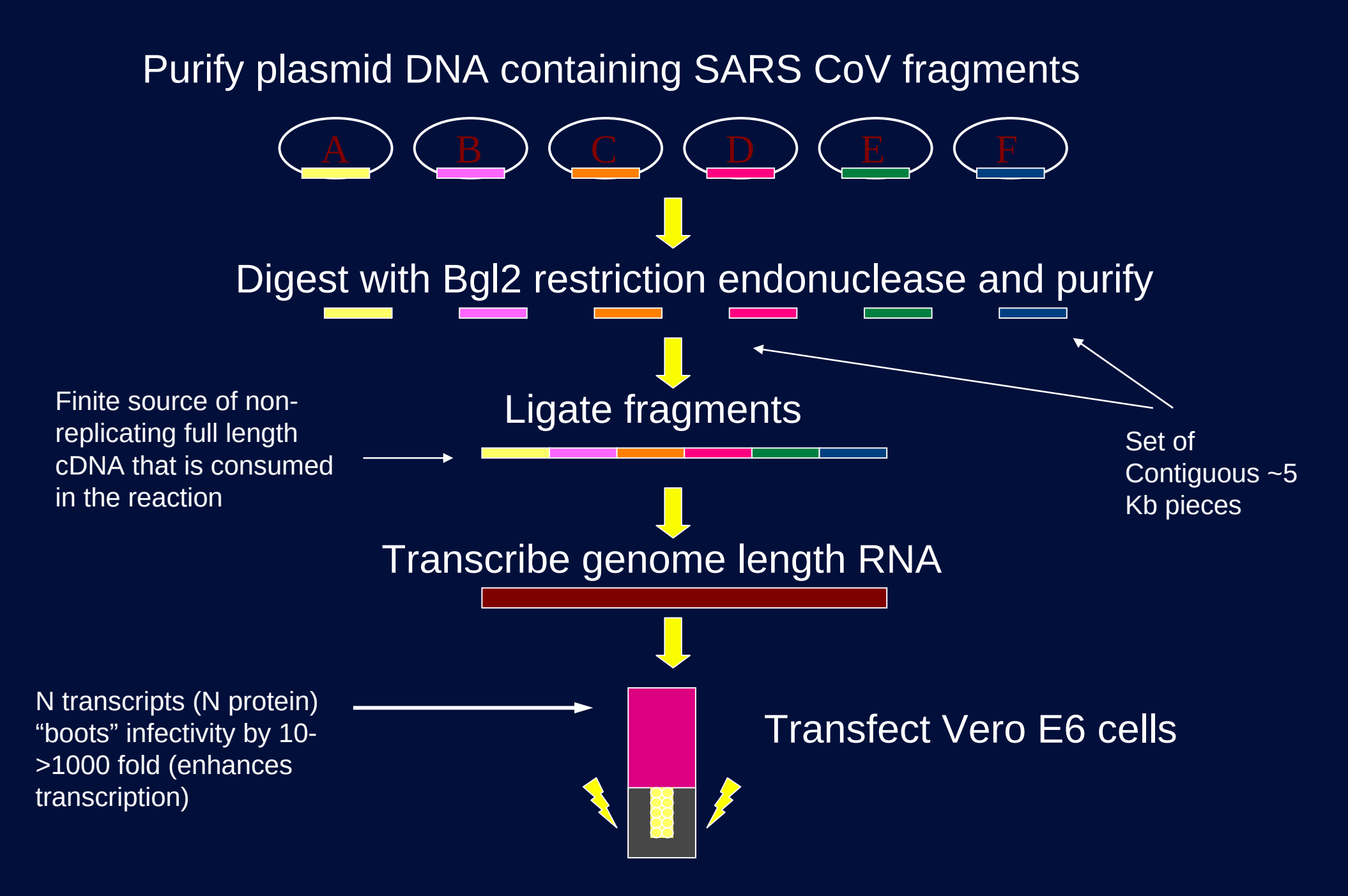


Yount et al., 2003. PNAS USA 100, 12995-13000

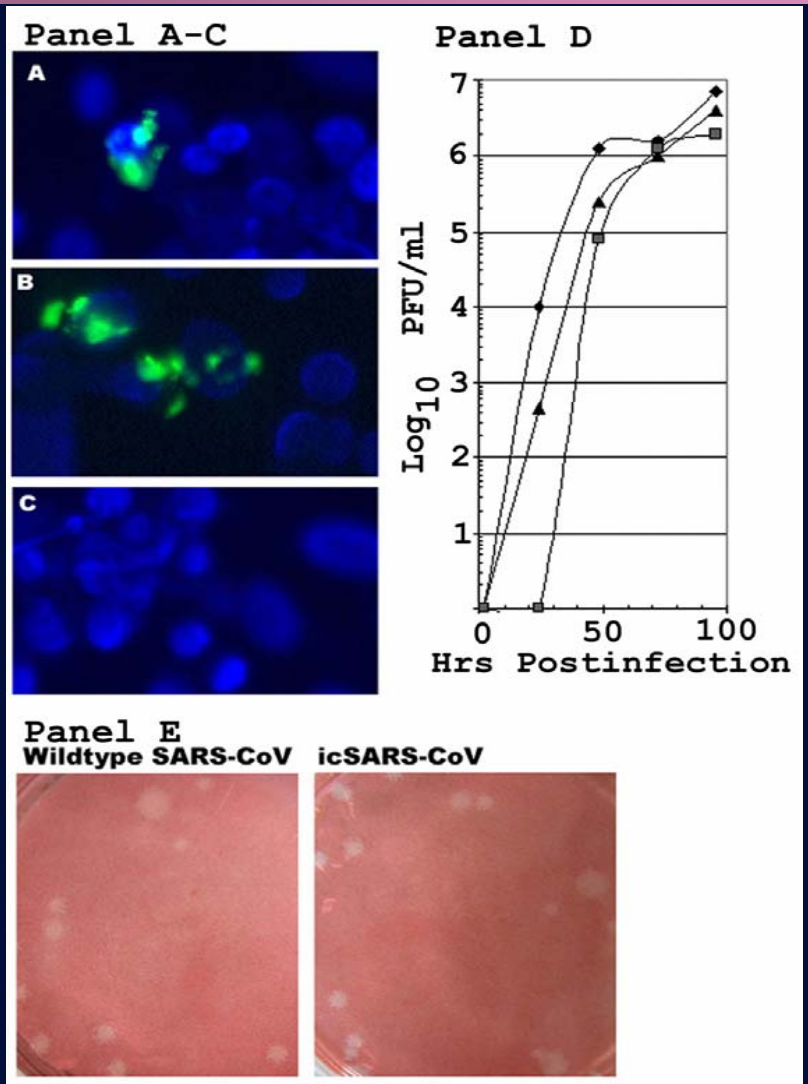
Systematic assembly of contiguous cDNA clones

64 possible ends; most ends are not compatible with any other ends

Assemble 2^{64} fragments using a recursive assembly strategy


Purify plasmid DNA containing SARS CoV fragments
A
B
C
D
E
F
Digest with Bgl2 restriction endonuclease and purify
Finite source of non-replicating full length cDNA that is consumed in the reaction
Ligate fragments
Set of Contiguous ~5 Kb pieces
Transcribe genome length RNA
N transcripts (N protein) "boots" infectivity by 10->1000 fold (enhances transcription)
Transfect Vero E6 cells

SARS Reverse Genetics



Built full length constructs and performed in vitro transcription with T7 RNA polymerase

Electroporated cells, performed FA at about 24 hrs post-transfection

+/- N transcripts

Virus yields determined by plaque assay

icSARS-CoV Pathogenesis

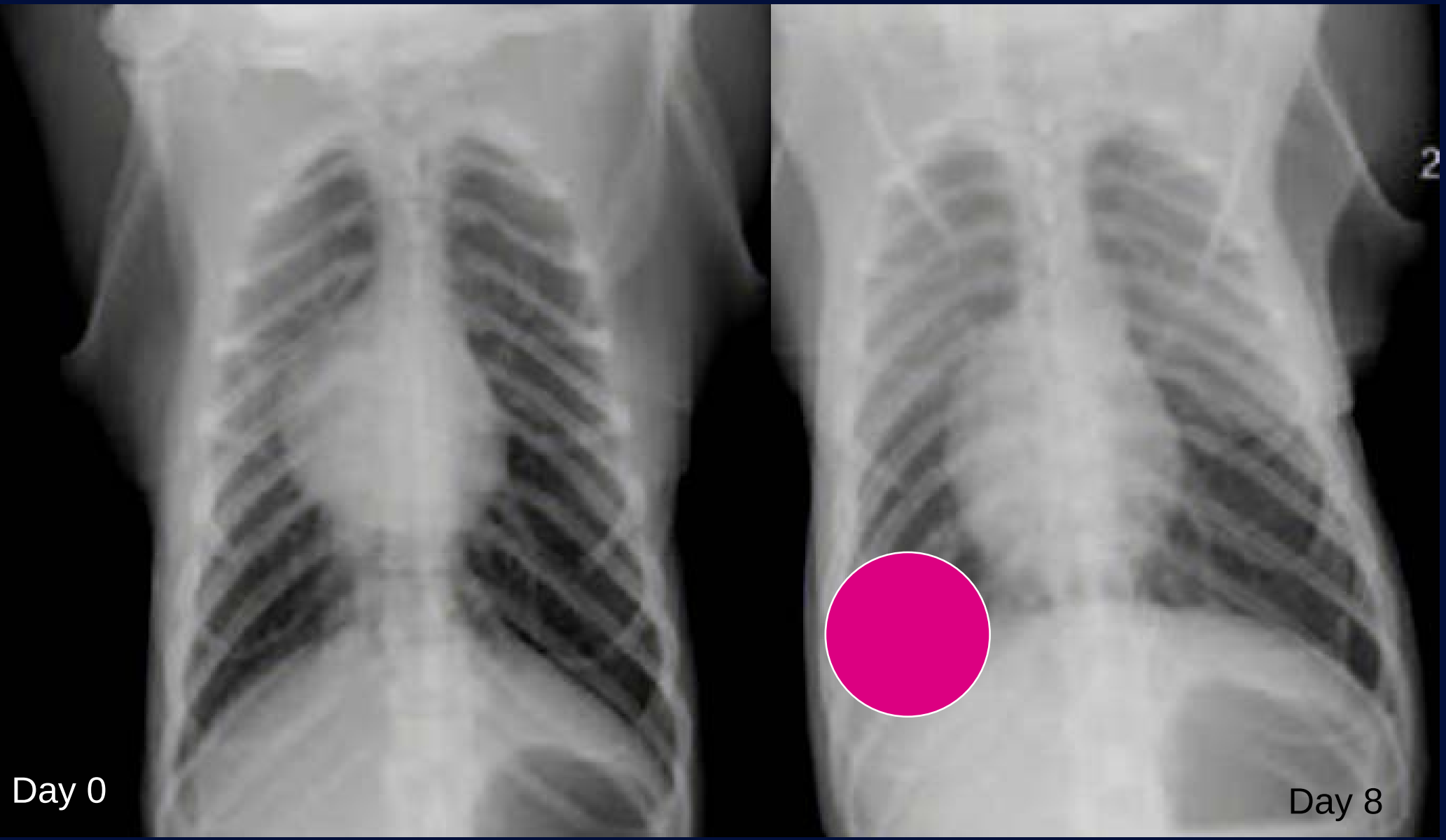
- 2 NHPs (Cynomolgus macaques) where infected by bronchial instillation and intranasal routes with icSARS-CoV ($3x10^6$ PFU/ml); 2 NHPs inoculated with wildtype SARS Urbani
- NHPs where monitored daily for clinical signs of disease
- Even days chest radiographic films were taken (AP and Lateral)
- Even days nostrils, throat, blood, urine, and feces were assayed for viral load by TaqMan RT-PCR

J.Paragas et al., unpublished

Wildtype and Recombinant icSARS-CoV NHP Results

- Recombinant icSARS-CoV and wildtype are comparable
- No clinical signs of disease
- No CBC or chemistry changes
- No temperature changes
- No reduction in oxygen saturation
- Viral load is pending
- RADIOGRAPHIC EVIDENCE OF MULTI-FOCAL LOWER LOBE PNEUMONIA in icSARS-CoV INFECTED ANIMALS

Jason Paragas et al, unpublished



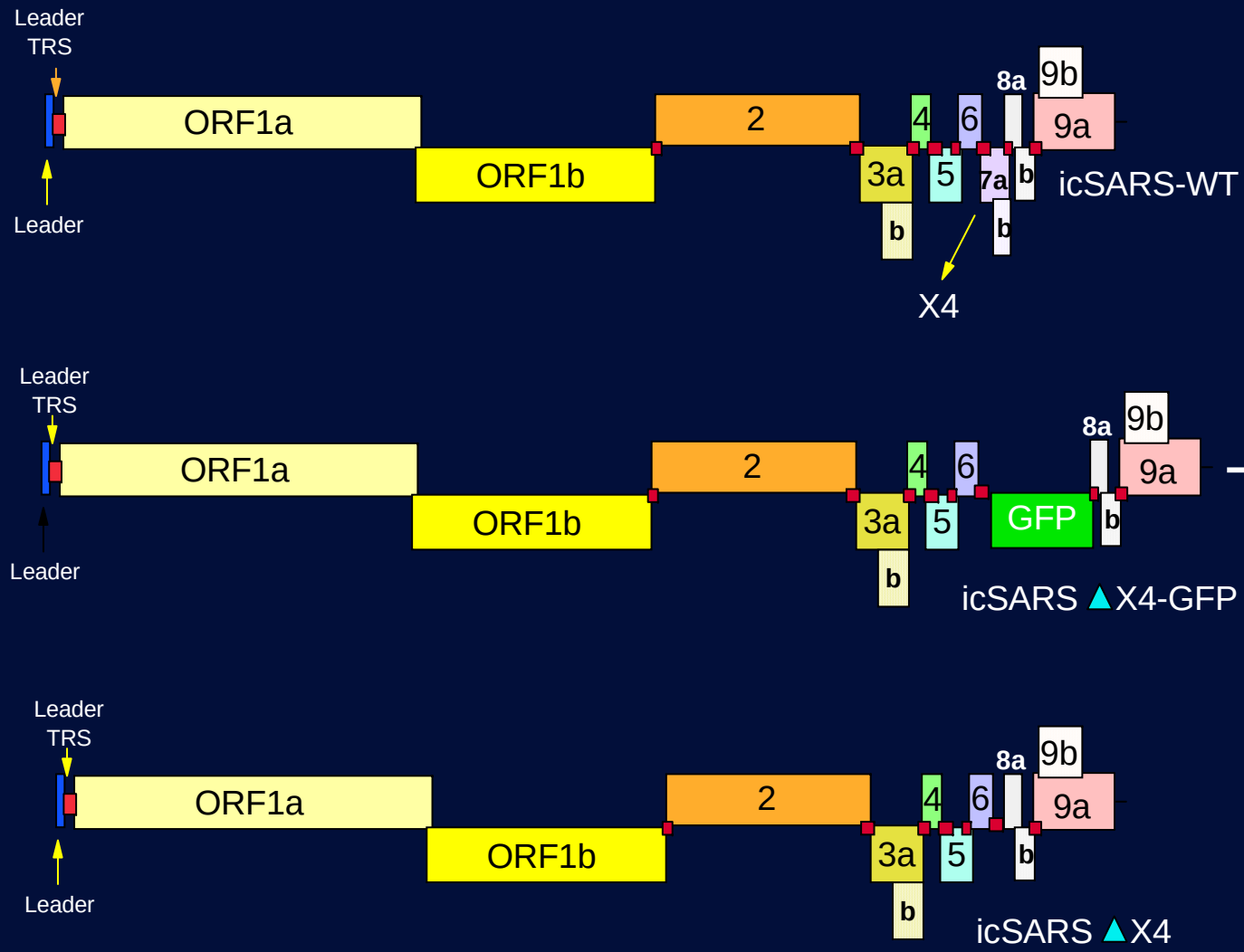
Right lower lobe pneumonia on X-Ray

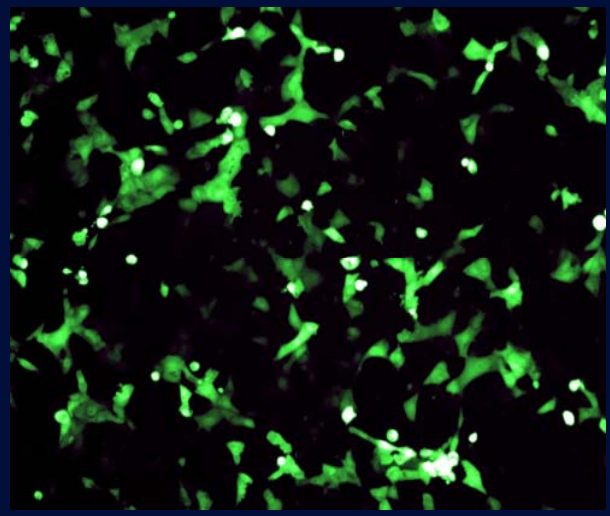
Summary SARS-CoV Pathogenesis

- icSARS-CoV produces radiographic pneumonia; reminiscent of clinical course of SARS in children less than 13 years of age.
  - Neutralization titers of 1:640 and 1:1280 against SARS-CoV
  - Animals are protected from wildtype challenge
- Results clearly not as dramatic as reported by other groups in the literature (**Fouchier RAM et al., Nature 2003)**.
- icSARS-CoV and wildtype virus pathogenesis are comparable in macaques
- icSARS-CoV and wildtype virus replicate to similar titers in the mouse

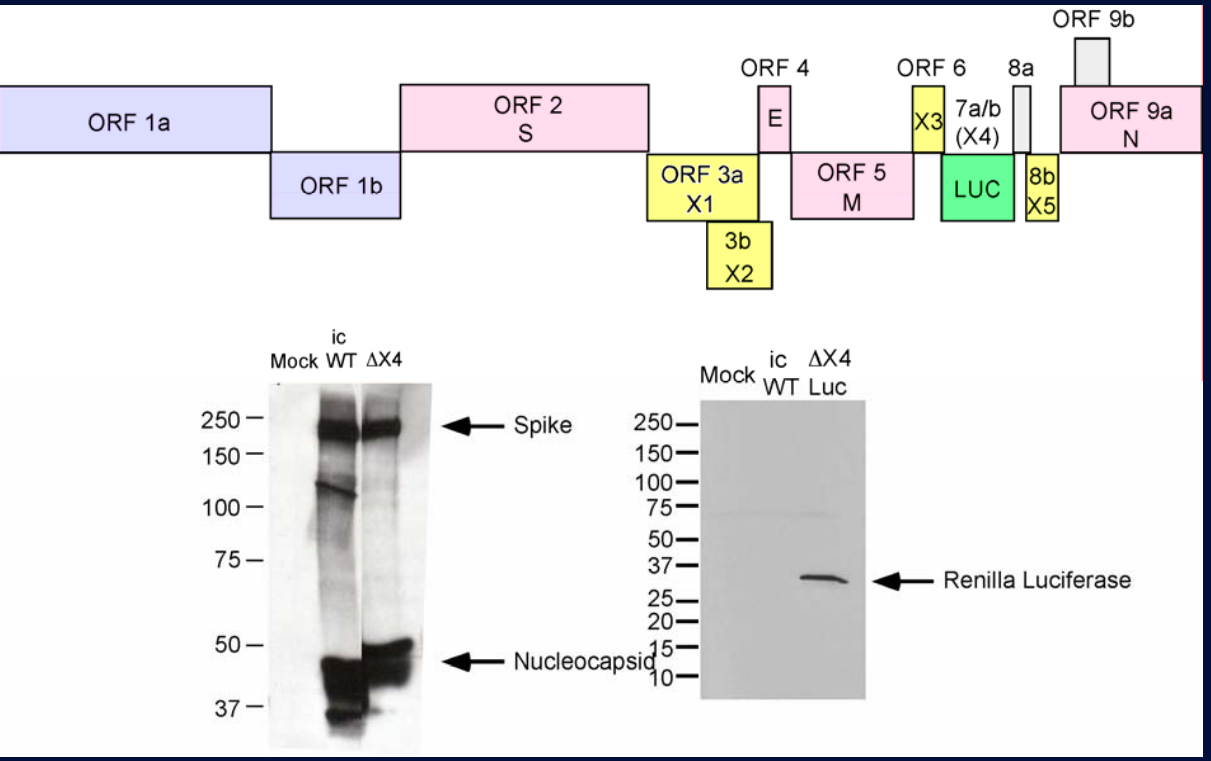
Accessory ORF function in replication and pathogenesis?

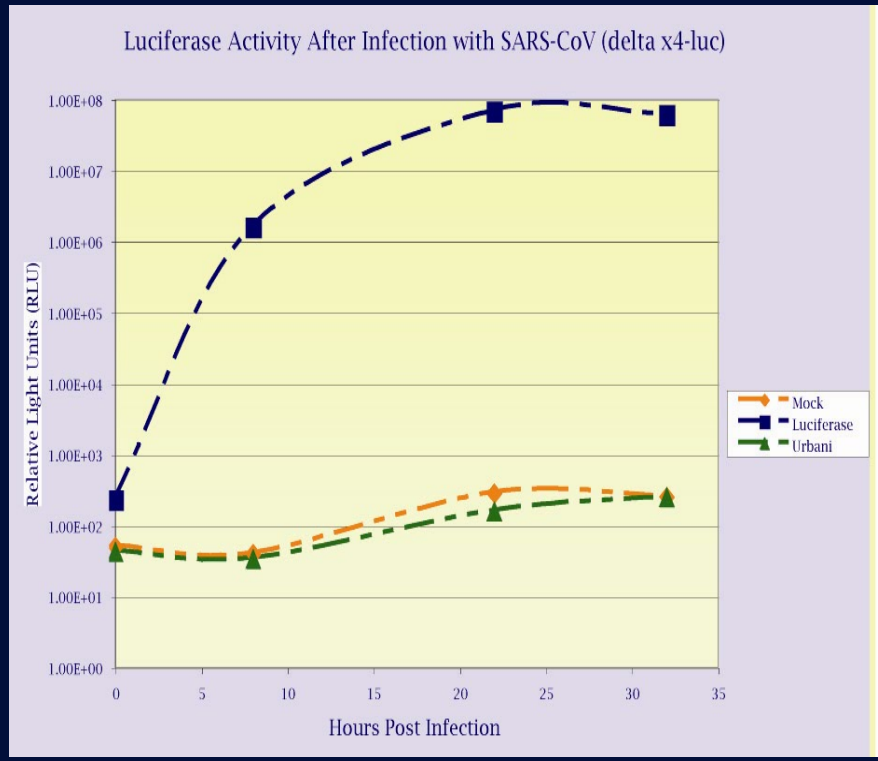
Recombinant SARS-CoV





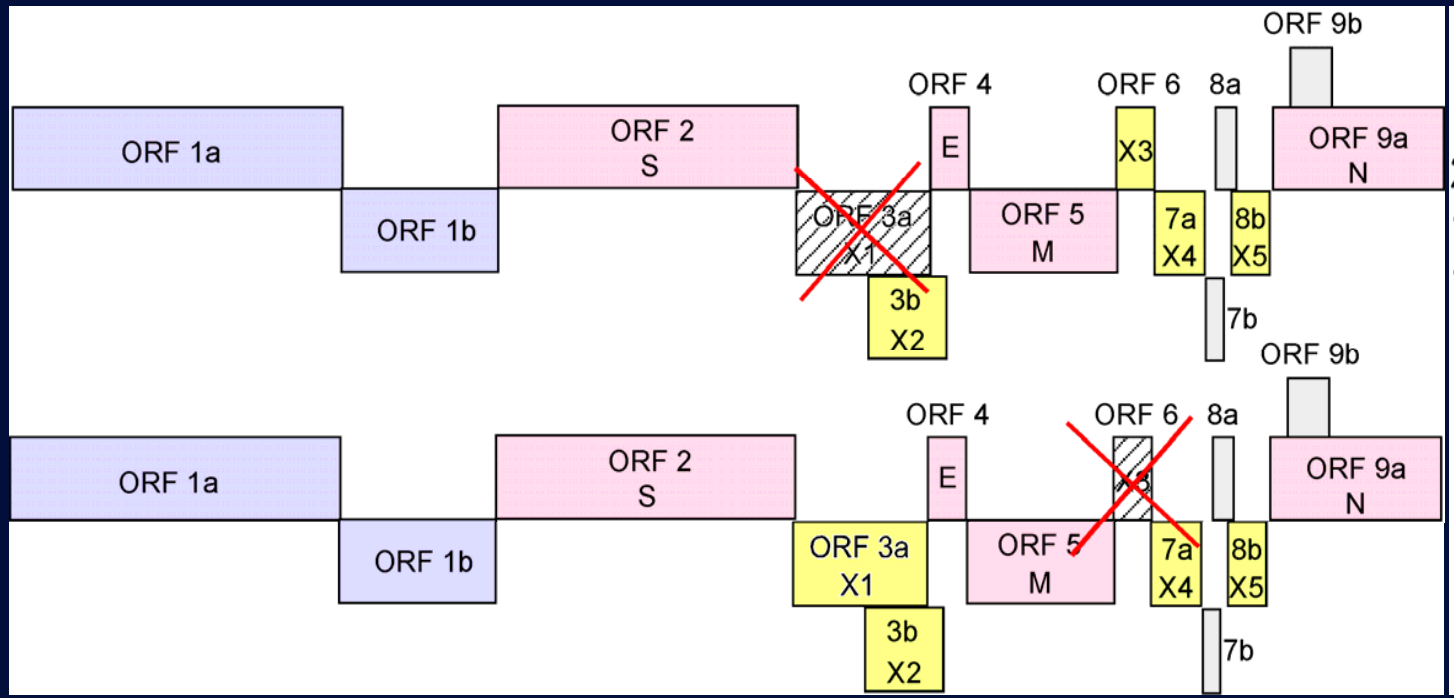
Indicator Virus to Screen Compounds with anti SARS- CoV Activity

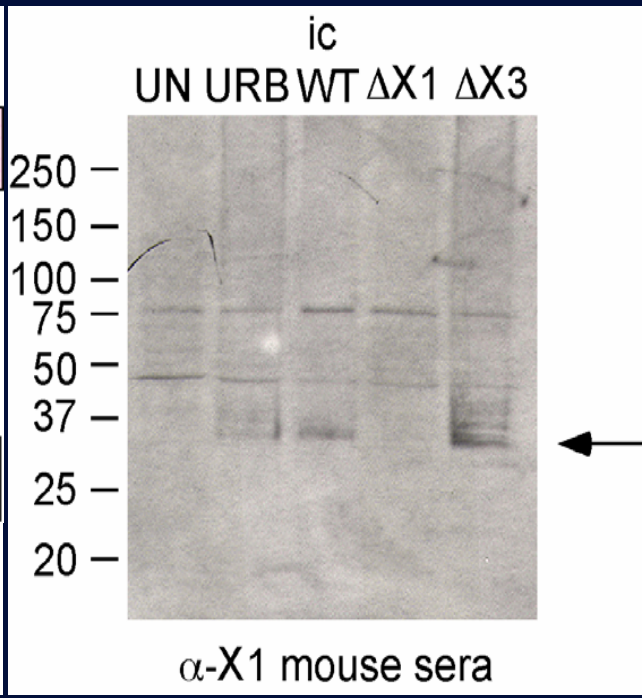




Platform for rapid screening of compounds with anti-SARS activity

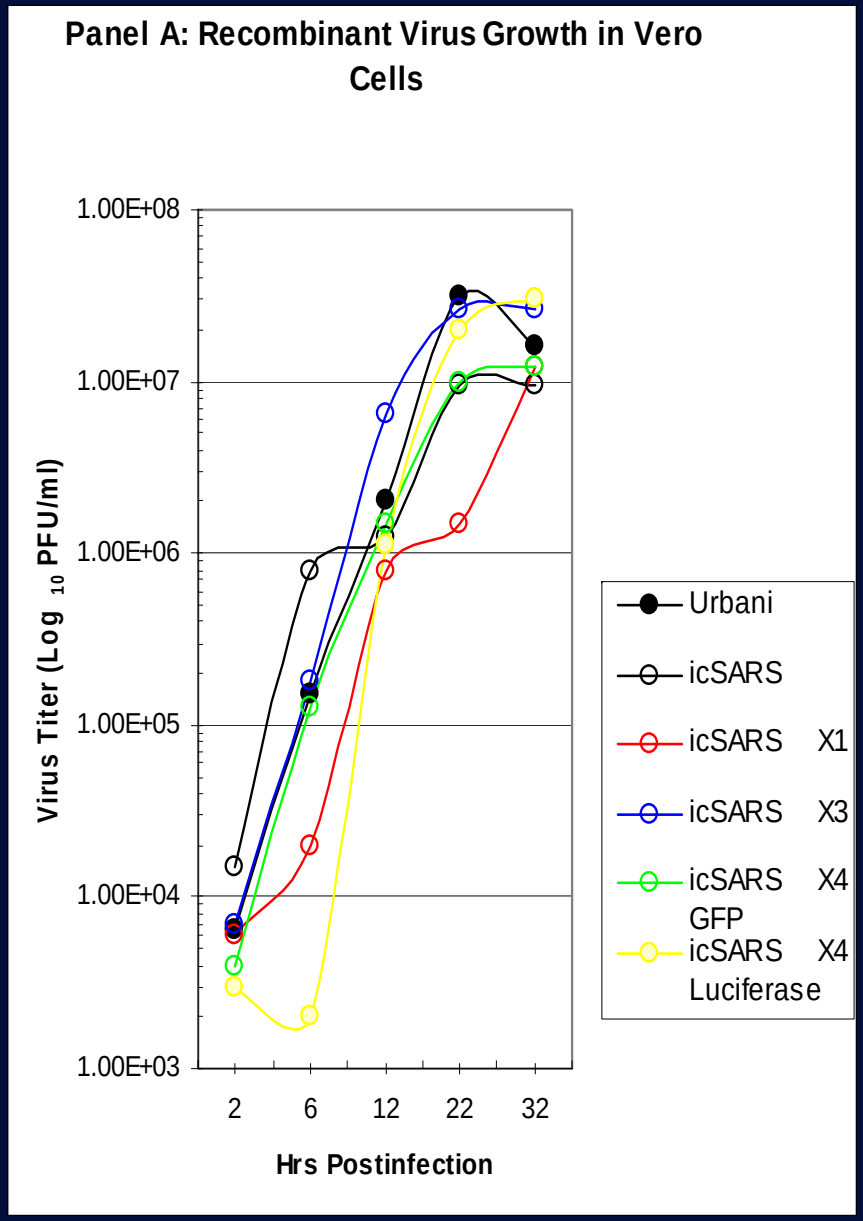
Are ORF3a and ORF6 accessory proteins essential?

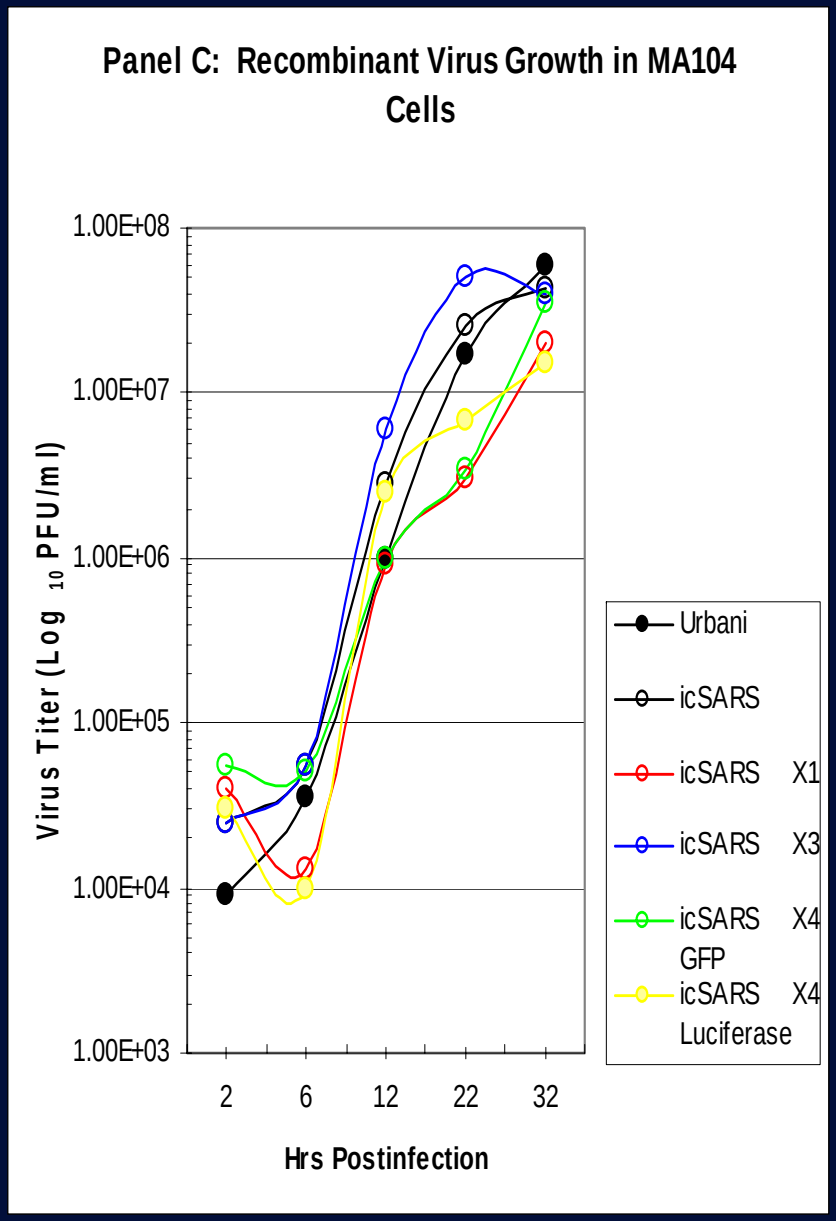




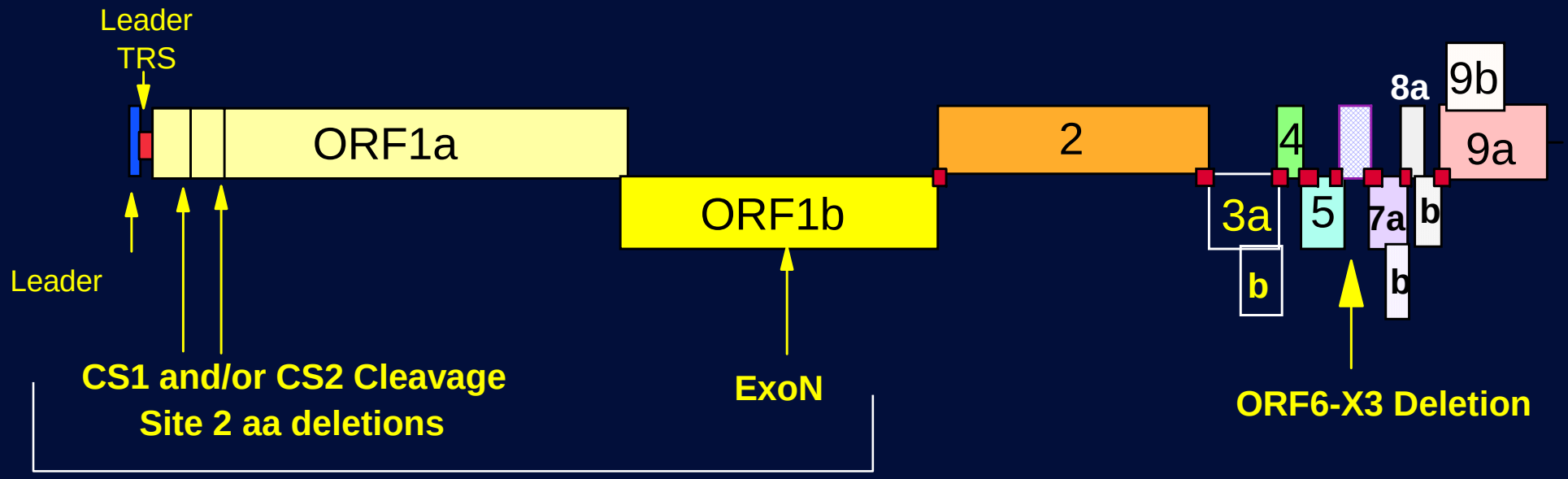
Why? Deletion of group specific genes attenuates all other coronaviruses tested but little effect on in vitro replication=candidate attenuated viruses and identification of virulence alleles

SARS-CoV Recombinant Virus Growth





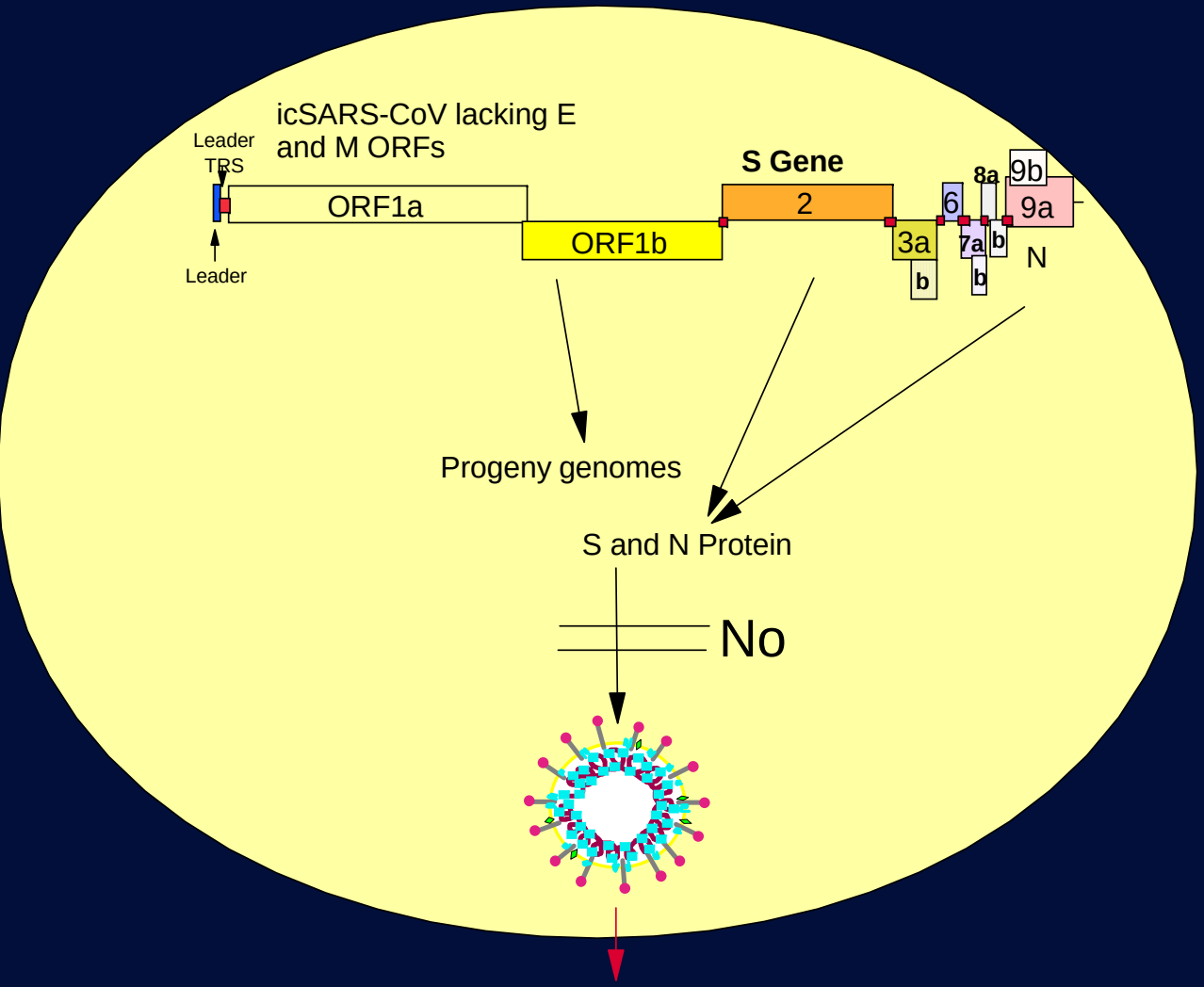
“Attenuated SARS-CoV or Safer Laboratory Strains”



Combinations:		Viable
7.5×10^6	X1, X2, X3	Yes
8.0×10^6	CS1, ExoN Y-H, X3	Yes
5.0×10^6	CS2, ExoN Y-H, X3	Yes

→ Live virus candidates or seed stocks for Killed Vaccines

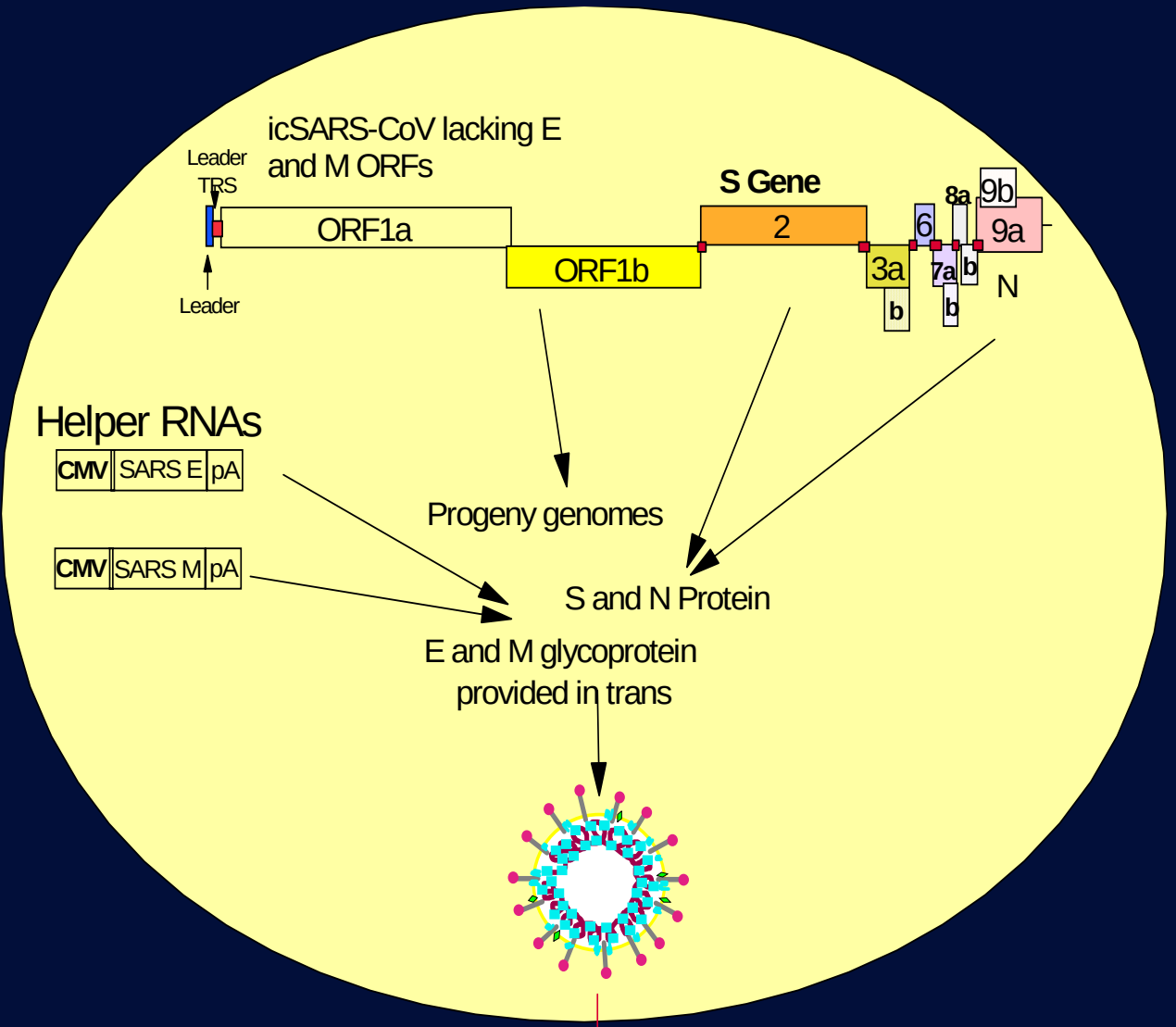
Replication Competent Propagation Deficient Coronaviruses



10^5-10^7 viral replicon particles (VRPs)

Curtis K et al., 2002; Ortego J. et al., 2002

Replication Competent Propagation Deficient Coronaviruses



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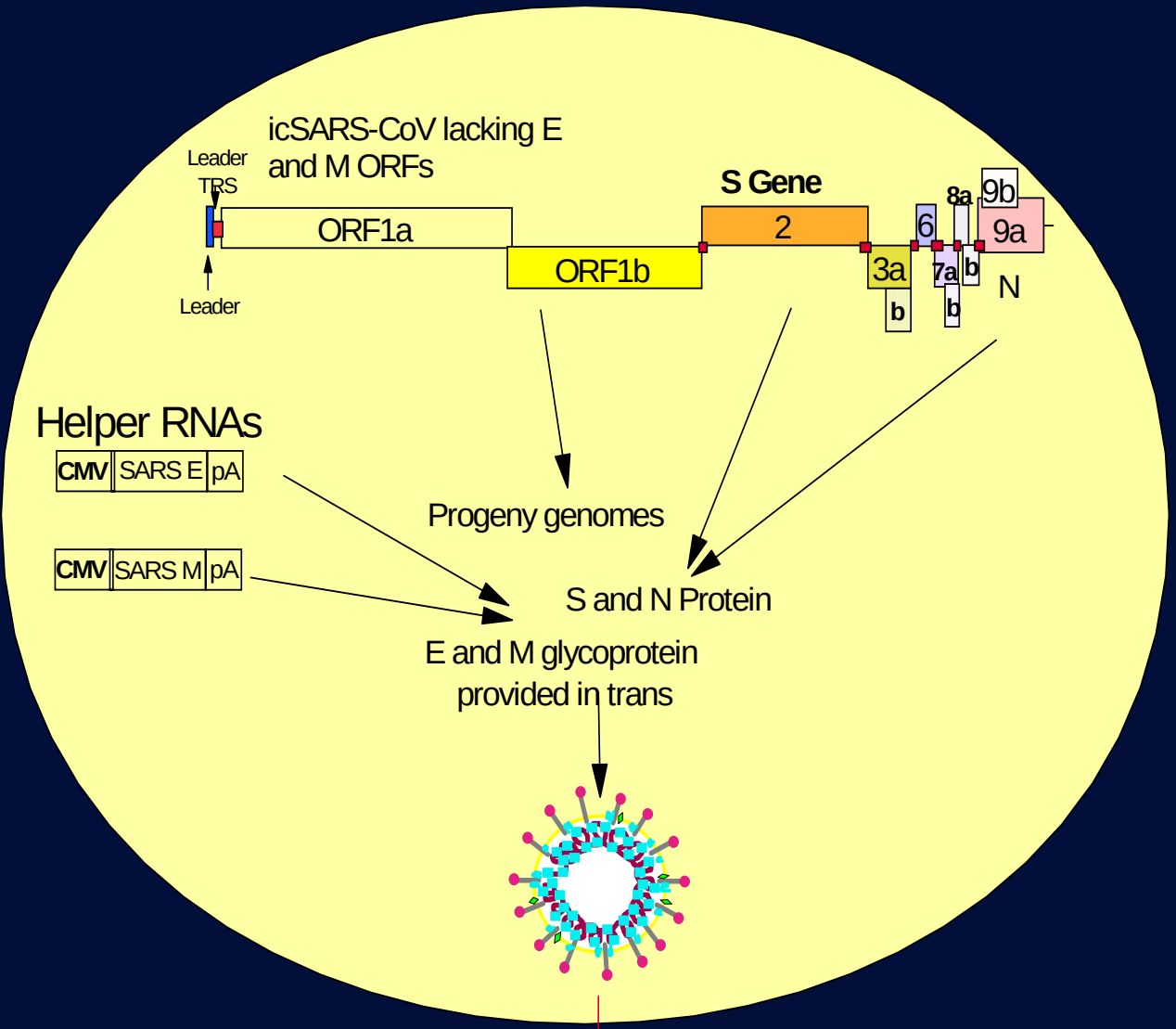

- E is absolutely essential for TGEV VRP formation, but not MHV
  - What is essential for SARS particle production?
- Most would likely agree that S, E, M deficient replication competent viruses would not produce progeny virions
  - Very Safe
  - Recombination repair?
    - Helper RNA or exogenous coronavirus
- 2/3 genome length is replicase in coronaviruses

10^5-10^7 viral replicon particles (VRPs)

Curtis K et al., 2002; Ortego J. et al., 2002

Safety Precautions

- Facility
- Personnel
- Community

Developed in collaboration with UNC Environmental Health Department (EHS), University Employee Occupational Health Center (UEOHC), Local and State Public Health Offices

Enhanced Features in the UNC SARS-CoV BSL3 Facility

- Shower in and out facilities, dual anteroom access
- HEPA filter exhaust
- Redundant exhaust fans
- Increased Security
  - Card key access, alarm system to Public Health/Campus Police, Lab controlled combination lock
- Techniplast Sealsafe™ Hepa filtered animal housing

Personnel Precautions
Observed in UNC SARS-CoV Facility

- Training Program
  - University safety training programs (BSL2 and BSL3 training, Laboratory Safety, chemical, radiation and Occupational health, Blood borne pathogen, etc.), Proficiency with numerous virological assays/mildly pathogenic coronaviruses, Proficiency with PAPR and protective gear, Partnered training in BSL3 (~6 months)
- PAPRs with Hepa filters and hoods worn at all times
- Yearly Flu vaccines
- Archived serum samples
- Protective disposable clothing:
  - Disposable coveralls
  - Shoe covers
  - Disposable wrap around gown
  - Double gloves

Worker/Community Protective Measures

- Cease all SARS-CoV work 14 days prior to any travel by mass transit
- High Risk Exposure Event (aerosol generating activity with failure of adequate work practices and/or PPE, needle stick or animal bite)
- Low Risk Exposure (e.g. no aerosol generating activity at time of failure of work practices and/or PPE):
- No Recognized Exposure Involving Breach of PPE or Inadequate Work Practices, but Employee Develops Respiratory Symptoms with Fever:

Response: Various times of self quarantine at home. Worker has mask and thermometer and is in daily contact with supervisor, EHS and physicians/staff at UEOHC.

Recombinant Coronaviruses

- Stability in nature?
  - Delete ORFs, rearrange ORFs alter replicase protein processing and expression, replication competent propagation defective (engineer to be fairly safe)
- Recombination with existing circulating strains?
  - Recombination repair of defective allele
  - Distribution of foreign sequences or virulence alleles into related coronaviruses
  - Recombination/reversion proof coronaviruses?
- Pathogenesis studies and identification of virulence alleles
- Platform for development of safe laboratory strains or attenuated viruses for live or killed vaccine applications, drug testing
- Recombinant coronavirus vaccines for humans and animals

SARS CoV-Related Research

NIH AI23946, AI059136, AI061819

- Baric Laboratory (UNC)
  - Boyd Yount
  - Kris Curtis
  - Will McRoy
  - Amy Sims
  - Damon Deming
  - Eric Donaldson
  - Tim Sheehan
- UNC UEOHC
  - Drs. Brian Boehlecke and David Weber
- UNC EHS Department
  - Ray Hackney
  - Deb Howard

- USAMRIID Group
  - Lisa Hensley
  - Elizabeth Fritz
  - Thomas Geisbert
  - Peter Jahrling
  - Jason Paragas, PhD
  - James Lawler, MD, LCDR,USN
  - Aura Garrison
  - Mallory Tate, DVM, MAJ, USA
  - John Huggins, PhD
  - Tim Endy, MD, COL, USA
- Denison Laboratory (Vanderbilt)
  - Mark Denison
  - Erik Prentice