

**RHU « CirB-RNA »**  
ANR-17-RHUS-0003

## International Workshop on Viral Biomarkers

September 7, 2023  
*Institut Lumiere, Lyon*



## The molecular biology of HBV RNAs: insights on new biomarkers

Massimo Levrero (Lyon, France)

**RHU « CirB-RNA »**  
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## The molecular biology of HBV RNAs: insights on new biomarkers

Massimo Levrero (Lyon, France)

*the CirB-RNA WP2 members*



Delphine  
Bousquet



DooHyun  
Kim



Hyoseon  
Tak



Alexia  
Paturel



Francesca  
Casuscelli



Xavier  
Grand



Vincenzo  
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Barbara  
Testoni



Massimo  
Levrero



Fabien  
Zoulim



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### The molecular biology of HBV RNAs: insights on new biomarkers

Massimo Levrero (Lyon, France)

*the CirB-RNA WP2 and WP3 Roche members*



Marintha Heil



Christian Voitenleitner



Aaron Hamilton



Debra Liggett



Beth Scott



Thomas Meister



Paul Dawson

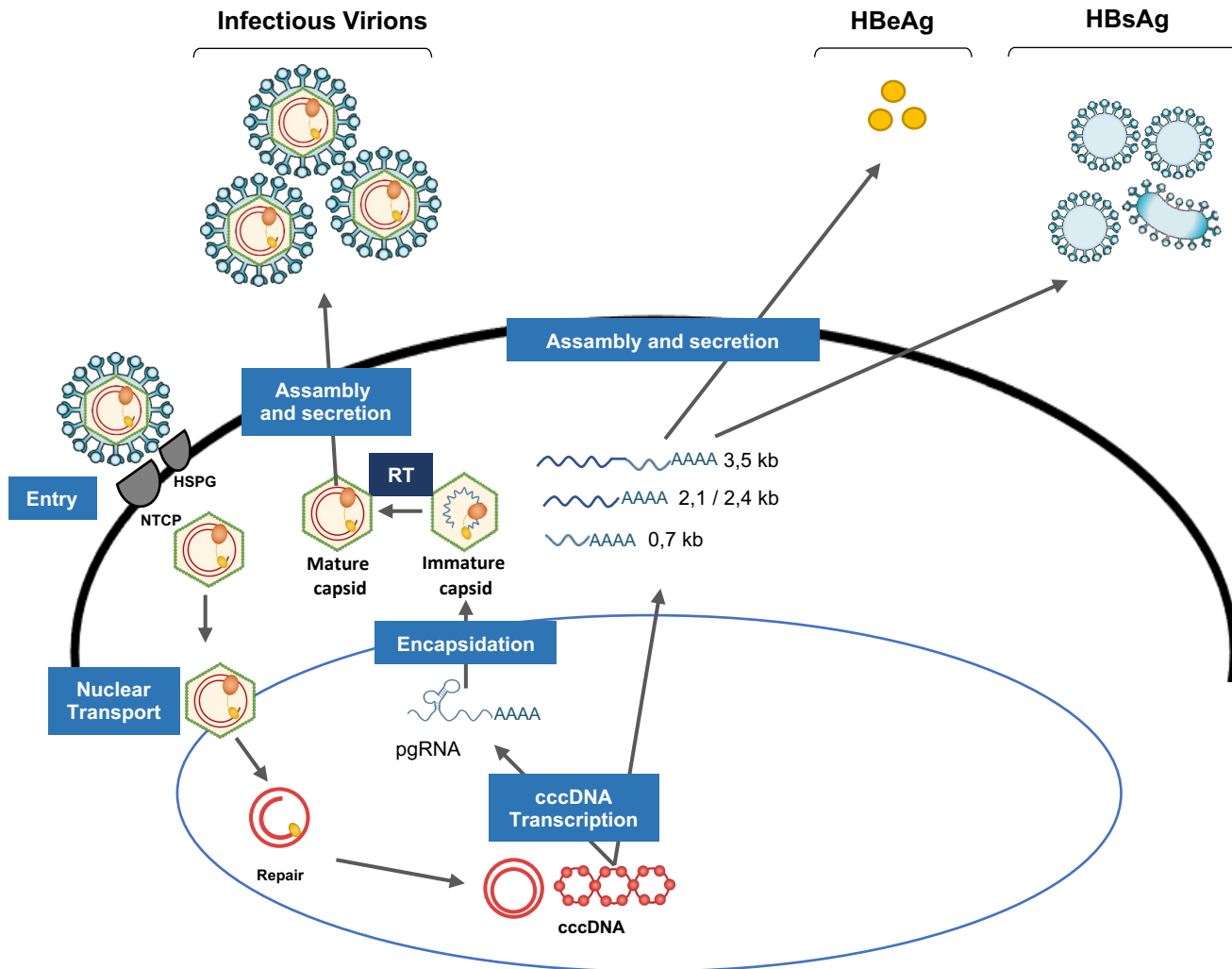


Michael Lobritz



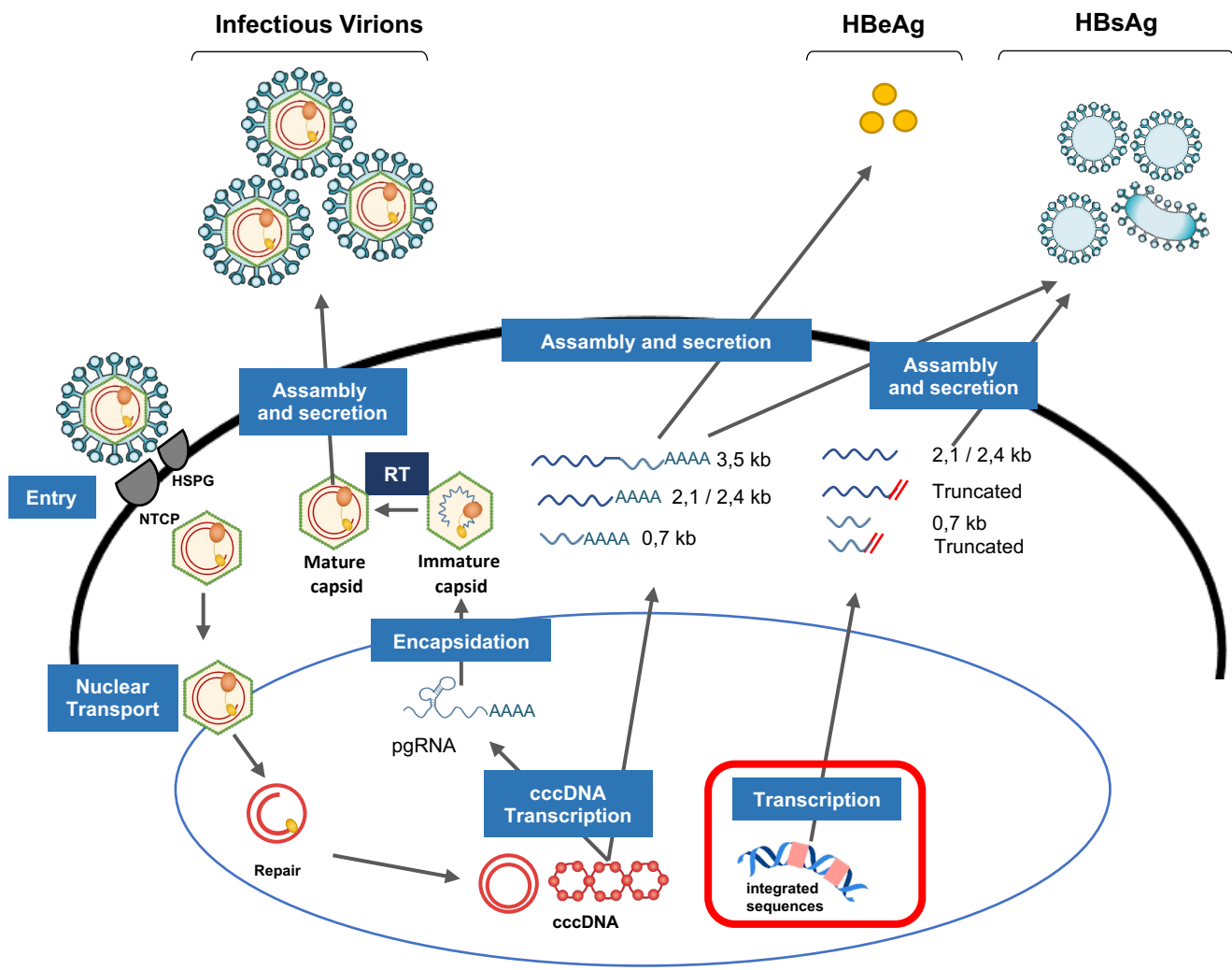
Alan Blair

# The molecular biology of HBV RNAs .1



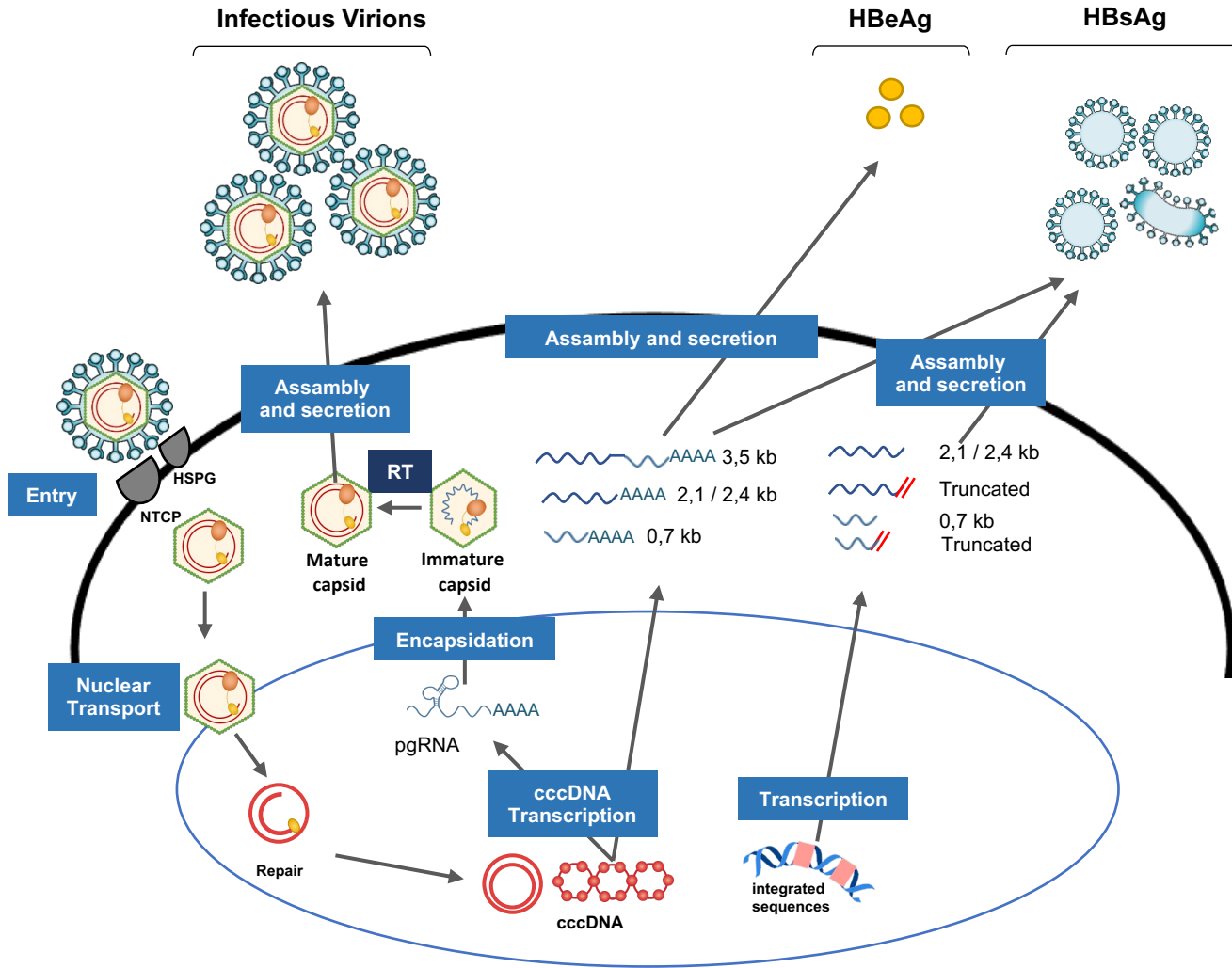
- 5 HBV RNAs :
  - 3.5 Kb (pgRNA, PreC)
  - 2.4 / 2.1 KB (PreS1, PreS/S)
  - 0.7 Kb (HBx)

# The molecular biology of HBV RNAs .1



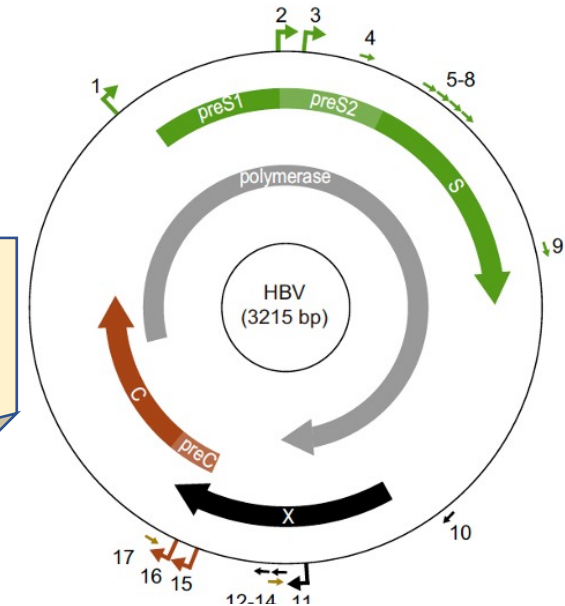
- 5 HBV RNAs :
  - 3.5 Kb (pgRNA, PreC)
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  - 0.7 Kb (HBx)

# The molecular biology of HBV RNAs .2



- 17 HBV RNAs :
  - 3.5 Kb (pgRNA, PreC)
  - 2.4 / 2.1 KB (PreS1, PreS/S, S)
  - 0.7 Kb (HBx)
  - AS

Liver:  
- 16 T/NT HCCs Pts  
Blood  
- 8 CHB Pts



Major peaks (>1,000 raw tags)

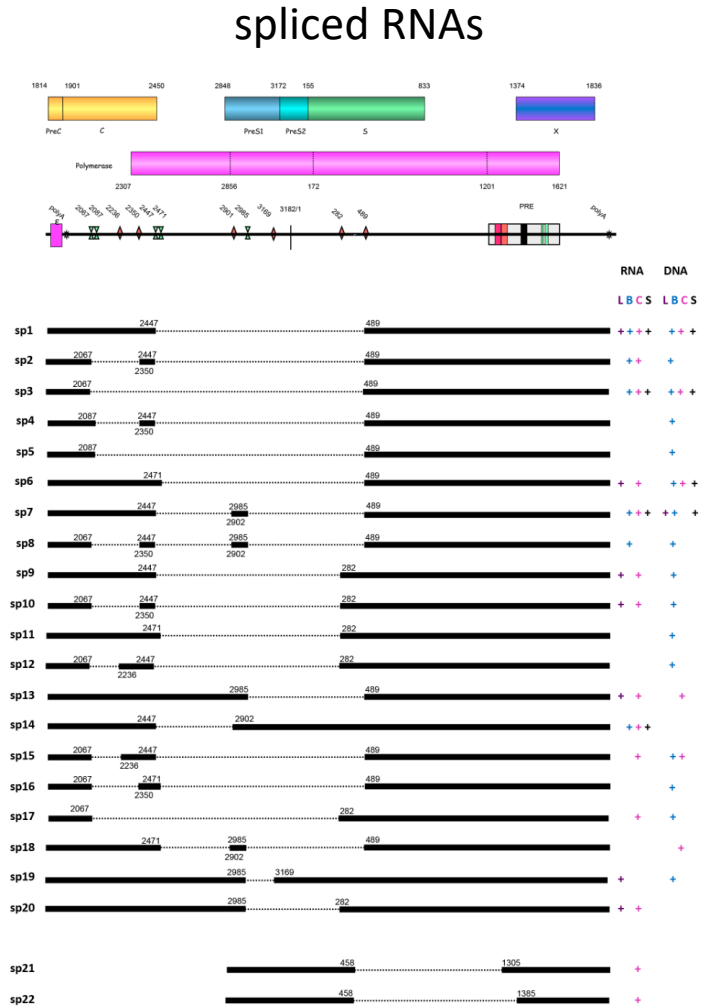
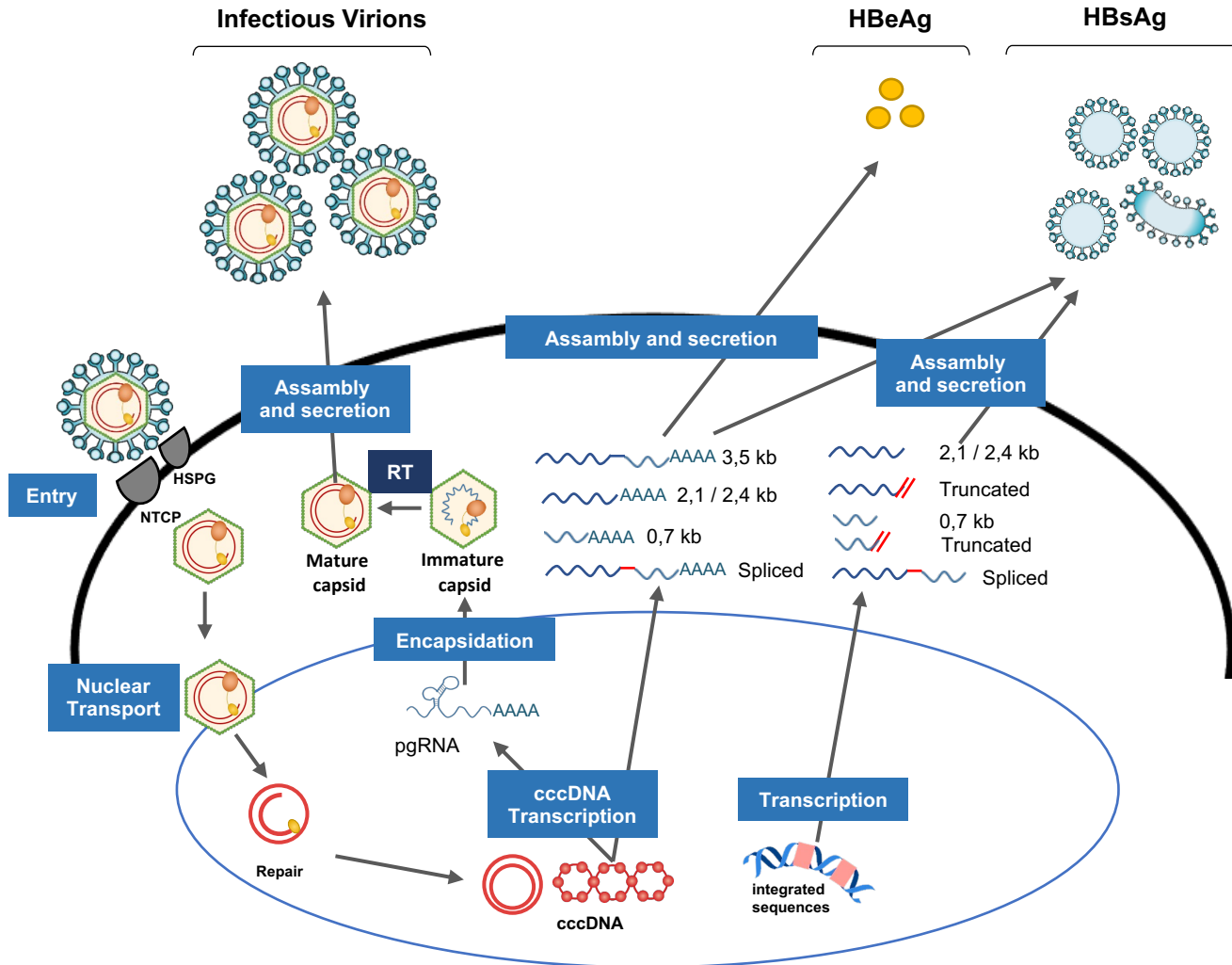
#	Gene	Start	End	Total count	Strand	Model
1	PreS1	2802	2807	12,748	+	Y
2	PreS/S	3183	19	201,883	+	Y
3	S	25	121	19,004	+	Y
11	X	1523	1524	1,013	+	Y
15	PreC/C	1745	1746	1,106	+	N
16	pgRNA	1816	1819	112,452	+	Y

Minor peaks (100 ~ 1,000 raw tags)

#	Gene	Start	End	Total count	Strand	Model
4	S	132	135	248	+	N
5	S	203	207	527	+	Y
6	S	258	262	282	+	N
7	S	284	285	528	+	Y
8	S	302	303	118	+	N
9	S	759	760	729	+	Y
10	X	1210	1211	124	+	Y
12	AS	1557	1610	157	-	Y
13	X	1572	1573	117	+	N
14	X	1655	1656	316	+	N
17	AS	1822	1834	207	-	N



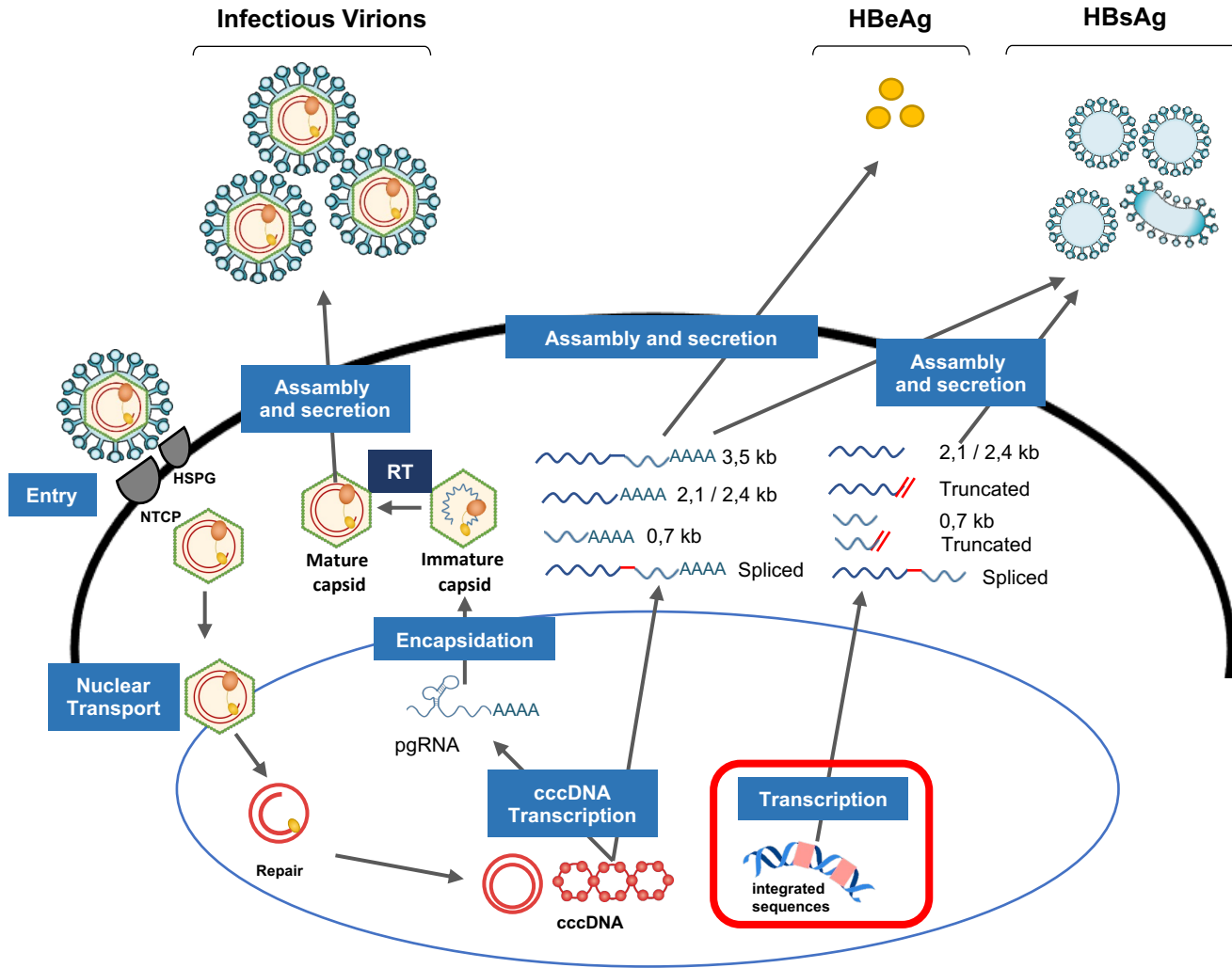
# The molecular biology of HBV RNAs .3



Kremsdorf et al. 2021

- 5 HBV RNAs :
  - 3.5 Kb (pgRNA, PreC)
  - 2.4 / 2.1 KB (PreS1, PreS/S)
  - 0.7 Kb (HBx)
- 22 spliced species

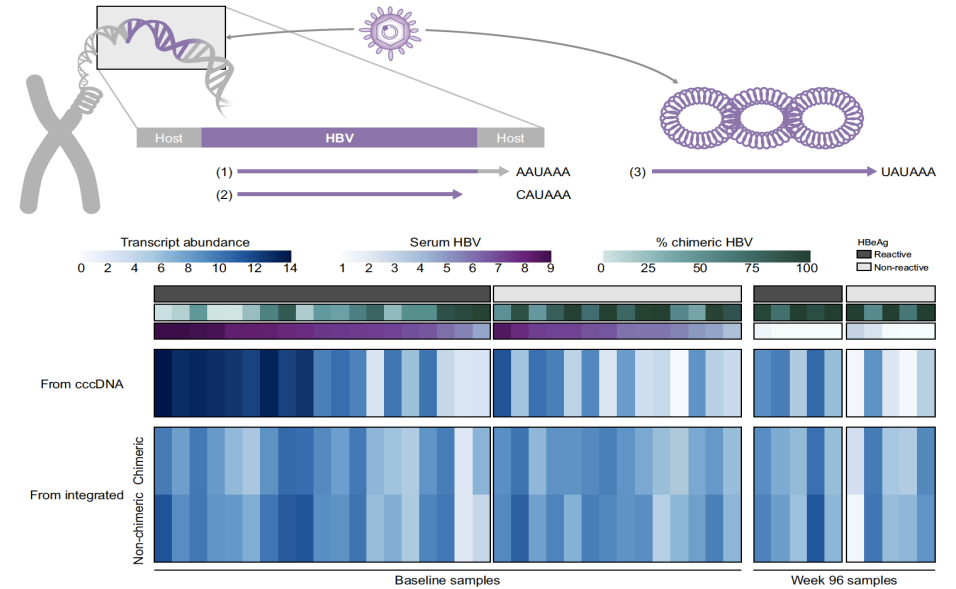
# The molecular biology of HBV RNAs .4



- 5 HBV RNAs :
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  - 0.7 Kb (HBx)

## Iso-Seq PacBio RNA sequencing

42 CHB patients from Gilead's study GS-US-174-0149

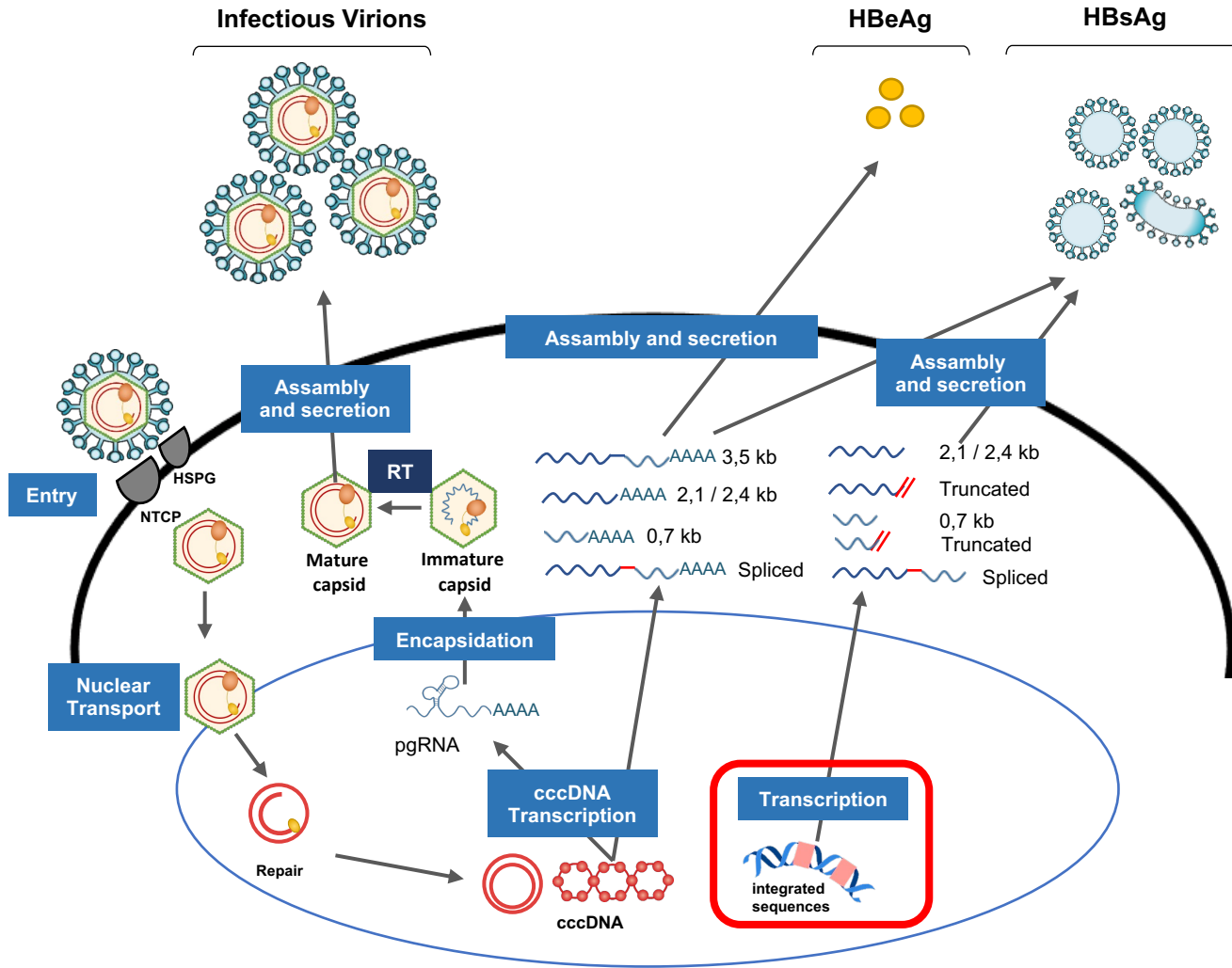


- The amount of transcription from integrations similar between HBeAg(+) and HBeAg(-) pts
- Lower transcription from cccDNA in HBeAg(-) pts
- Ratio of integrated HBV transcripts to cccDNA transcripts much higher in HBeAg(-) pts

Van Buuren, JHEP Reports 2022



# The molecular biology of HBV RNAs .4

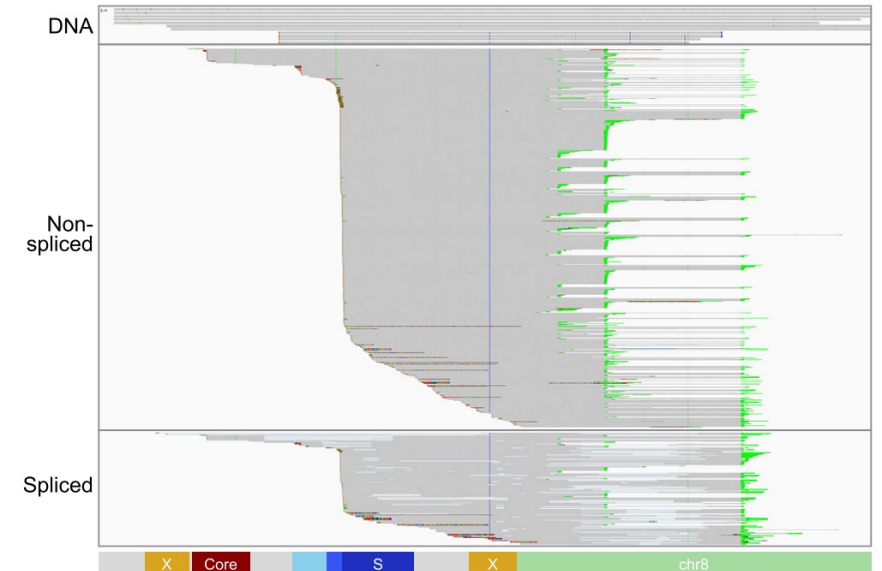


- 5 HBV RNAs :
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## Iso-Seq PacBio RNA sequencing

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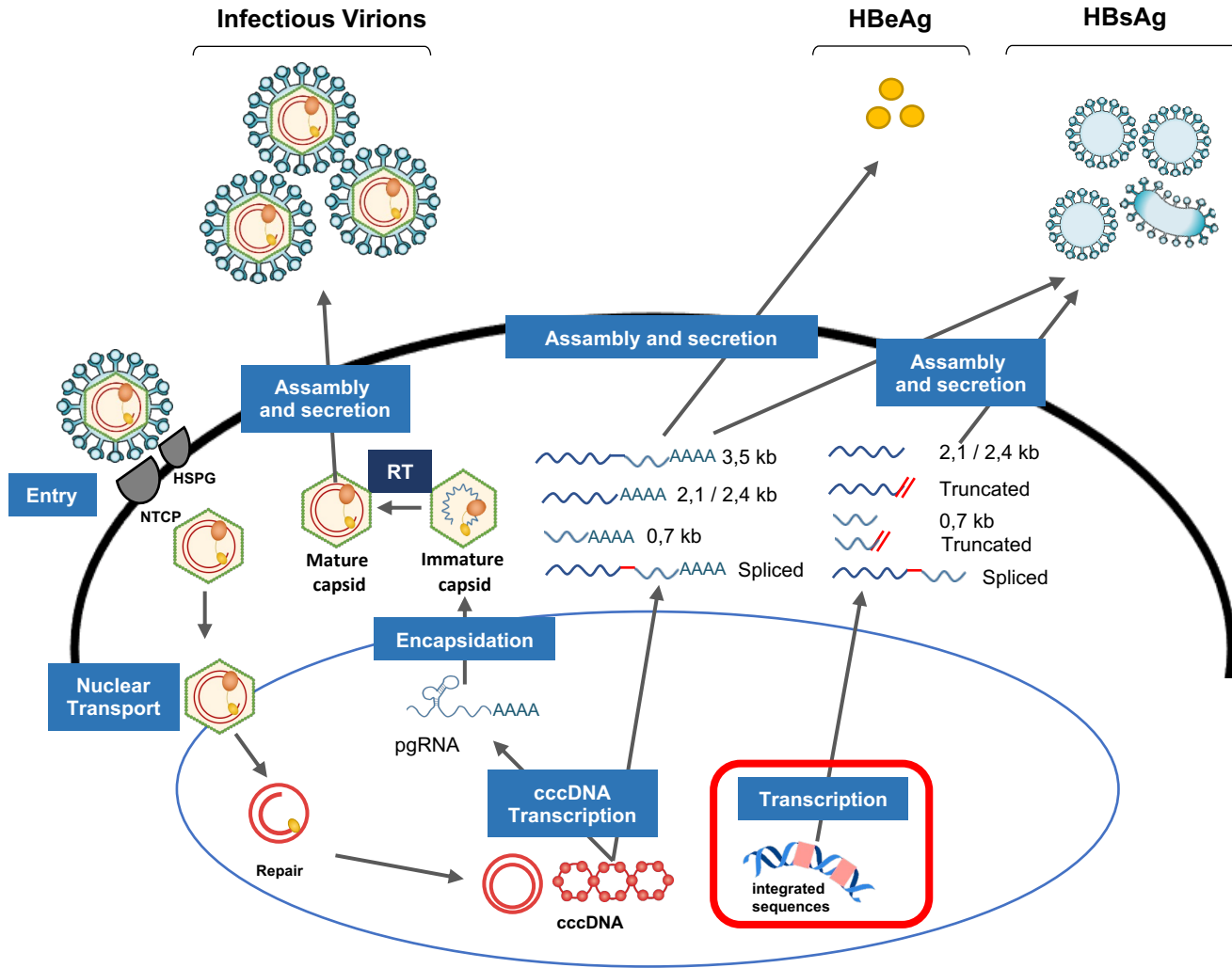
- an unusually long clonally expanded HBV sequence (4,119 bp), chimeric with chromosome 8 at the 3' end generates transcripts expressing all of Core, PreS1, and PreS2/S and utilises 3 different host poly(A) sequences.



- ratio of spliced to non-spliced HBV RNAs ranging from 2% to over 20%
- highest in pHBeAg-positive patients
- the majority of spliced HBV RNAs originated from cccDNA,

Van Buuren, JHEP Reports 2022

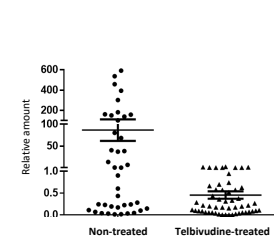
# The molecular biology of HBV RNAs .5



- 5 HBV RNAs :
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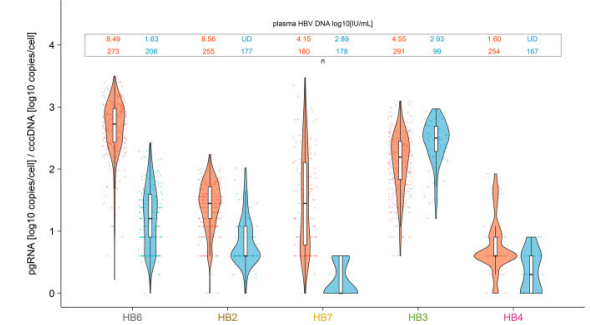
- NUCs decrease transcription from the cccDNA over time ...

3.5Kb RNA quantification



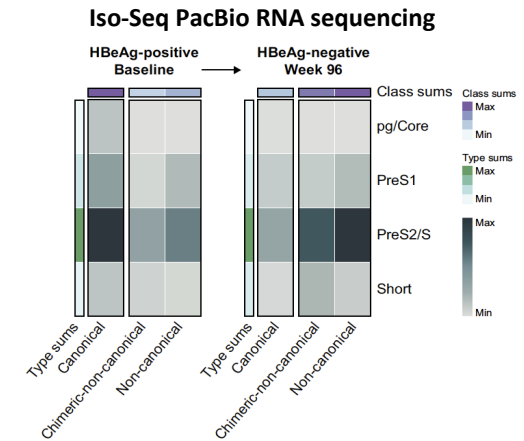
Lebossé, 2020

Paired biopsies during ttx



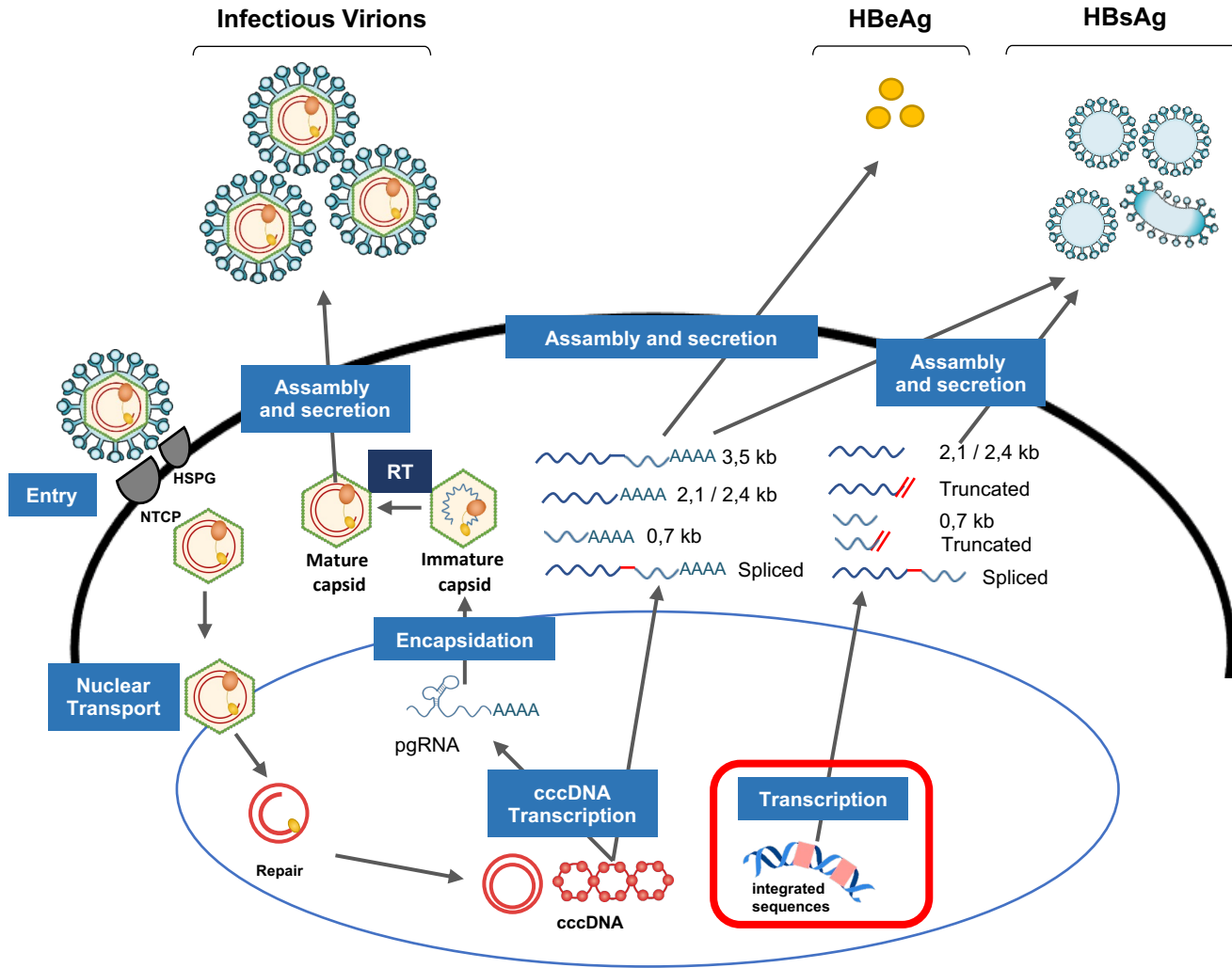
Balogopal, 2020

- .... but do not affect transcription from HBV integrants

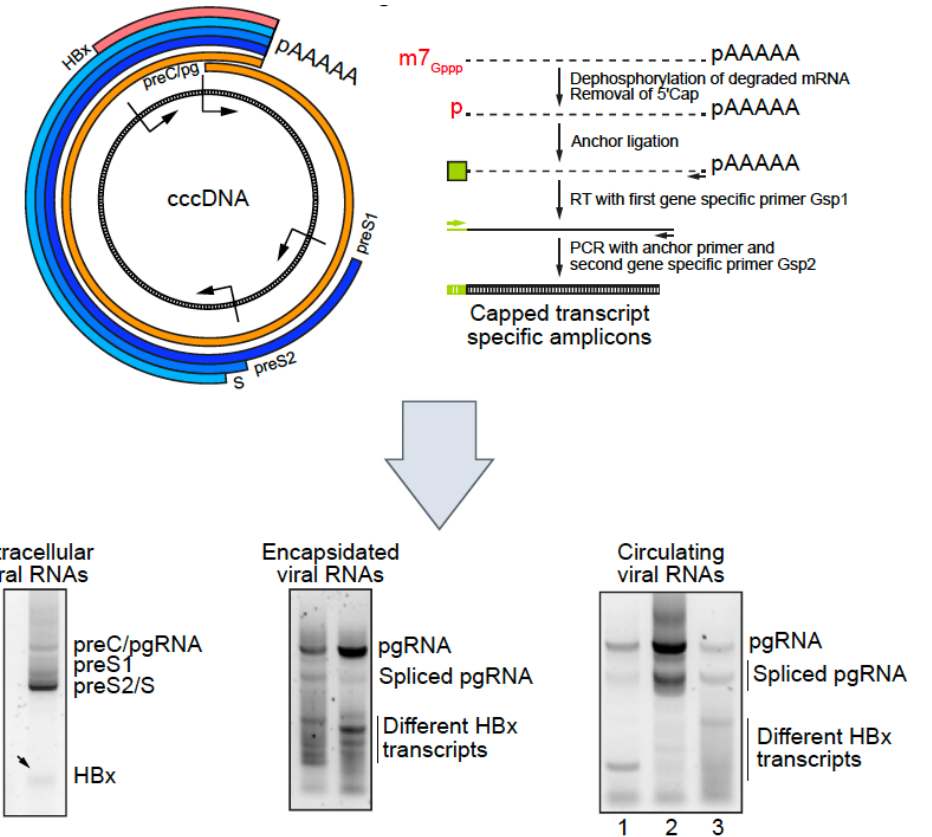


Van Buuren, 2022

# The molecular biology of HBV RNAs .6



- Full-length 5' RACE to identify HBV transcripts in HBV-infected hepatocytes ... and patient serum

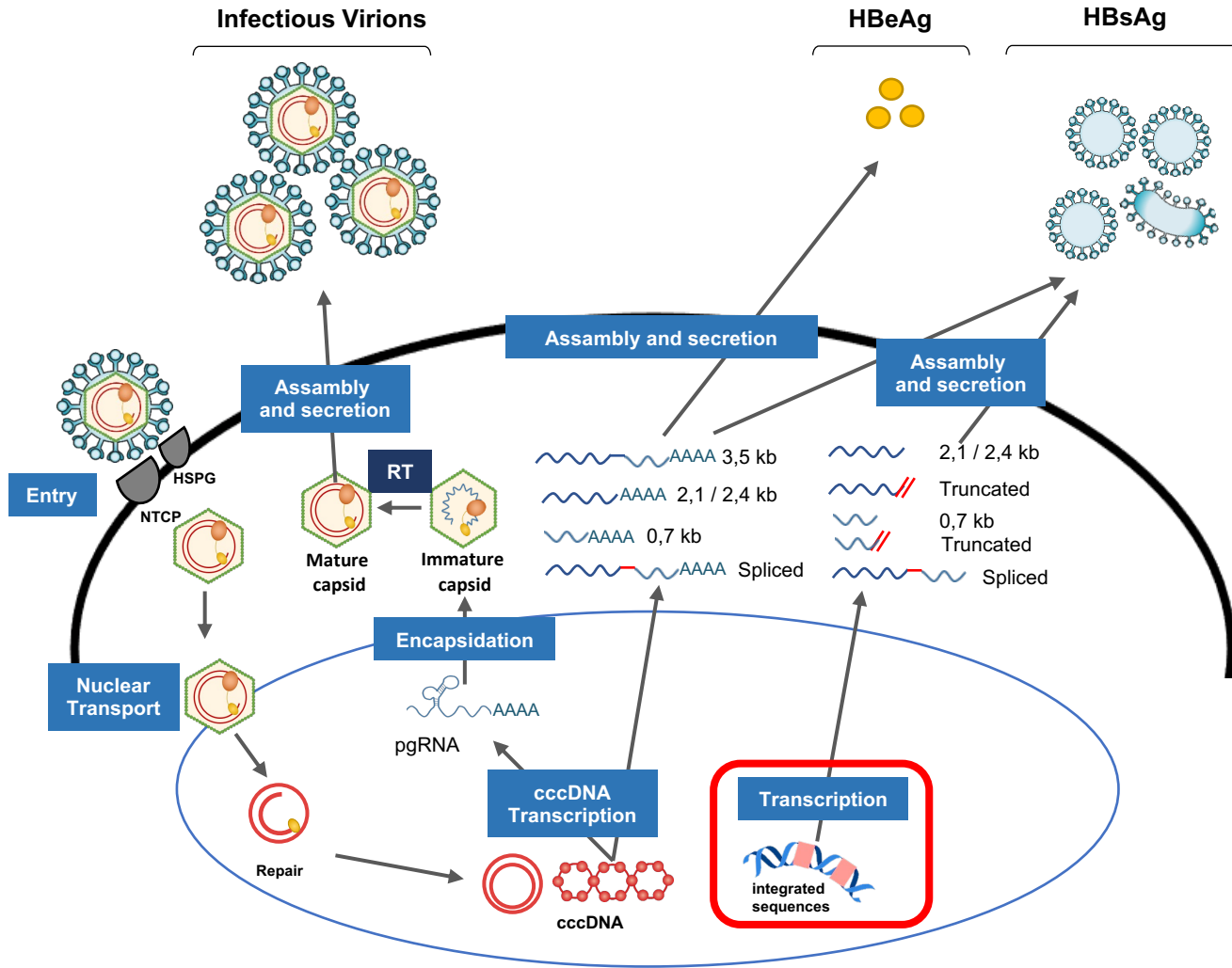


Stadelmayer B et Al, J Hepatol 2020

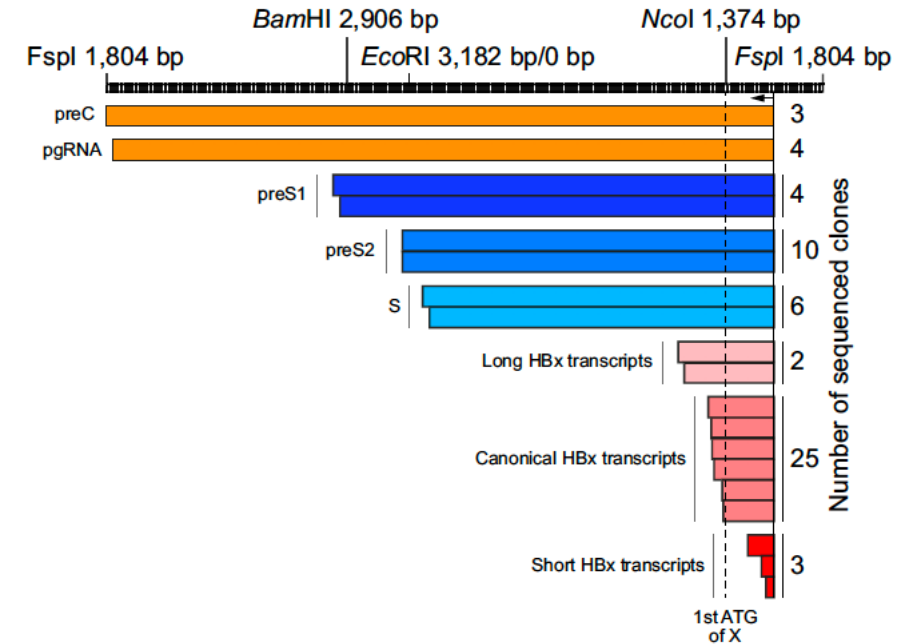
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# The molecular biology of HBV RNAs .6



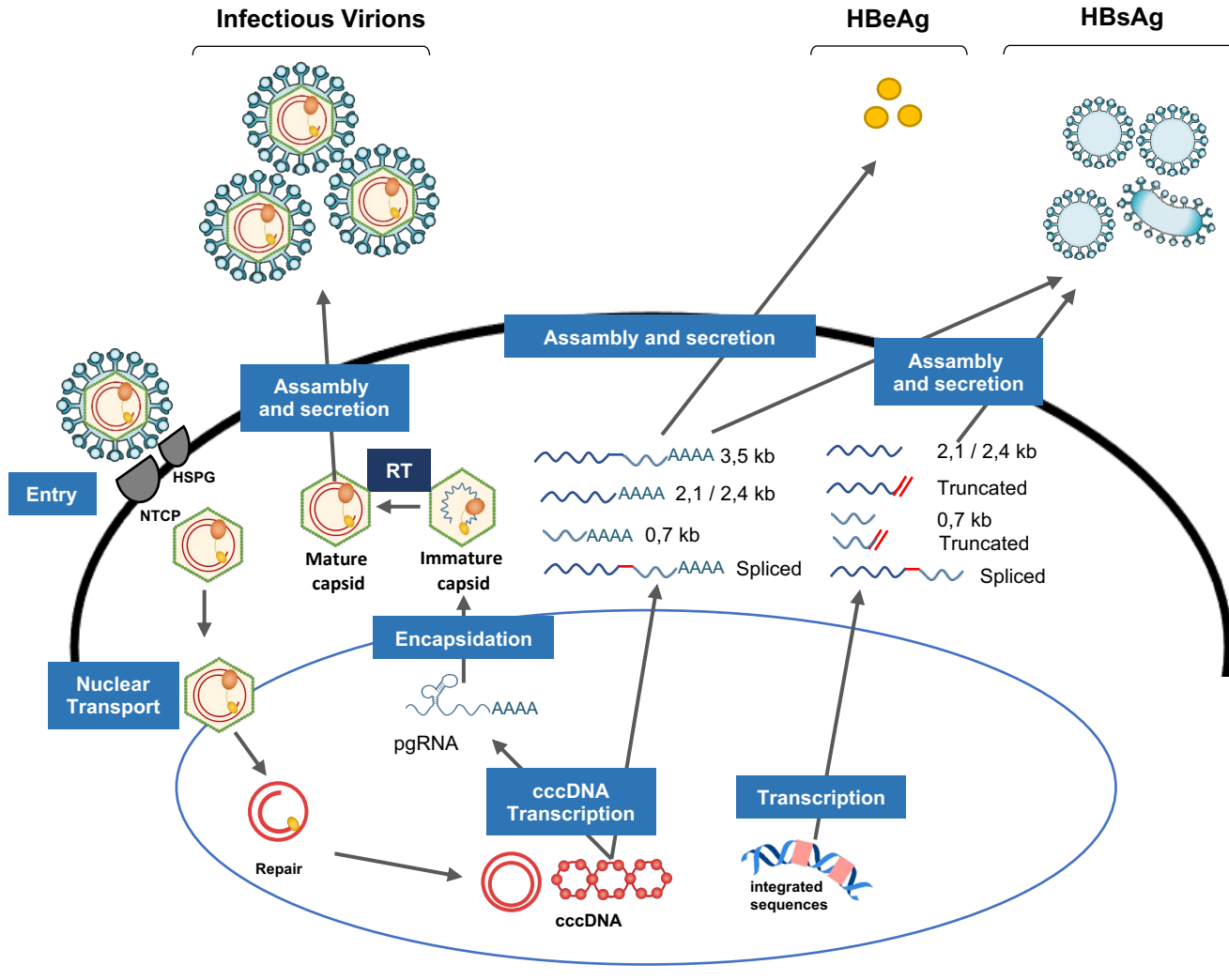
- Full-length 5'RACE to identify HBV transcripts in HBV-infected hepatocytes ... and patient serum



Stadelmayer B et Al, J Hepatol 2020

- 5 HBV RNAs :
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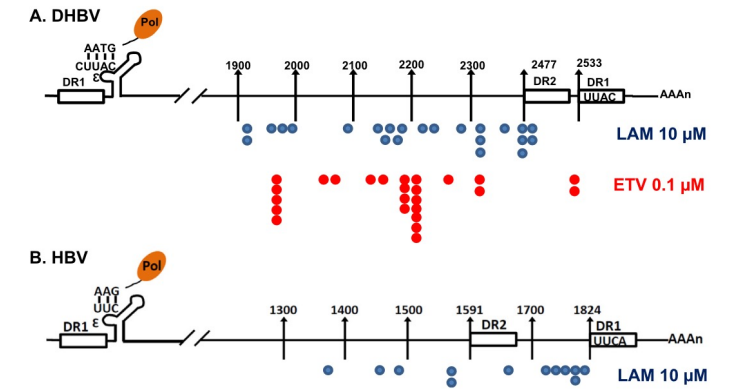
# The molecular biology of HBV RNAs .7



- 5 HBV RNAs :
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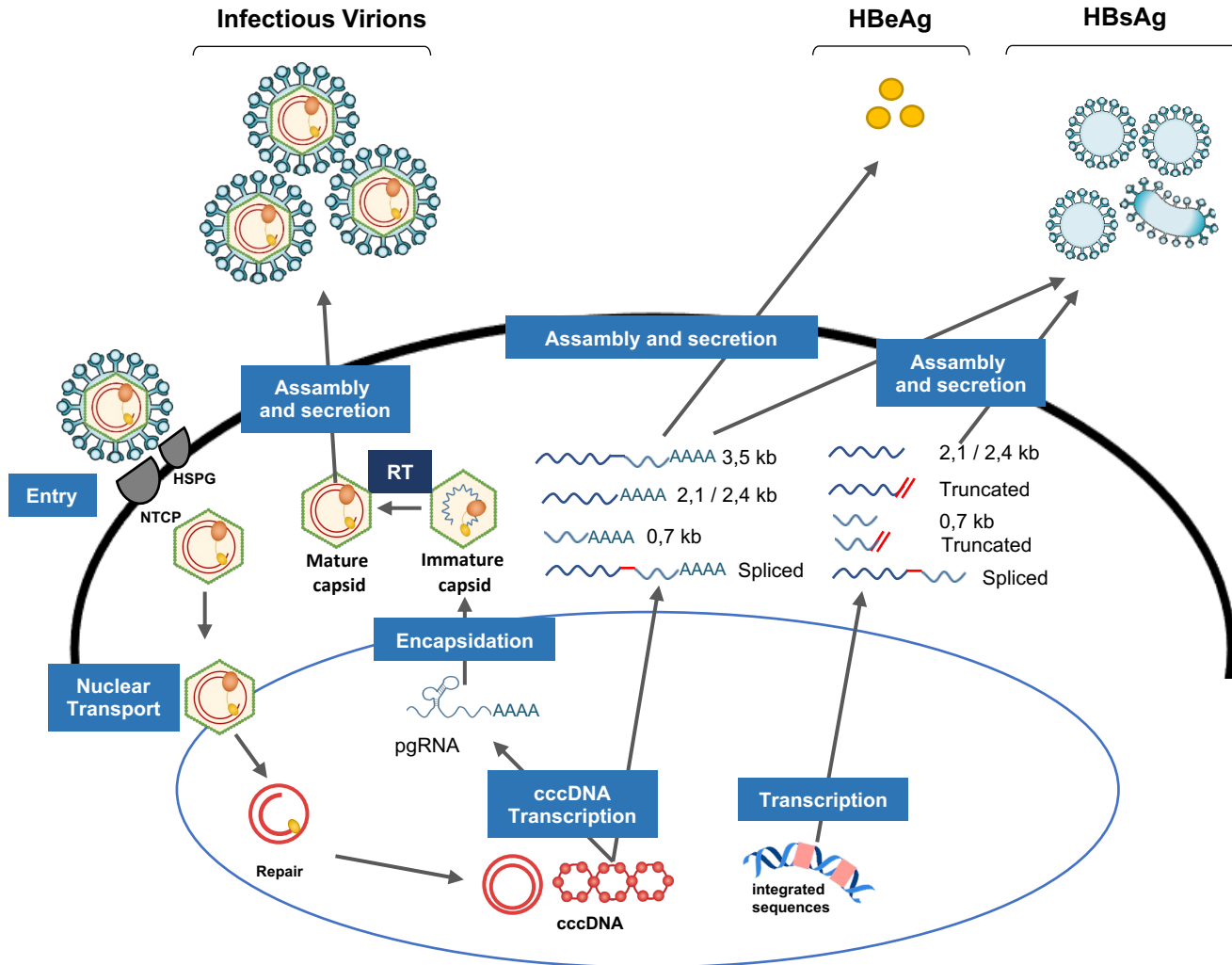
## pgRNA variants

- 3' truncated transcript resulting from a cryptic polyadenylation signal (cryptic trRNA), thought to arise from integrated HBV genomes
  - Hilger, 1991*
  - van Bömmel et al., 2015*
- pgRNA spliced variants
  - Bayliss, 2013*
  - Betz-Stablein, 2016*
  - Lim, 2021*
- non-polyadenylated 3' truncated variant transcripts (incomplete inhibition of the RNase H) during NA therapy



*Zhang et al., 2016*

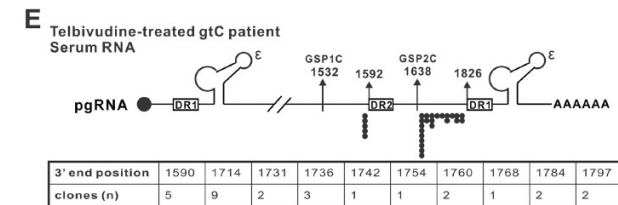
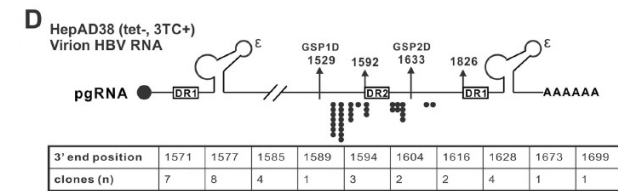
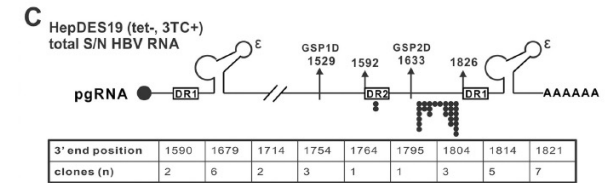
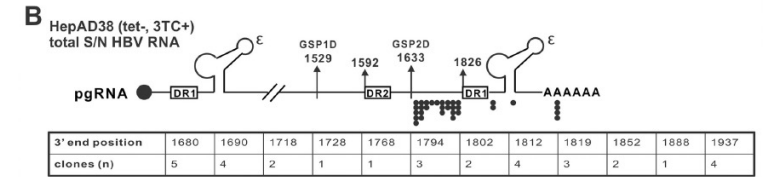
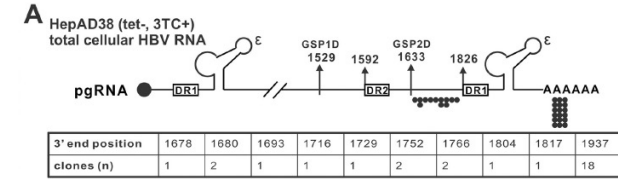
# The molecular biology of HBV RNAs .7



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## pgRNA variants

- non-polyadenylated 3' truncated variant transcripts (incomplete inhibition of the RNase H) during NA therapy

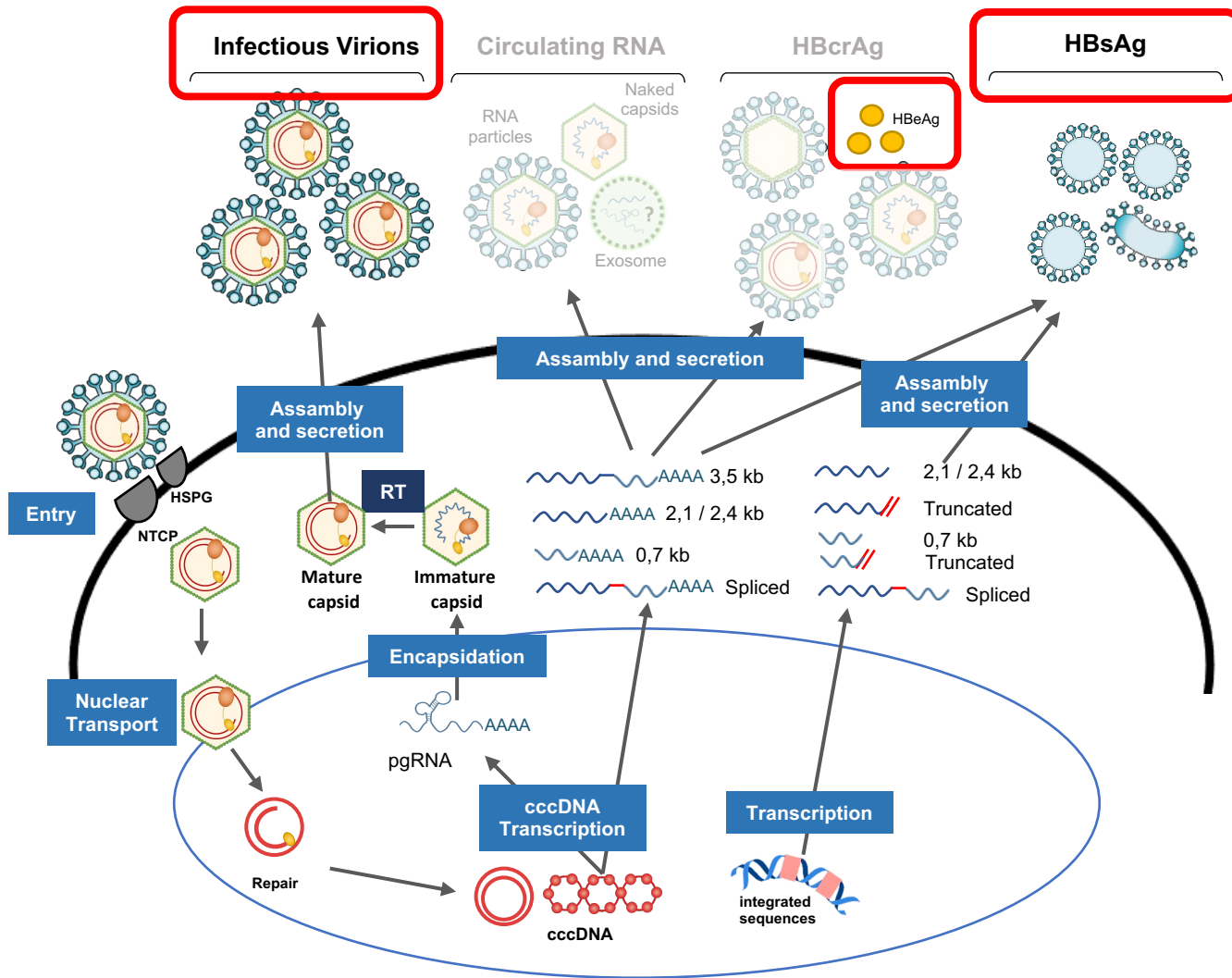


Shen et al., 2020



# The molecular biology of HBV RNAs: insights on new biomarkers

What old HBV biomarkers cannot tell us?

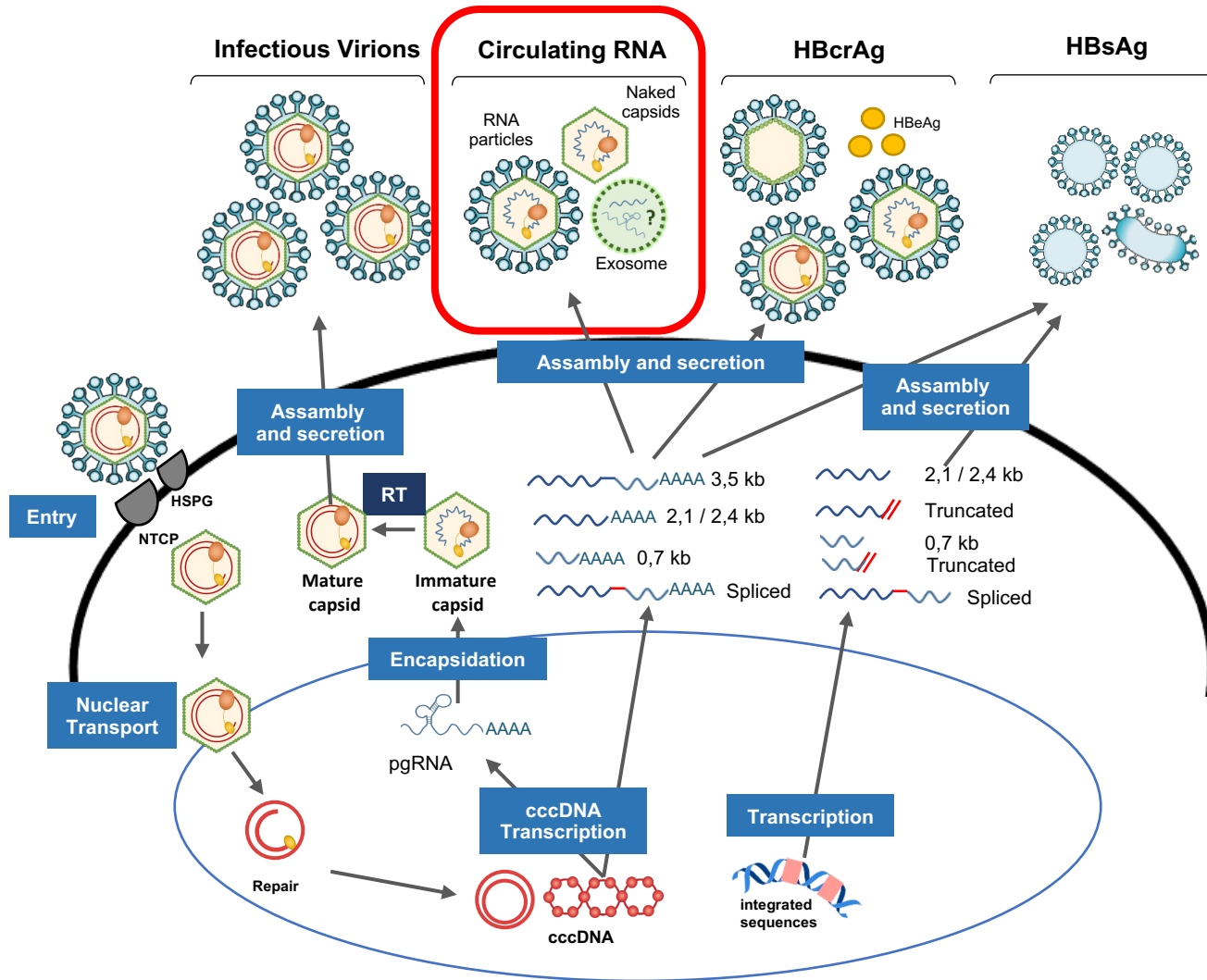


rcDNA contained in virions can originate only from cccDNA, but NUC-induced serum HBV DNA suppression does not eliminate cccDNA  
 → **Not useful in combination therapies !**

HBeAg reflects HBV replication and cccDNA transcription, but HBeAg can disappear from the serum because of the selection of HBeAg-defective viruses  
 → **Not sufficient !**

HBsAg can originate from cccDNA AND integrated HBV DNA  
 → **Not specific to cccDNA**  
 → **HBsAg loss reflects both cccDNA loss and cccDNA inactivation**  
 → **qHBs decline kinetic is too slow to be informative**

# The molecular biology of HBV RNAs: insights on new biomarkers

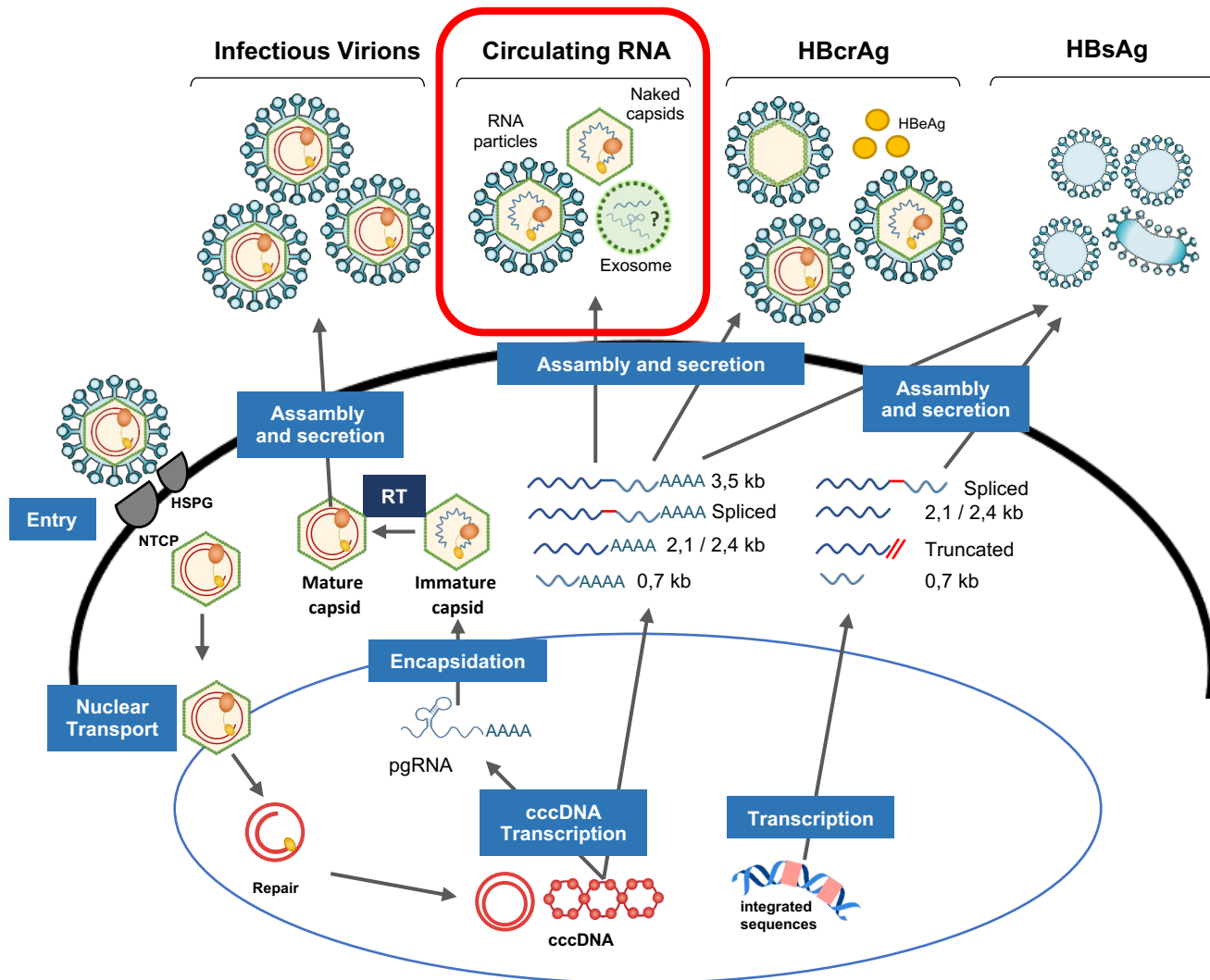


## serum HBV-RNAs as a viral biomarker

- clinical question asked
  - reflecting virus / cccDNA activity
  - detecting drugs target engagement
  - predicting clinical endpoints
- biomarker biology
  - which RNA species
  - which vehicle (RNA particles, naked capsids, exosomes, EV)
 and their impact on detection and significance
- contribution of HBV integrations
  - chimeric transcripts
- detection and quantification strategy
  - assay design and performances

**According to the different quantification strategies, the biological/clinical significance may be significantly different!**

# The molecular biology of HBV RNAs: insights on new biomarkers



## serum HBV-RNAs as a viral biomarker

- pgRNA is transcribed (only) from cccDNA
- pgRNA well correlates with intrahepatic cccDNA activity in Hu-Hep mouse model *Giersch, 2017*
- serum HBV RNA correlates with intrahepatic cccDNA levels/activity *Huang, 2017*  
*Wang, 2021*  
*Testoni, 2023*

...but

- serum HBV RNA levels varies in the different CHB phases *Wang, 2018*
- pgRNA is not the only circulating HBV RNA species...

*Wang J, 2016*  
*Jansen L, 2016*  
*Prakash K, 2018*  
*Lam AM, 2017*  
*Stadlmayer B, 2020*  
*and others...*



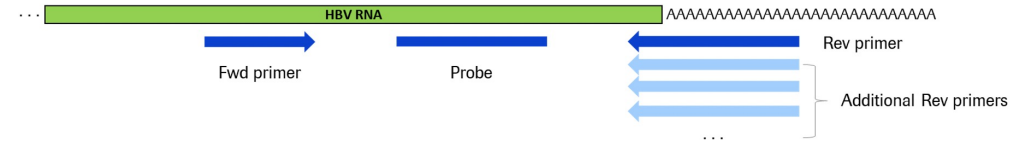
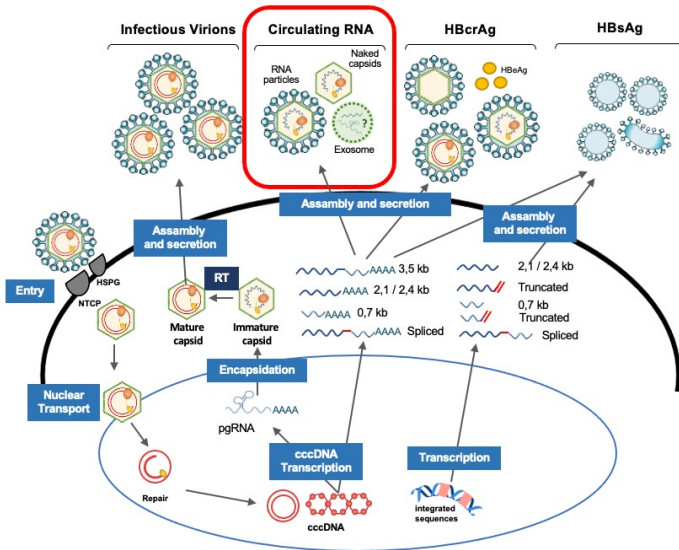


# The molecular biology of HBV RNAs: insights on new biomarkers

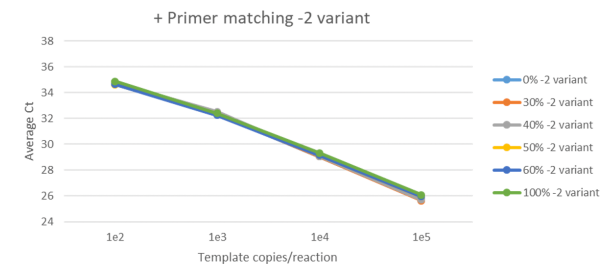
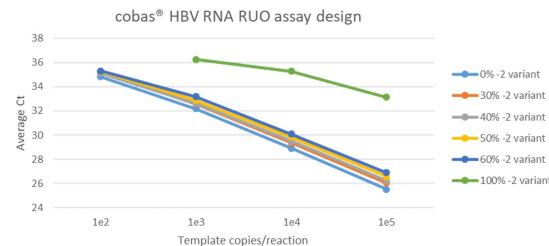
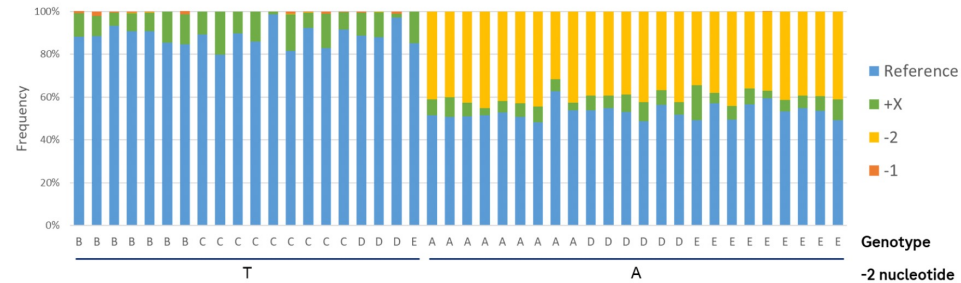
serum HBV-RNAs isoform composition

HBV capture + Illumina NGS

- Performance of the cobas® HBV RNA assay for use on the cobas® 5800/6800/8800 Systems (RUO) against genomic variants and transcript heterogeneity



Poly-A start site	
Reference	G A A T T T G G A G C T T C A A A A A A A A A A A A A A A A A A
-2	G A A T T T G G A G C T T A A A A A A A A A A A A A A A A A A
-1	G A A T T T G G A G C T T A A A A A A A A A A A A A A A A A A
+1	G A A T T T G G A G C T T C T A A A A A A A A A A A A A A A A A
+4	G A A T T T G G A G C T T C T G T G A A A A A A A A A A A A A
+5	G A A T T T G G A G C T T C T G T G G A A A A A A A A A A A A A
+9	G A A T T T G G A G C T T C T G T G G A A G T T A A A A A A A A

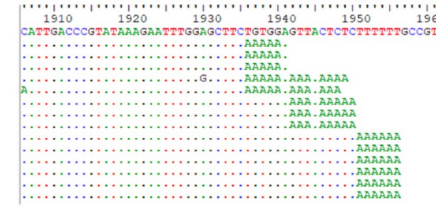


# The molecular biology of HBV RNAs: insights on new biomarkers

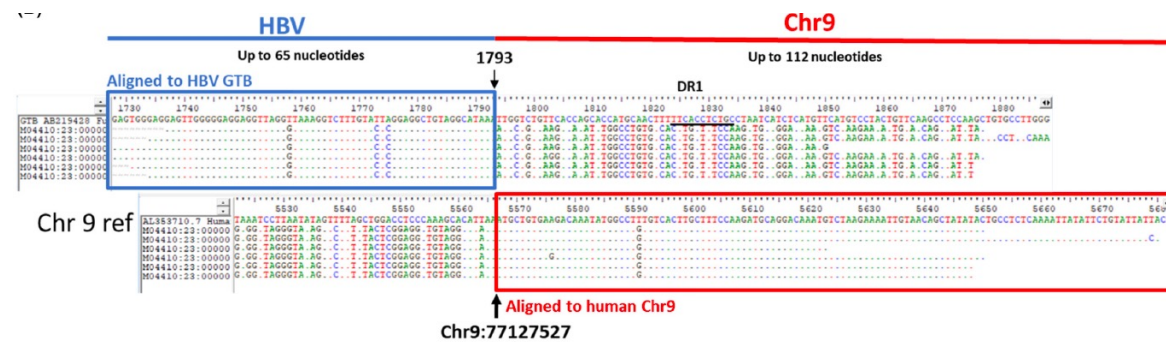
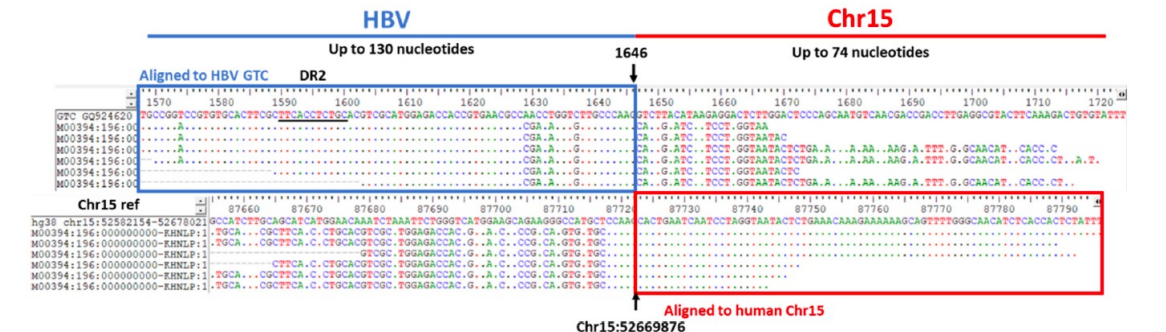
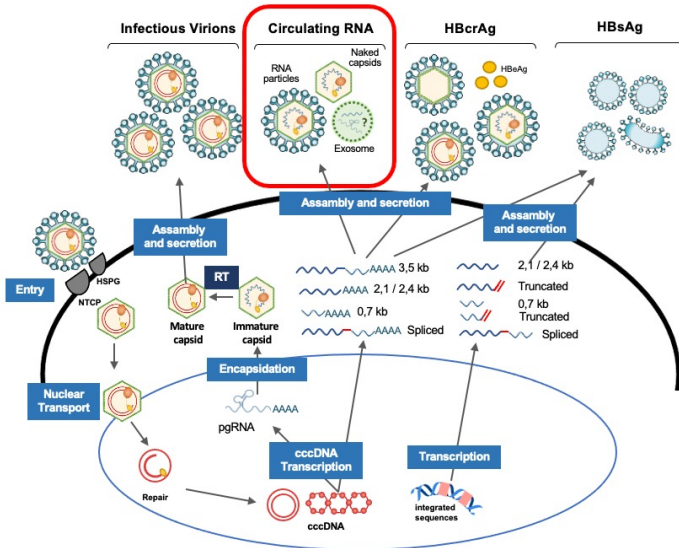
serum HBV-RNAs isoform composition

total RNA amplification coupled to NGS

- splice variants and poly-A tail variability



- 6 HBV-Human chimeric junction observed in 2 RNA samples from patient plasma



Chang, 2022

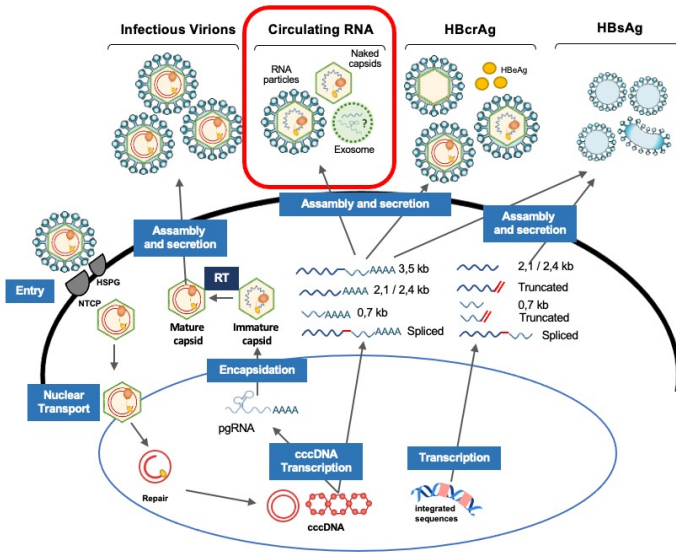


# The molecular biology of HBV RNAs: insights on new biomarkers

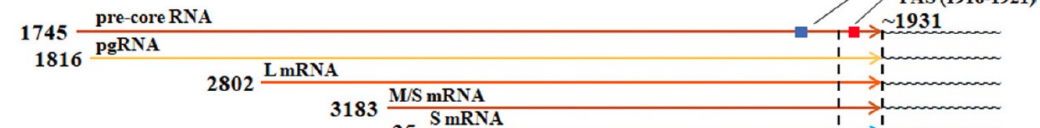
serum HBV-RNAs isoform composition

HBV RNA amplification coupled to NGS

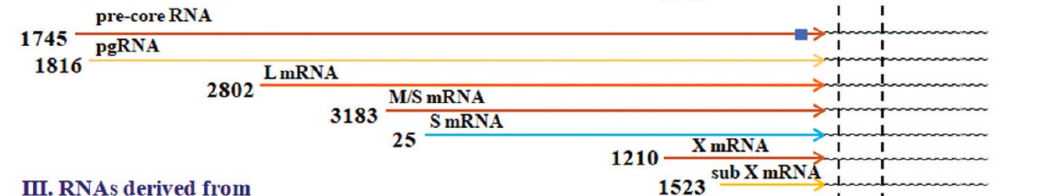
- both HBV replication-derived RNAs (rd-RNAs) and few (6) RNAs transcribed from integrated HBV DNA, including 5'-HBV-human-3' RNAs (integrant-derived RNAs [id-RNAs]) and 5'-human-HBV-3' transcripts,
- spliced HBV RNAs abundant in 50% of analyzed samples
- most serum rd-RNAs were polyadenylated via the conventional poly-A signal
- pregenomic RNA (pgRNA) as the major component
- the vast majority of rd-RNAs and pgRNA are associated with HBV virions but not with unenveloped capsids, exosomes, classic microvesicles, or apoptotic vesicles and bodies



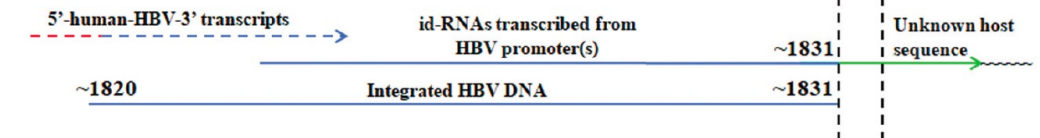
## I. rd-RNAs polyadenylated via conventional poly(A) signal



## II. cps-RNAs polyadenylated via cryptic poly(A) signal



## III. RNAs derived from integrated HBV DNA



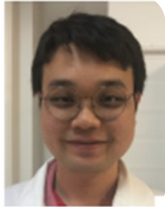
Gudima, 2023

# The molecular biology of HBV RNAs: insights on new biomarkers

Characterization of circulating HBV RNAs in CHB patients and mechanism of cellular export



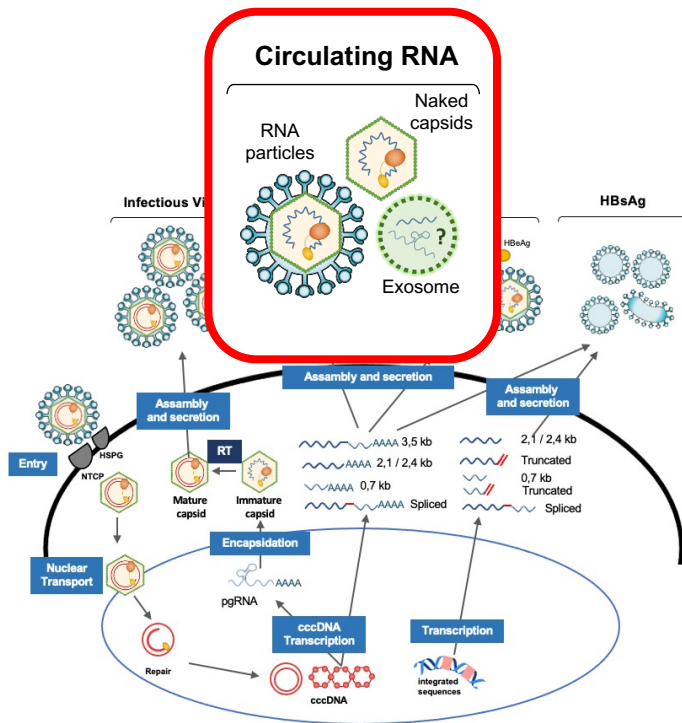
Delphine Bousquet



DooHyun Kim



Hyoseon Tak



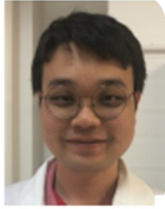
RNA Species	Reference
<b>pgRNA</b>	<ul style="list-style-type: none"> <li>Wang J, 2016</li> <li>Jansen L, 2016</li> <li>Prakash K, 2018</li> <li>Lam AM, 2017</li> <li>Stadelmayer B, 2020</li> </ul>
<b>Spliced Variants</b>	<ul style="list-style-type: none"> <li>Lam AM, 2017</li> <li>Wang J, 2017</li> <li>Stadelmayer B, 2020</li> </ul>
<b>3' Truncated</b>	<ul style="list-style-type: none"> <li>Hacker HJ, 2004</li> <li>Wang J, 2017</li> </ul>
<b>HBx RNA</b>	<ul style="list-style-type: none"> <li>Niu C, 2017</li> <li>Stadelmayer B, 2020</li> </ul>

Where ?	Reference
<b>Virion « Like » Particles</b>	<ul style="list-style-type: none"> <li>Wang J, 2016</li> <li>Bai L, 2018</li> </ul>
<b>Naked Capsids</b>	<ul style="list-style-type: none"> <li>Bai L, 2018</li> </ul>
<b>Capsid Antibody Complexes</b>	<ul style="list-style-type: none"> <li>Bai L, 2018</li> </ul>
<b>No Free</b>	<ul style="list-style-type: none"> <li>Jansen L, 2016</li> </ul>

# The molecular biology of HBV RNAs: insights on new biomarkers



Delphine Bousquet



DooHyun Kim



Hyoseon Tak

Characterization of circulating HBV RNAs in CHB patients and mechanism of cellular export

## Species?

pgRNA



Spliced pgRNA



HBs RNA



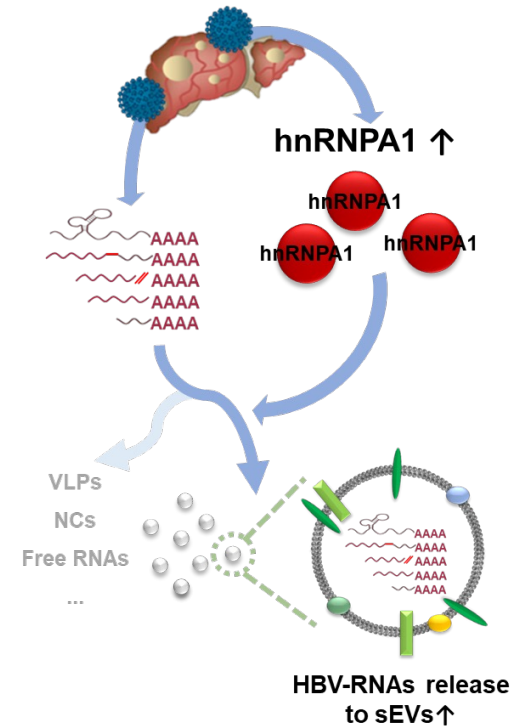
HBx RNA



Differ according to CHB stage

## mechanism?

HBV infection



## Distribution?



VLPs

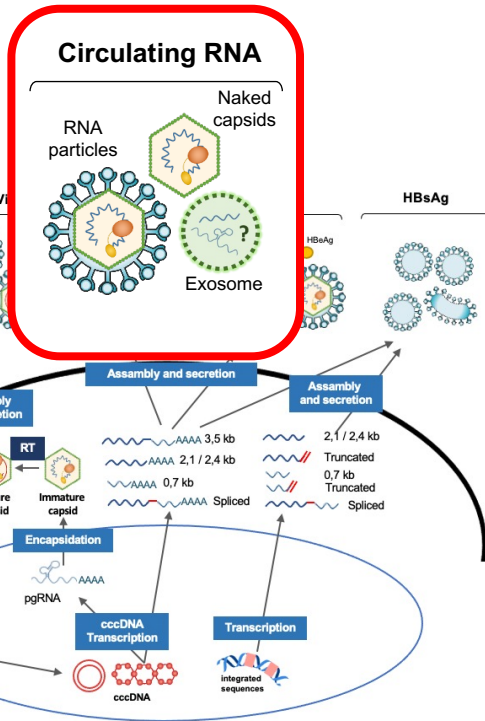
NUCs treatment



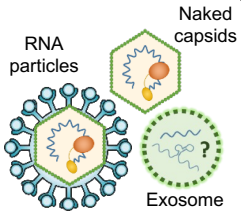
sEVs

## Vehicles?

- High viral load & HBsAg: **VLPs**, **Evs**, and **NCs**
- Low viral load & HBsAg: **EVs**



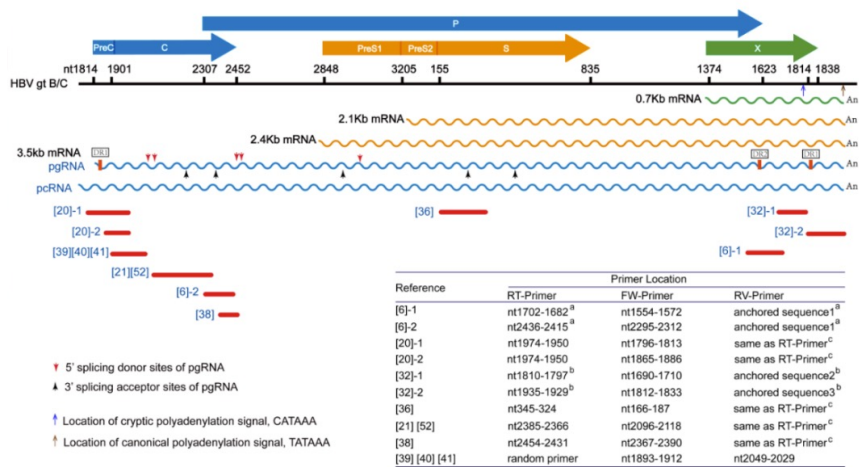
### Circulating RNA



# The molecular biology of HBV RNAs: insights on new biomarkers

Multiple assays : different performances ? comparable results?

## 1. Academic / home made assays



Adapted from Liu, Hepatology 2019

## 2. Industrial assays

### Abbott ROU investigational assay (IA)

HEPATOLOGY

HEPATOLOGY, VOL. 00, NO. 00, 2018  
doi: 10.1002/hep.30262. [Epub ahead of print]

### Hepatitis B Virus Serum DNA and RNA Levels in Nucleos(t)ide Analog-Treated or Untreated Patients During Chronic and Acute Infection

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### calibration: WHO HBV DNA

### Roche @Cobas 6800/800 investigational assay (IA)



Journal of Clinical Virology

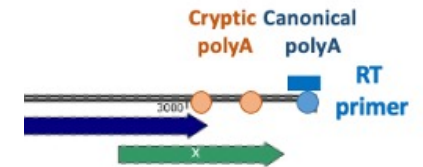
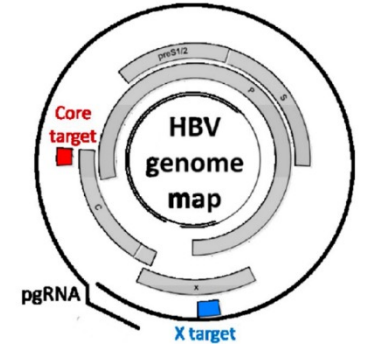
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Performance of the cobas® HBV RNA automated investigational assay for the detection and quantification of circulating HBV RNA in chronic HBV patients

Caroline Scholtès<sup>a, b, c</sup>, Aaron T. Hamilton<sup>d</sup>, Marie-Laure Plissonnier<sup>a</sup>, Caroline Charre<sup>a, b</sup>, Beth Scott<sup>d</sup>, Ling Wang<sup>d</sup>, Françoise Berby<sup>a</sup>, Janine French<sup>f</sup>, Barbara Testoni<sup>a</sup>, Alan Blair<sup>d</sup>, Miroslava Subic<sup>f</sup>, Matthias Hoppler<sup>g</sup>, Andreas Lankenau<sup>g</sup>, Andreas Grubenmann<sup>g</sup>, Massimo Levvero<sup>a, b, f, g</sup>, Marantha L. Heil<sup>d</sup>, Fabien Zoulim<sup>a, b, f</sup>

### calibration: « armored RNAs »





# The molecular biology of HBV RNAs: insights on new biomarkers

Multiple assays : different performances ? comparable results?

## 1. Academic / home made assays



HBV genome map showing the PreC, C, and S regions. The genome is 3.2 kb long. The RNA structures shown include 3.5kb mRNA, pgRNA, and pcRNA. The 5' and 3' splicing donor and acceptor sites are indicated. The location of cryptic polyadenylation signals (CATAAA) and canonical polyadenylation signals (TATAAA) are also shown.

**Alexia Paturel**

**Francesca Casuscelli**

*Hepatology* 2019

Development and characterization of a stable clonal cell line secreting HBV-RNAs as a potential RNA standard for (all) HBV RNA assays

## 2. Industrial assays

Abbott ROU investigational assay (IA)

HEPATOLOGY

Hepatitis B Virus Serum DNA and RNA Levels in Nucleos(t)ide Analog-Treated or Untreated Patients During Chronic and Acute Infection

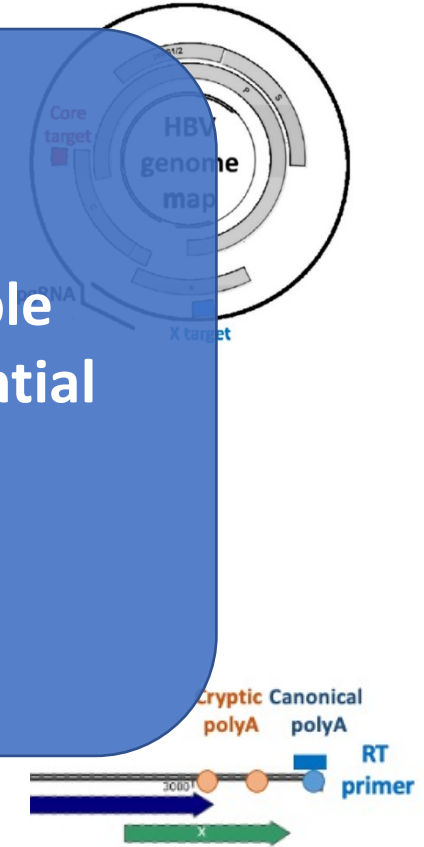
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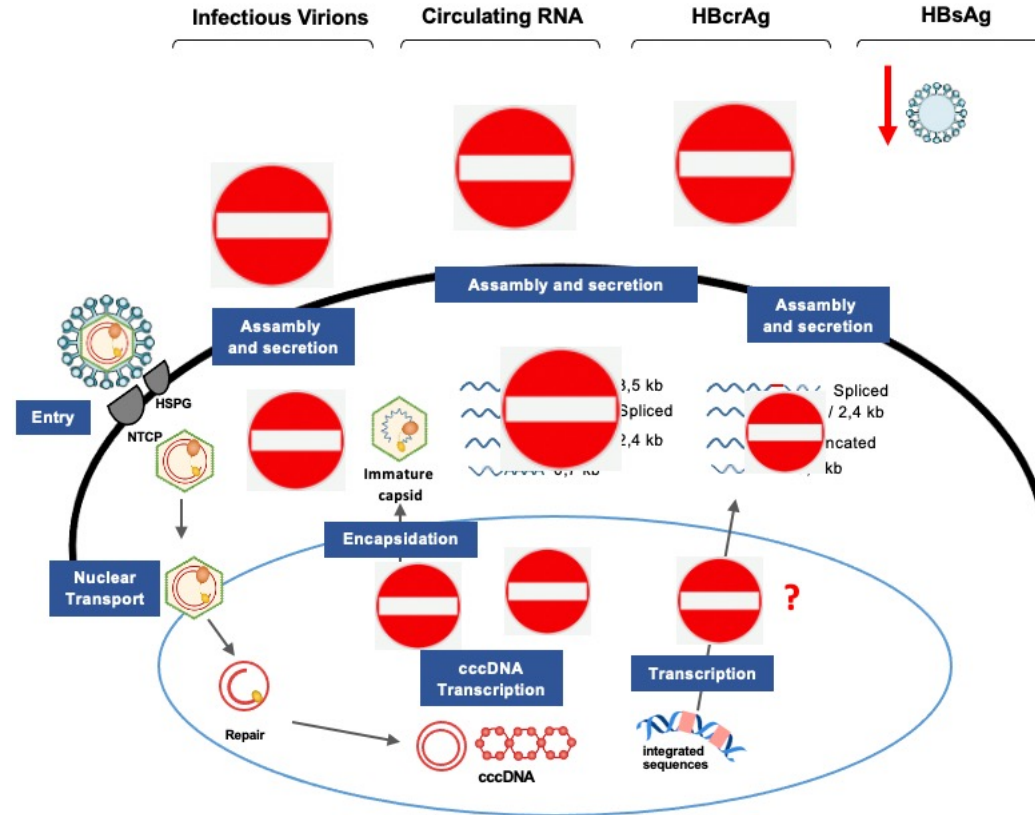
calibration: « armored RNAs »



# The molecular biology of HBV RNAs: insights on new biomarkers

*Serum HBV RNAs in CHB treated patients: target engagement vs endpoints prediction*

## IFN $\alpha$ (IFN $\lambda$ )

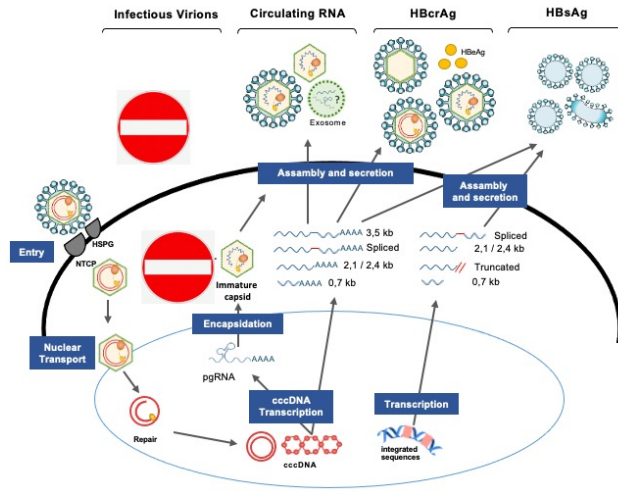


- **direct targeting cccDNA transcription**
- direct effect on capsid (and HBV replication)
- cccDNA destabilization not proven in patients
- contribution of immuno-modulation
- variable from patient to patient

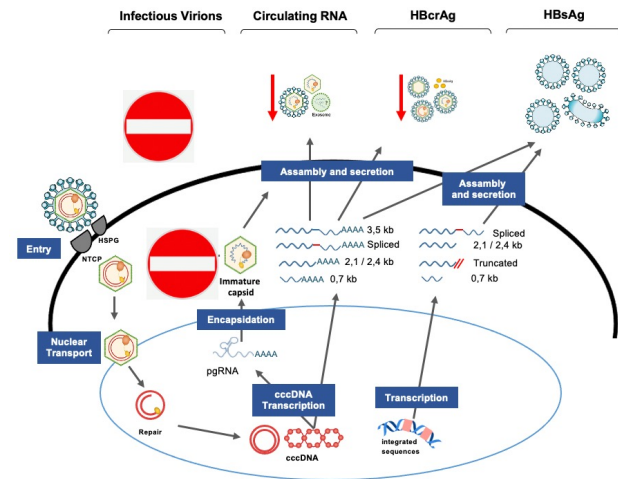
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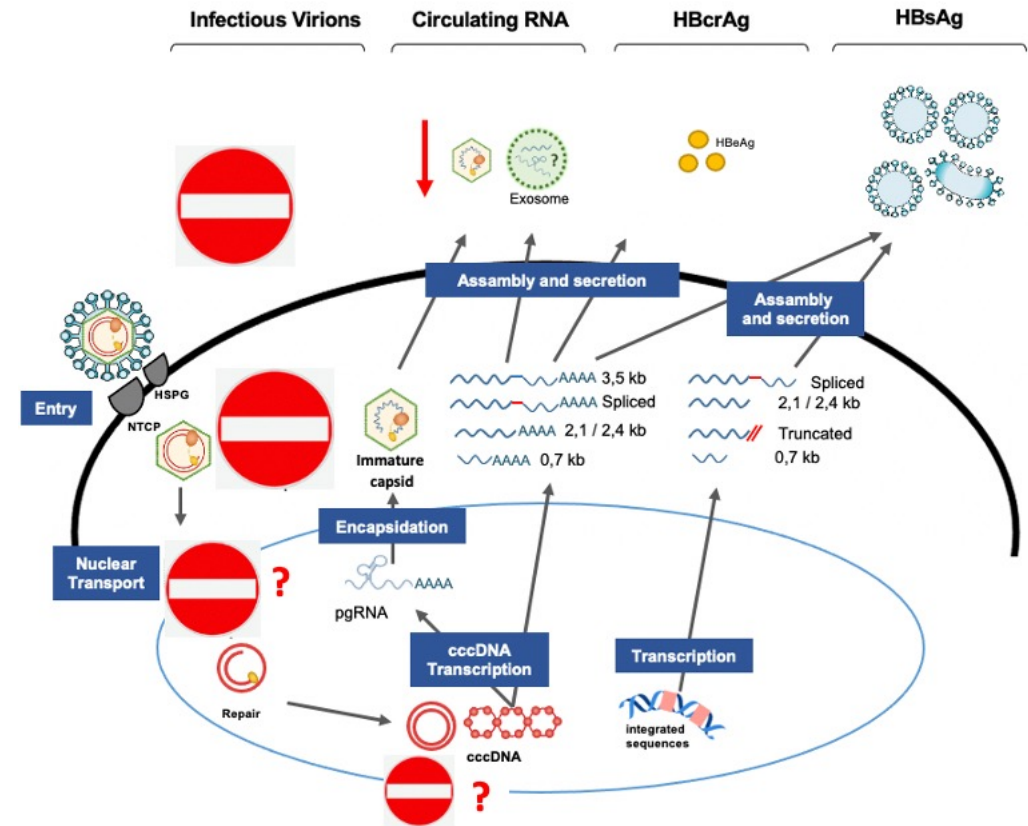
## NUCs (early)



## NUCs (late)



## CAMs

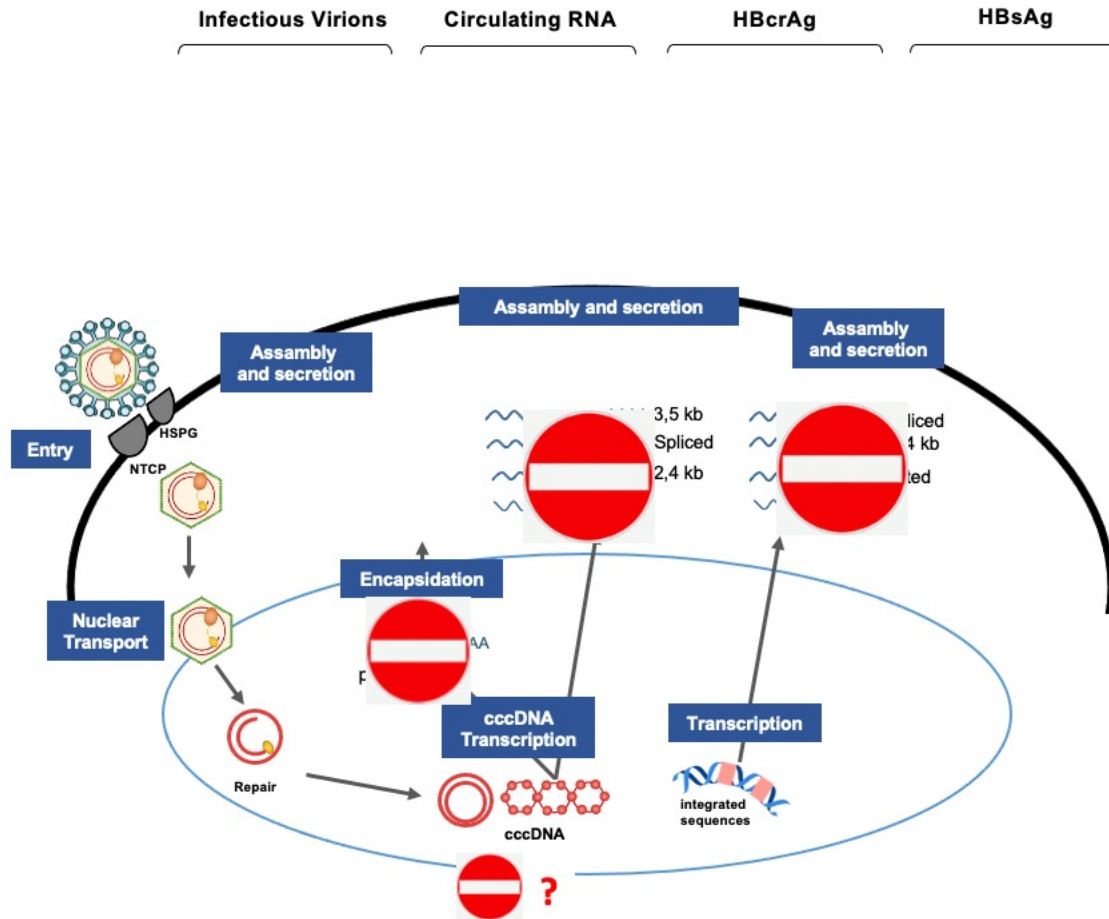


- mainly target engagement
- reduction of cccDNA pool over time
- direct effect on cccDNA activity not established

# The molecular biology of HBV RNAs: insights on new biomarkers

*Serum HBV RNAs in CHB treated patients: target engagement vs endpoints prediction*

## siRNAs (ASO, RNA destabilizers)



- **target engagement**
- effect on cccDNA pool size not established
- effect on cccDNA activity not established

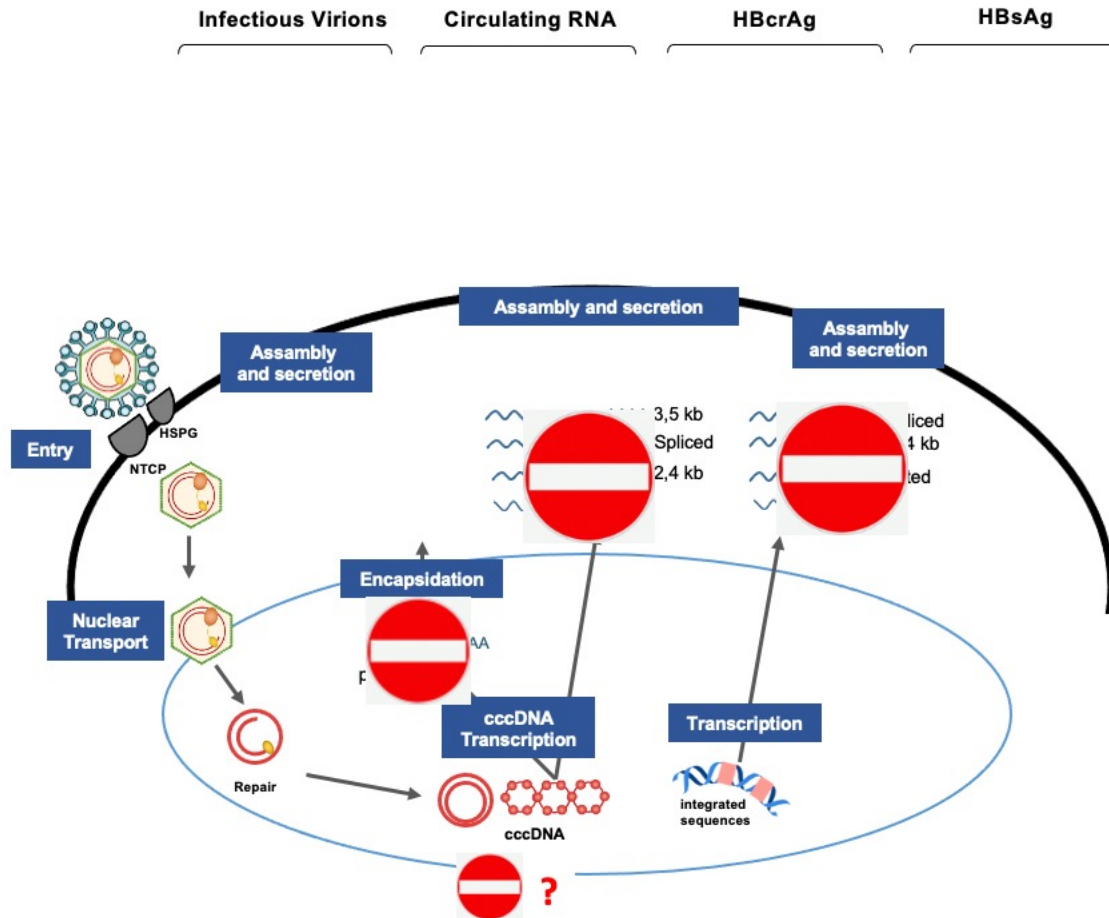


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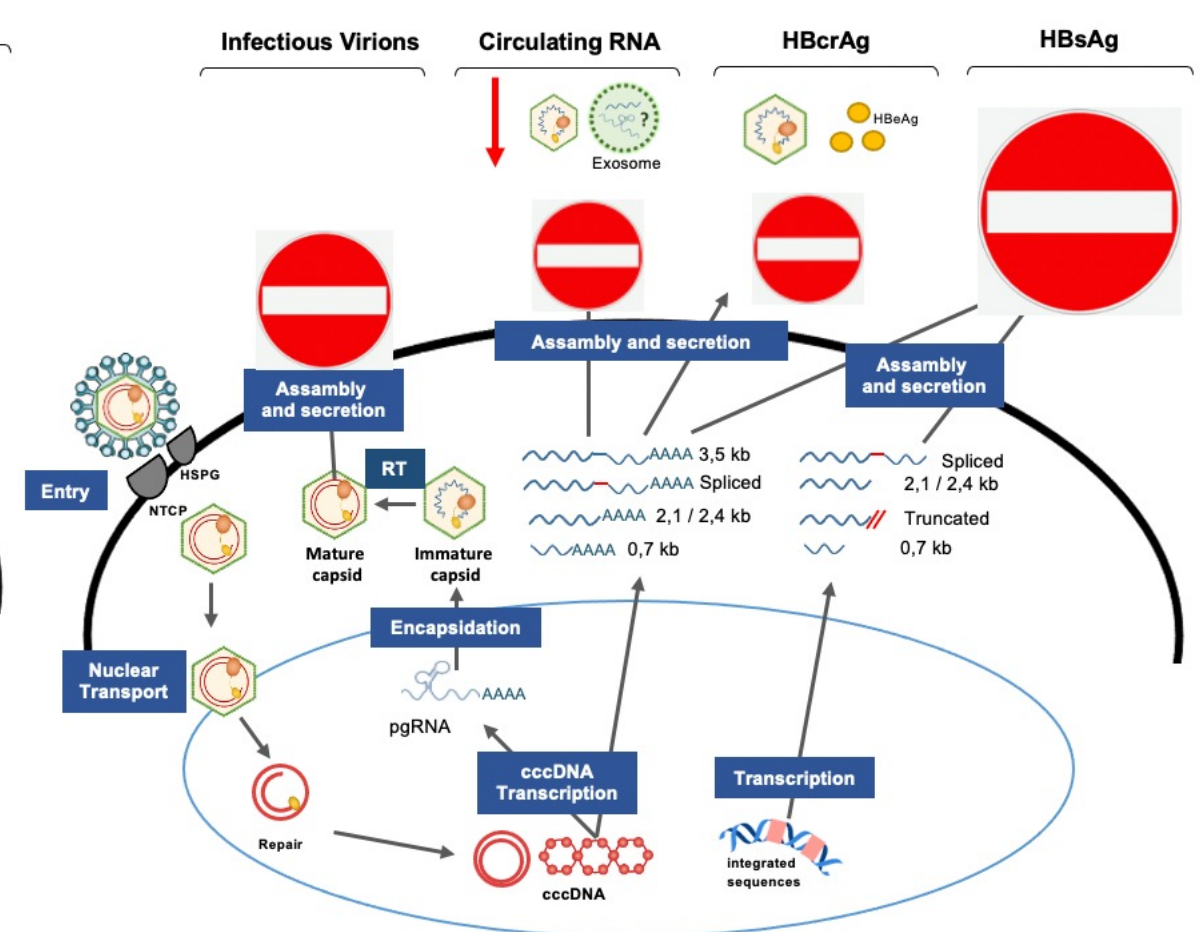
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**siRNAs (ASO, RNA destabilizers)**

**Inhibitors of HBsAg release**



- **target engagement**
- effect on cccDNA pool size not established
- effect on cccDNA activity not established



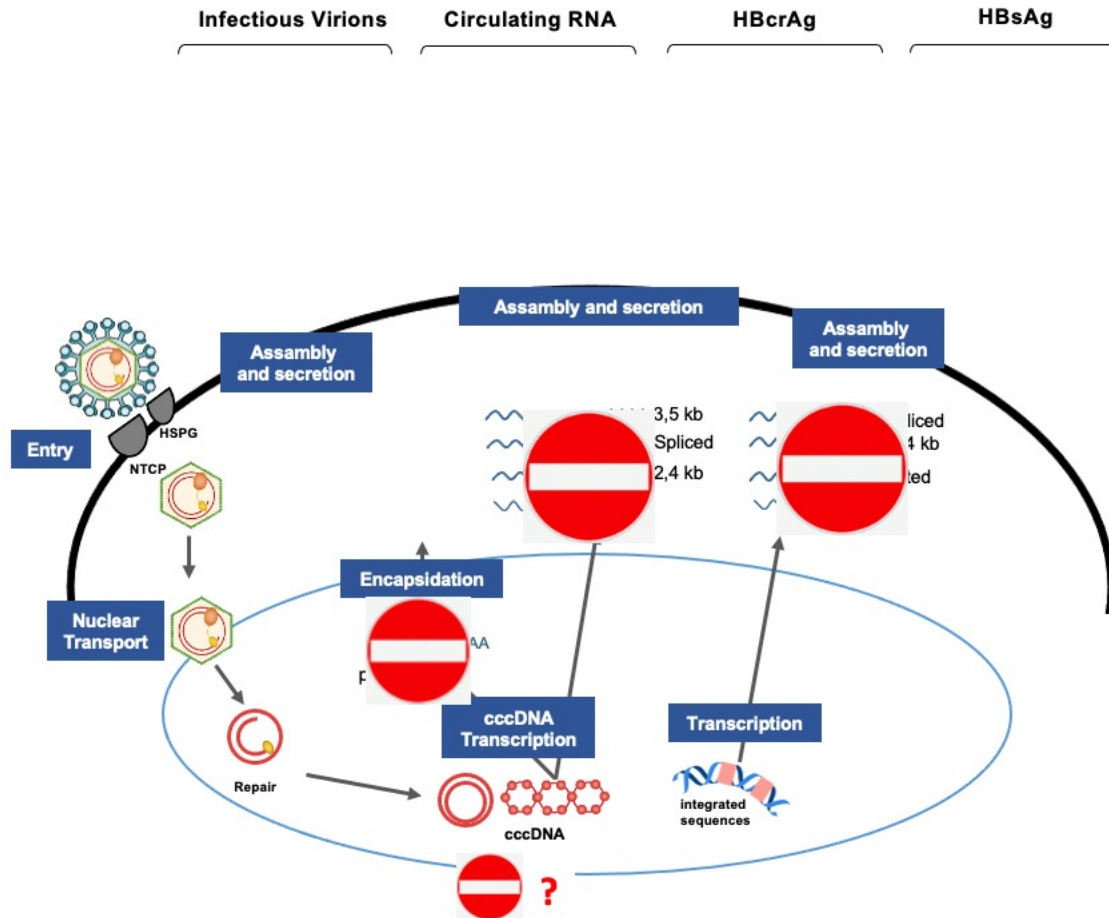
- **mainly target engagement**
- effect on cccDNA pool size not established
- effect on cccDNA activity not established

# The molecular biology of HBV RNAs: insights on new biomarkers

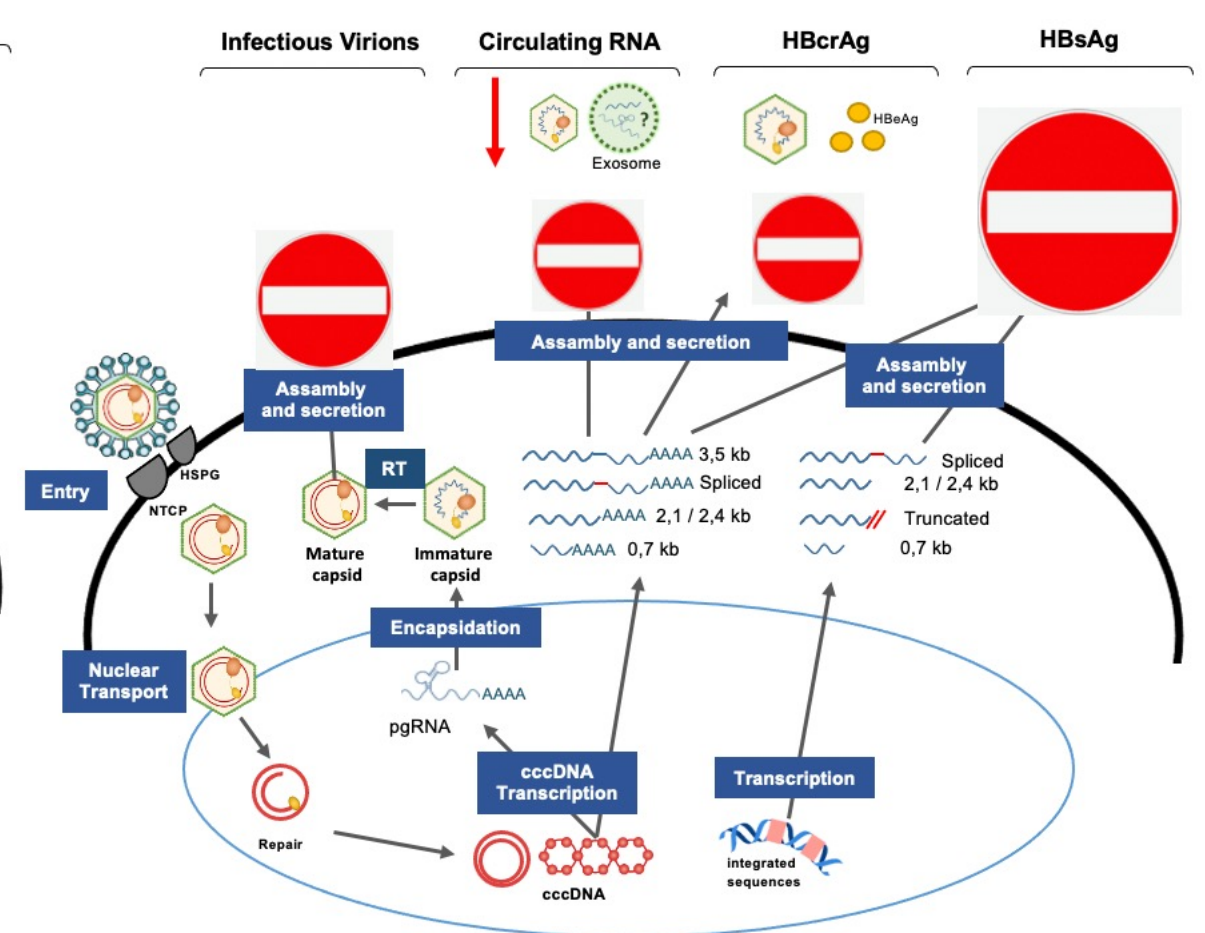
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**siRNAs (ASO, RNA destabilizers)**

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- **target engagement**
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- **mainly target engagement**
- effect on cccDNA pool size not established
- effect on cccDNA activity not established

**RHU « CirB-RNA »**  
ANR-17-RHUS-0003

## International Workshop on Viral Biomarkers

September 7, 2023  
*Institut Lumiere, Lyon*

**Thank you for your attention**