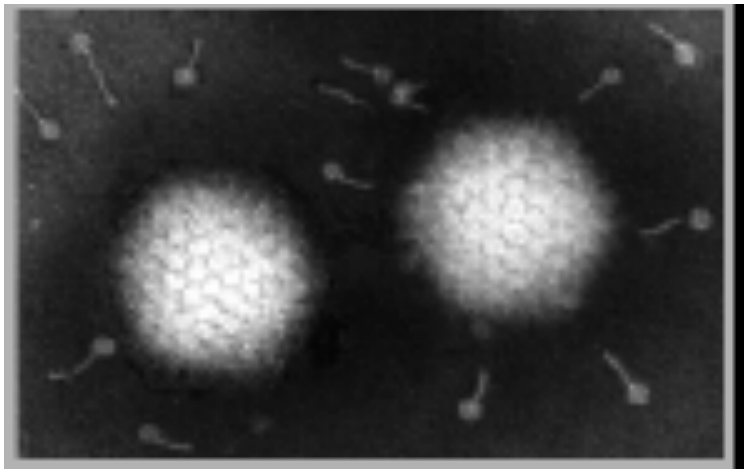
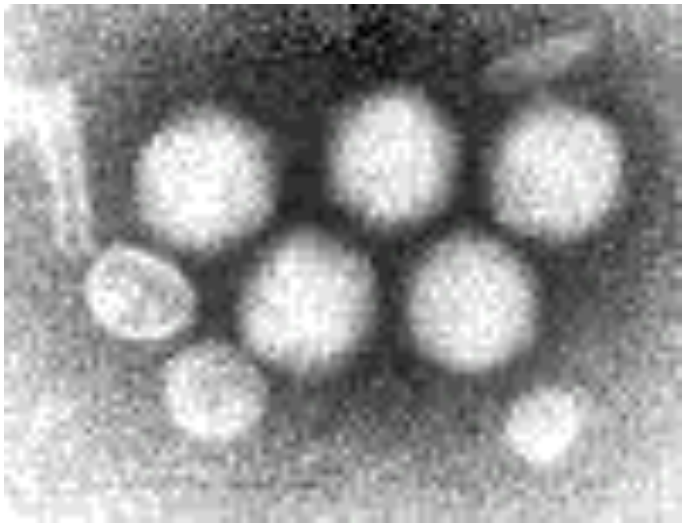
VIROLOGIA

Engineering Viral Genomes: Adenovirus Vectors

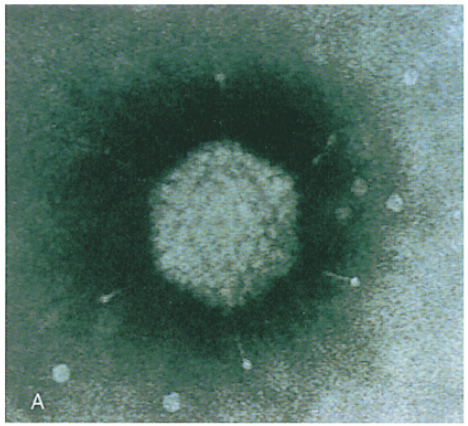
Viral vectors

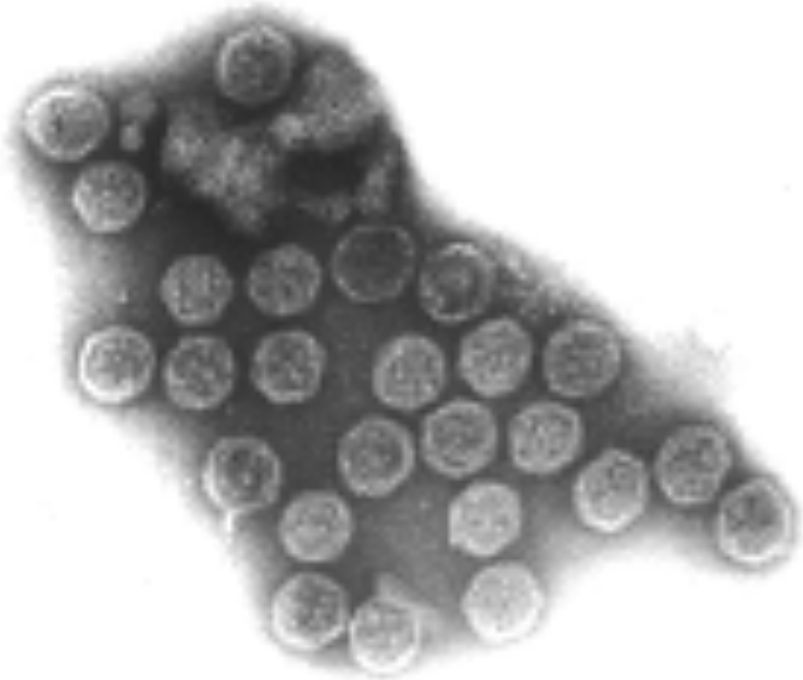
Virus	Insert size	Integration	Duration of expression	Advantages	Potential disadvantages
Adeno-associated virus	~4.5–9 (?) kb	Low efficiency	Long	Nonpathogenic, episomal, infects nondividing cells	Immunogenic, toxicity, small packaging limit
Adenovirus	2–38 kb	No	Short	Efficient gene delivery, infects nondividing cells	Transient, immunogenic
Alphavirus	~5 kb	No	Short	Broad host range, high level expression	Virulence
Epstein-Barr virus	~120 kb	No; episomal	Long	High capacity, episomal, long-term expression	
Gammaretrovirus	1–7.5 kb	Yes	Shorter than formerly	Stable integration	May rearrange genome, insertional mutagenesis require cell division
Herpes simplex virus	~30 kb	No	Long in central nervous system, short elsewhere	Infects nondividing cells; neurotropic, large capacity	Virulence, persistence in neurons, immunogenic
Lentivirus	7–18 kb	Yes	Long	Stable integration; infects nondividing and terminally differentiated mammalian cells	Insertional mutagenesis
Poliovirus	~300 bp for helper-free virus; ~3 kb for defective virus	No	Short	Excellent mucosal immunity	Limited capacity; reversio to neurovirulence
Rhabdovirus	Unknown	No	Short	High-level expression, rapid cell killing	Virulence, highly cytopathic
Vaccinia virus	At least ~25 kb, probably ~75–100 kb	No	Short	Wide host range, ease of isolation, large capacity, high-level expression	Transient, immunogenic



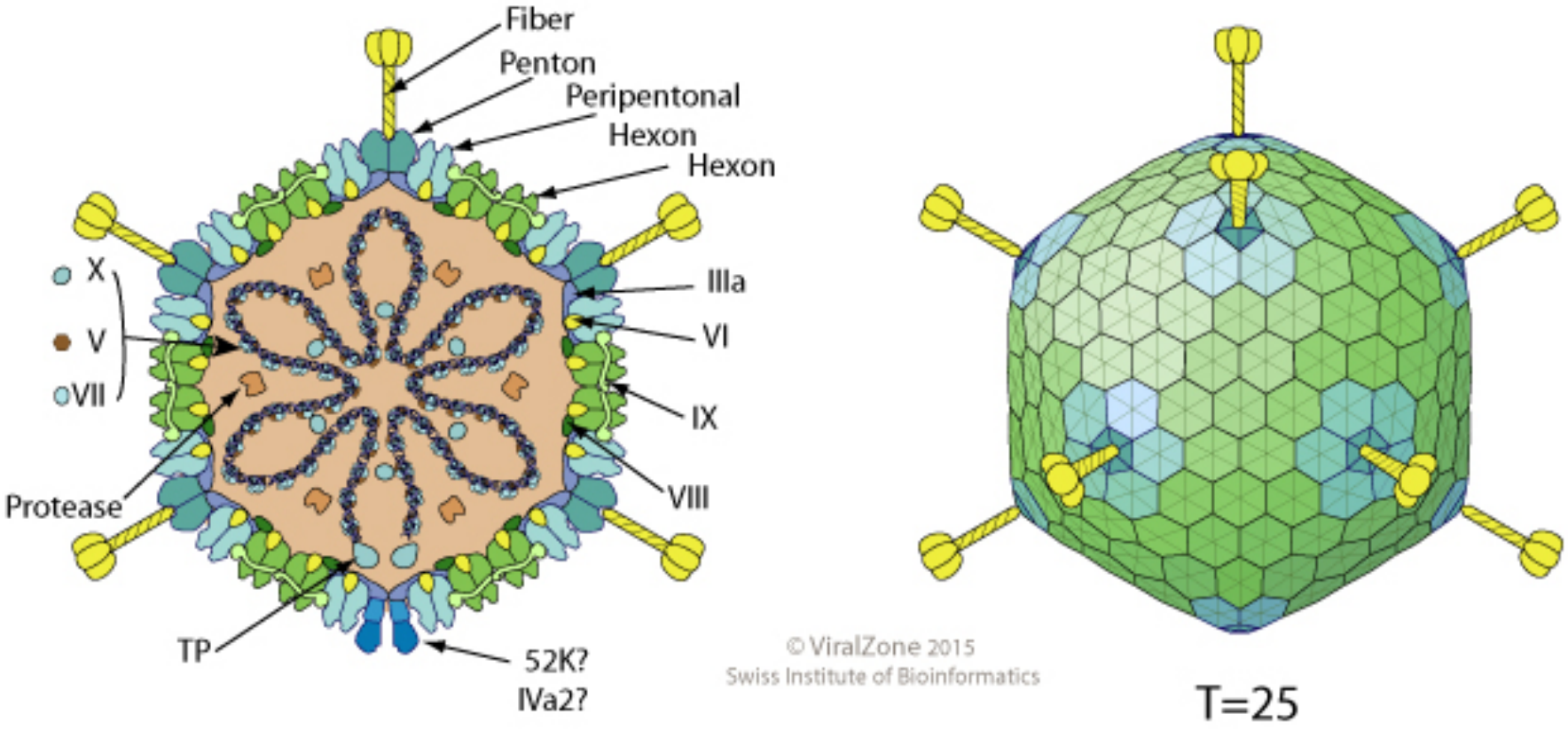


ADENOVIRUSES

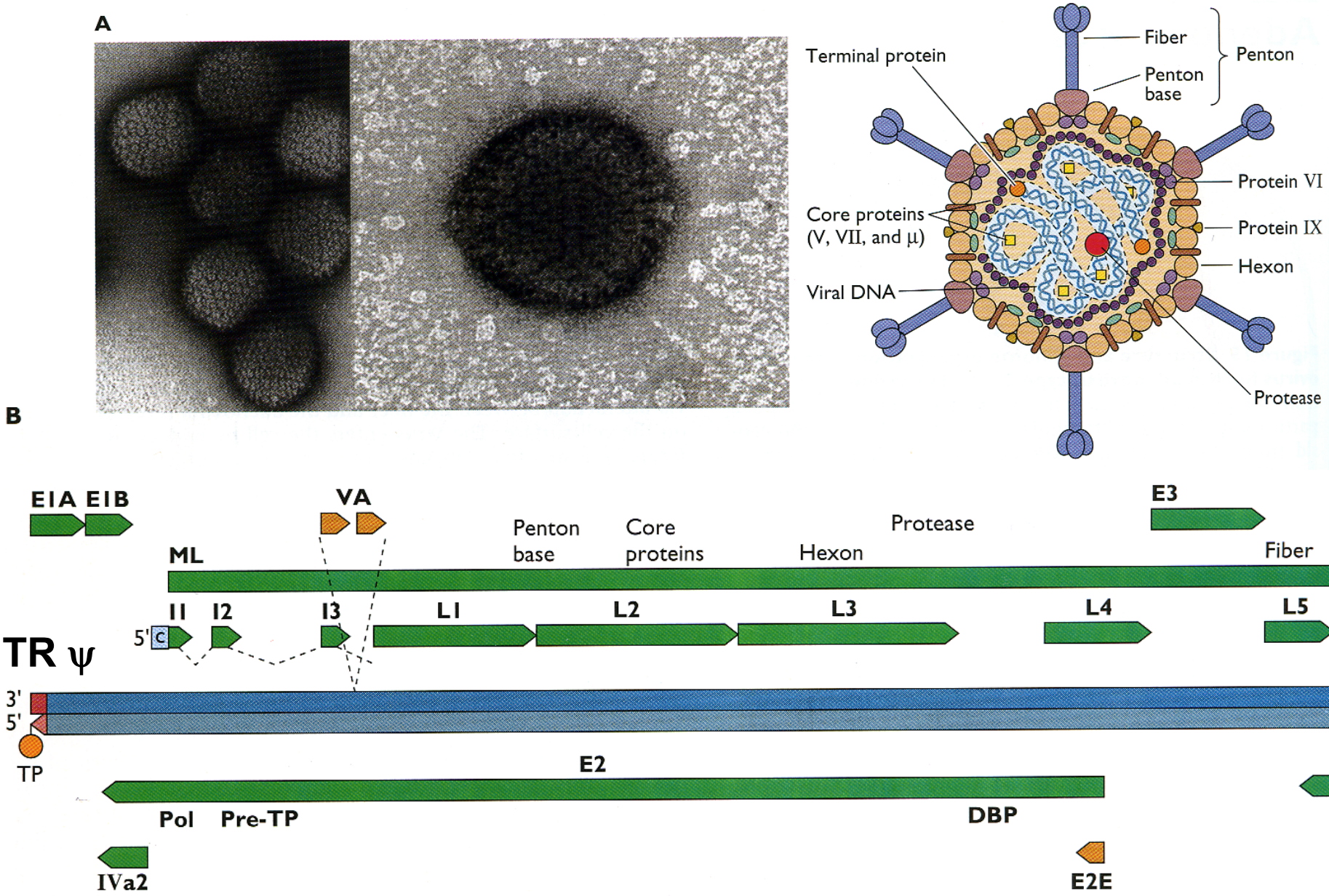




Structural model of the adenovirus virion



Structure and genome organization of the human adenovirus type 2



Nobelprize.org

The Official Web Site of the Nobel Prize

The Nobel Prize in Physiology or Medicine 1993
Richard J. Roberts, Phillip A. Sharp

The Nobel Prize in Physiology or Medicine 1993

Nobel Prize Award Ceremony

Richard J. Roberts

Phillip A. Sharp

Richard J. Roberts

Phillip A. Sharp

The Nobel Prize in Physiology or Medicine 1993 was awarded jointly to Richard J. Roberts and Phillip A. Sharp *"for their discoveries of split genes"*

BOX 10.4 EXPERIMENTS

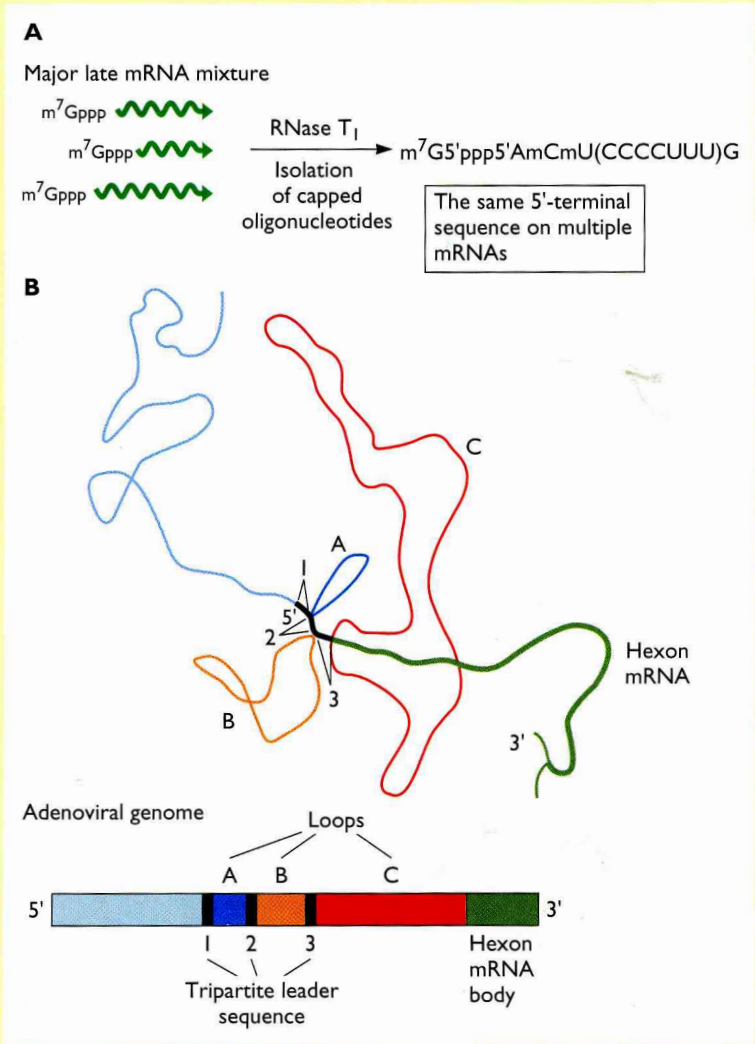
Discovery of the spliced structure of adenoviral major late mRNAs

(A) Digestion of adenoviral major late mRNAs with RNase T_1, which cleaves after G, and isolation of the capped 5′ oligonucleotides indicated the **same** 11-nucleotide sequence was present at the 5′ ends of several different mRNAs. This observation was surprising, and puzzling. Hybridization studies indicated that these 5′ ends were not encoded adjacent to the main segments of major late mRNAs. Direct visualization of such mRNAs hybridized to viral DNA provided convincing proof that their coding sequences are dispersed in the viral genome. **(B)** Schematic diagram of one major late mRNA (hexon mRNA) hybridized to a complementary adenoviral DNA fragment extending from the left end of the genome to a point within the hexon coding sequence. Three loops of unhybridized DNA (thin lines), designated A, B, and C, bounded or separated by three short segments (1, 2, and 3) and one long segment (hexon mRNA) of DNA-RNA hybrid (thick lines) were observed. Other adenoviral late mRNAs examined yielded the same sets of hybridized and unhybridized viral DNA sequences at their 5′ ends, but differed in the length of loop C, and the length and location of the 3′-terminal RNA-DNA hybrid. It was therefore concluded that the major late mRNAs contain a common 5′-terminal segment (segments 1, 2, and 3) built from sequences encoded at three different sites in the viral genome, and termed the tripartite leader sequence. This sequence is joined to the mRNA body, a long sequence complementary to part of the hexon coding sequence in the example shown. (B) Adapted from S. M. Berget et al., *Proc. Natl. Acad. Sci. USA* **74:**3171–3175, 1977, with permission.

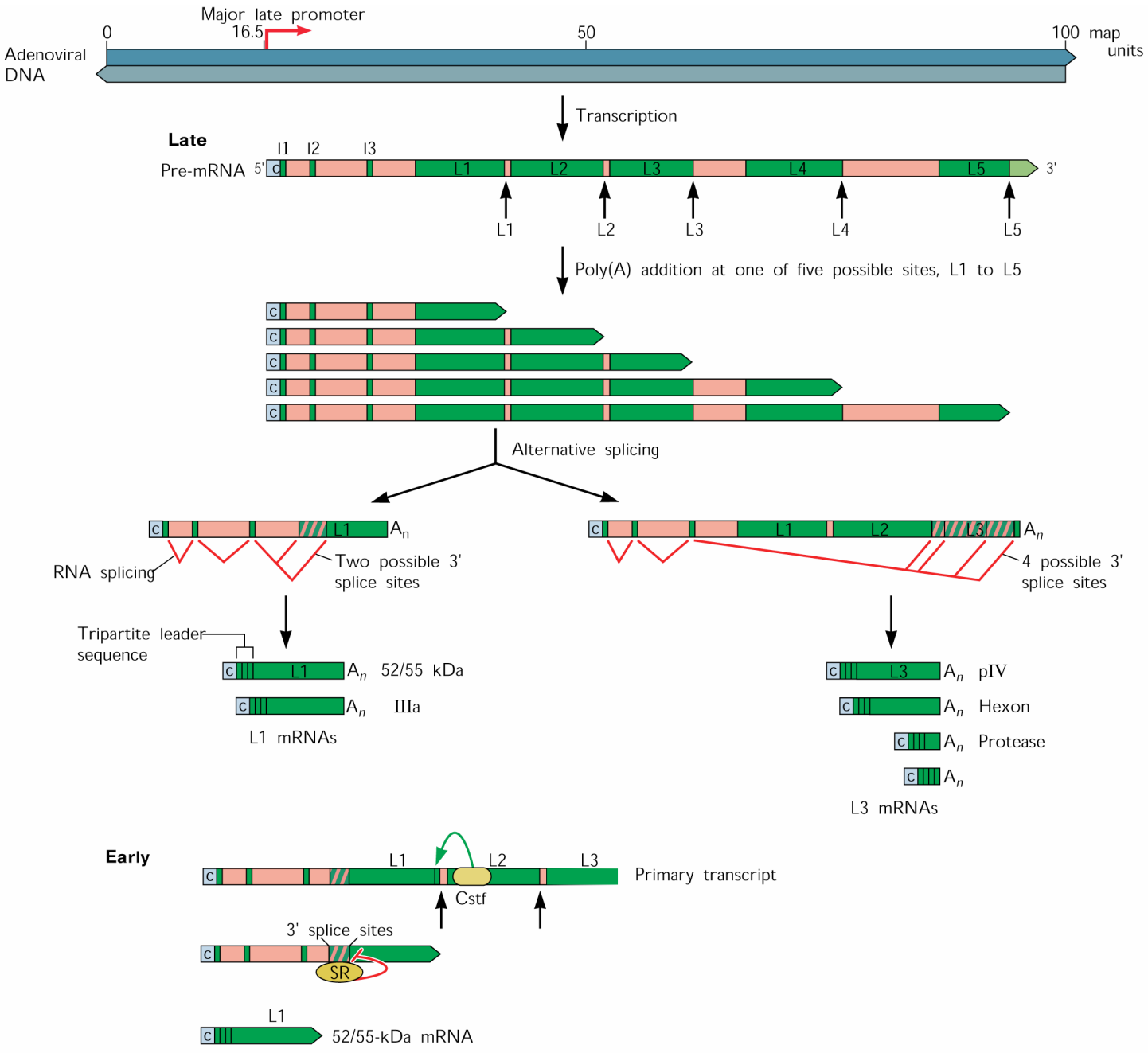
Berget, S. M., C. Moore, and P. A. Sharp. 1977. Spliced segments at the 5′ terminus of adenovirus 2 late mRNA. *Proc. Natl. Acad. Sci. USA* **74:**3171–3175.

Chow, L. T., R. E. Gelinas, T. R. Booker, and R. J. Roberts. 1977. An amazing sequence arrangement at the 5′ ends of adenovirus 2 messenger RNA. *Cell* **12:**1–8.

Gelinas, R. E., and R. J. Roberts. 1977. One predominant undecanucleotide in adenovirus late messenger RNAs. *Cell* **11:**533–544.



Alternative polyadenylation and splicing of adenoviral major late transcripts

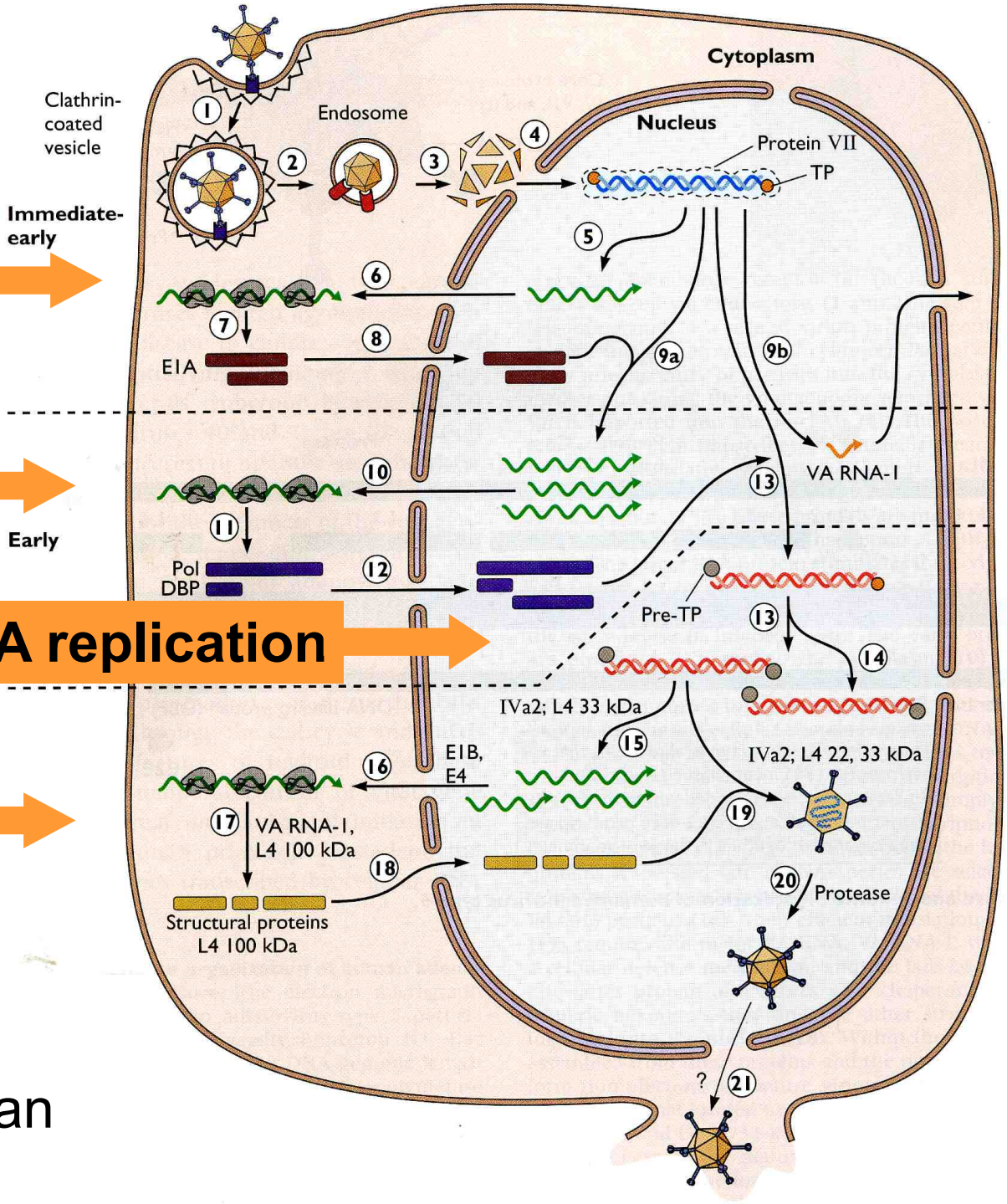


Immediate Early

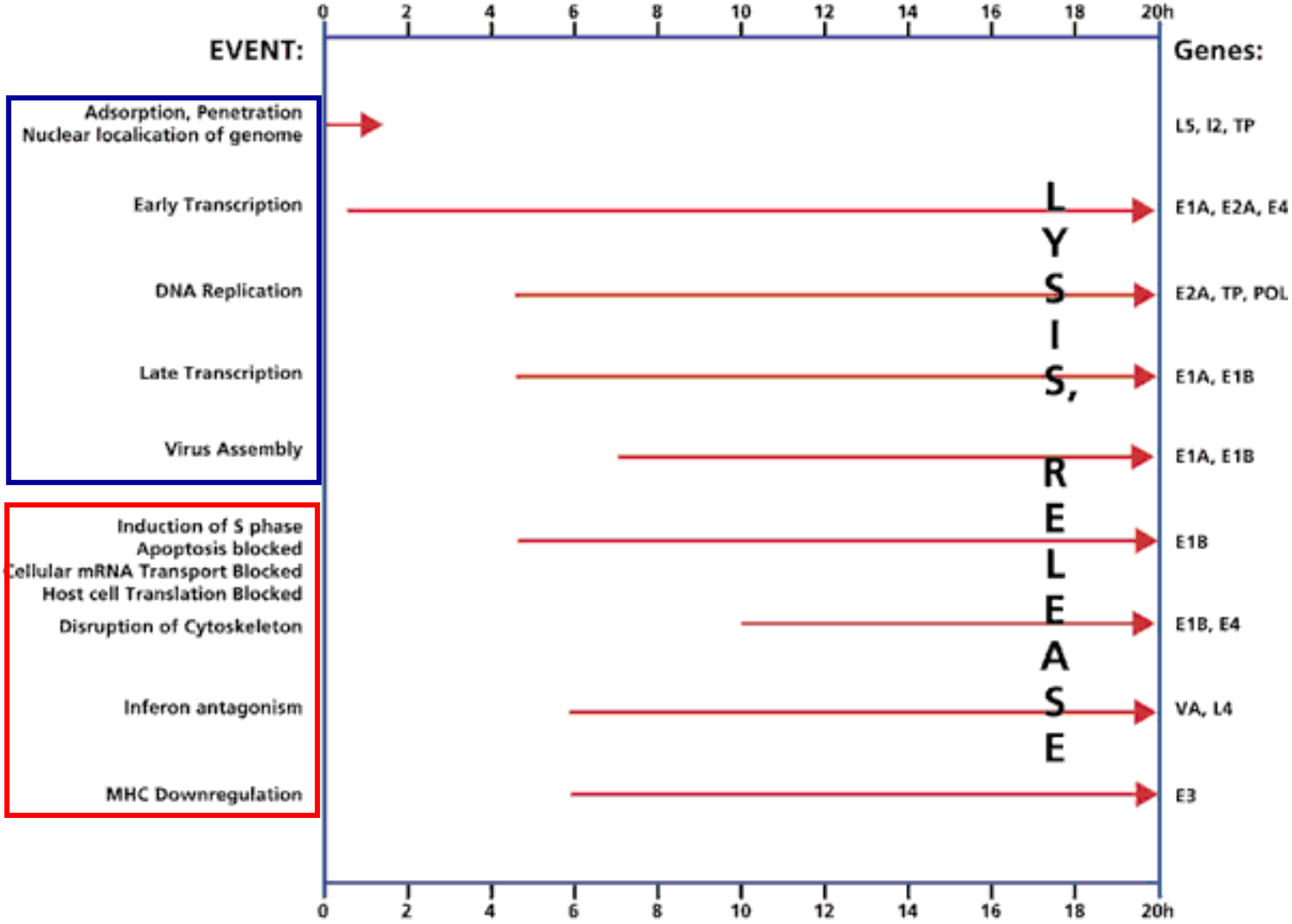
Early

DNA replication

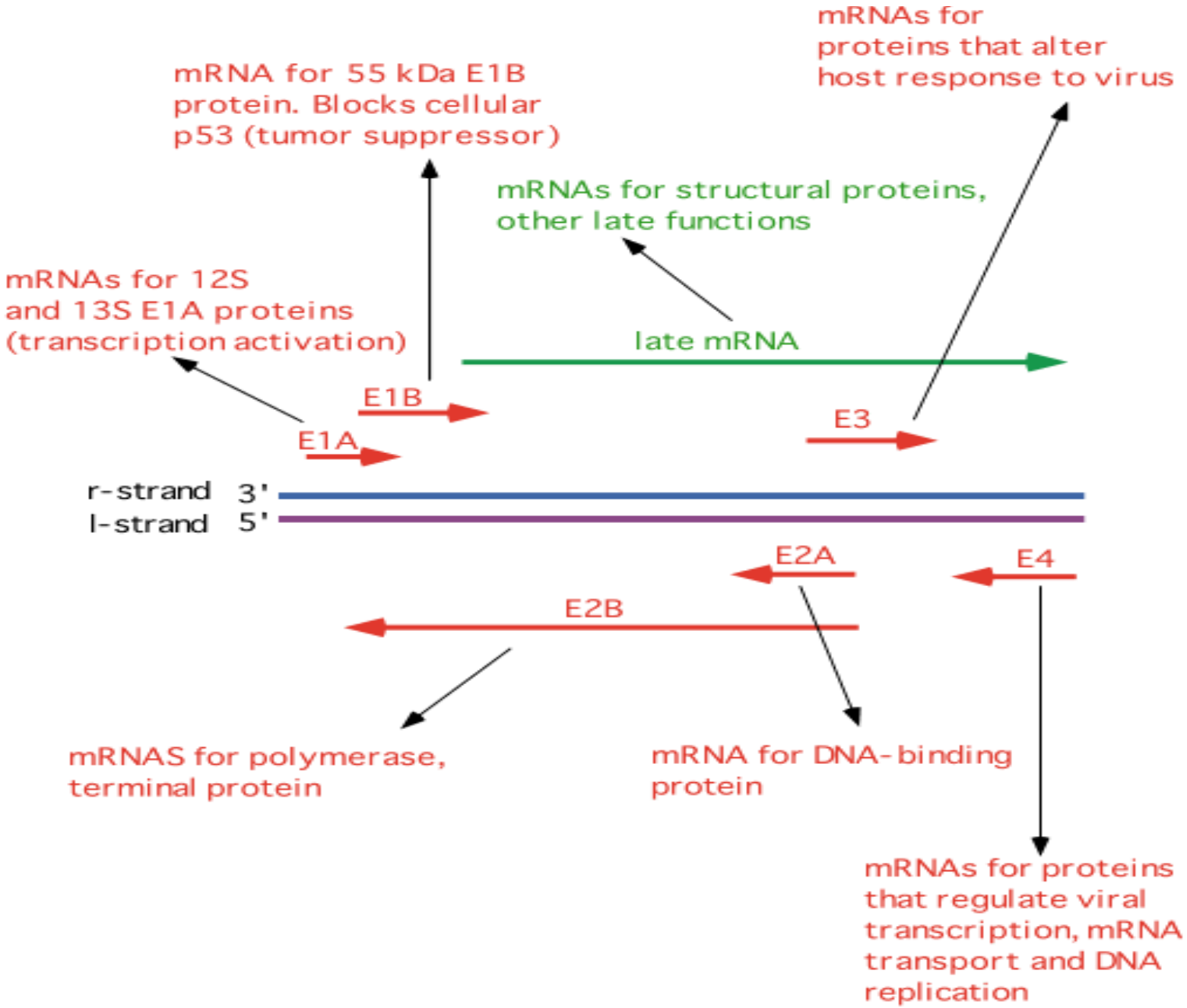
Late

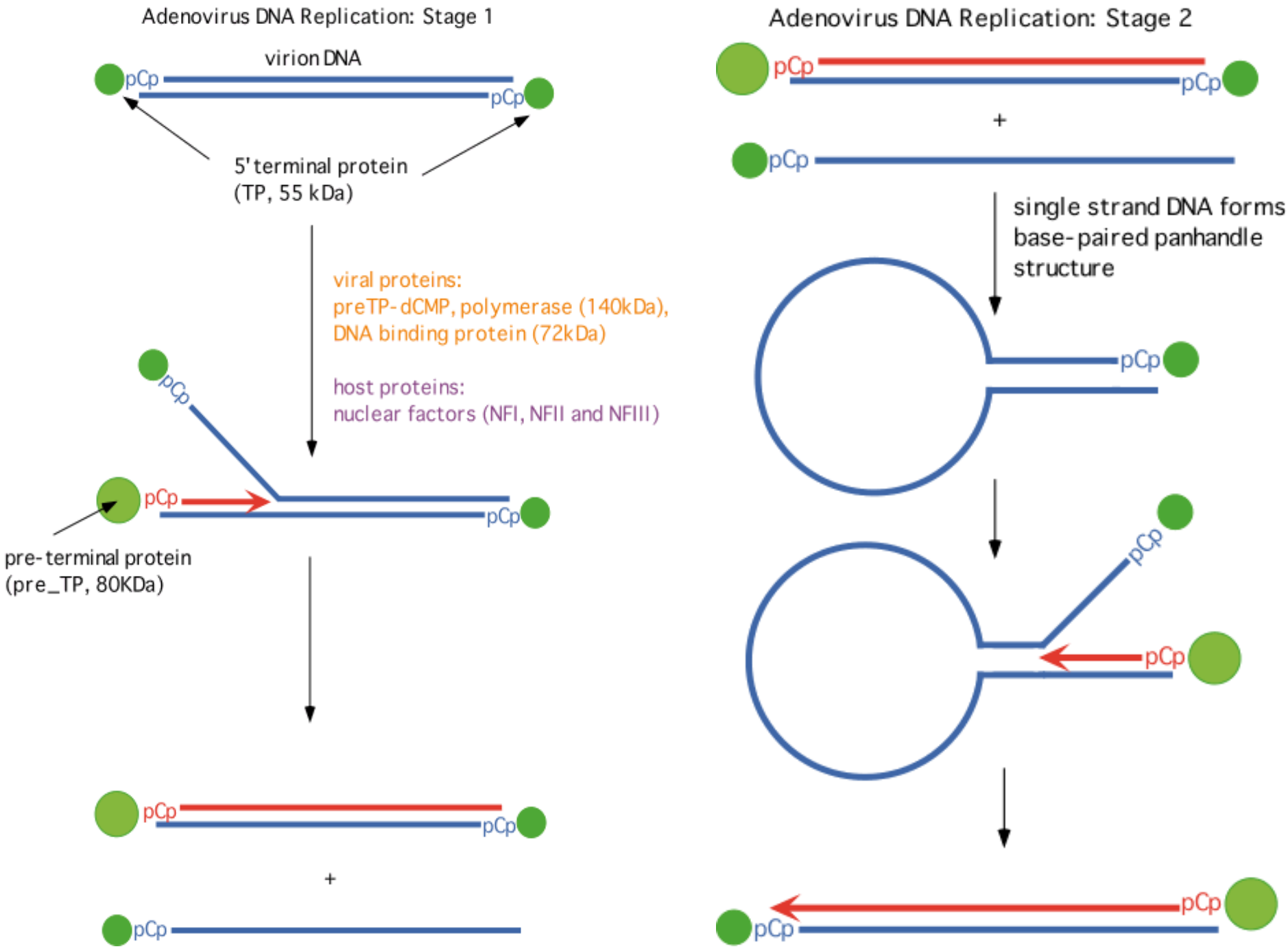


Replication of the human **adenovirus** type 2


0
2
4
6
8
10
12
14
16
18
20h
EVENT:
Genes:
Adsorption, Penetration
Nuclear localication of genome
L5, I2, TP
Early Transcription
E1A, E2A, E4
DNA Replication
E2A, TP, POL
Late Transcription
E1A, E1B
Virus Assembly
E1A, E1B
Induction of S phase
Apoptosis blocked
Cellular mRNA Transport Blocked
Host cell Translation Blocked
E1B
Disruption of Cytoskeleton
E1B, E4
Inferon antagonism
VA, L4
MHC Downregulation
E3
LYSIS,
RELEASE

Adenovirus transcription and post-transcriptional processing




Adenovirus DNA Replication: Stage 1
virion DNA
pCp
pCp
5' terminal protein
(TP, 55 kDa)
viral proteins:
preTP-dCMP, polymerase (140kDa),
DNA binding protein (72kDa)
host proteins:
nuclear factors (NFI, NFII and NFIII)
pCp
pCp
pCp
pre-terminal protein
(pre_TP, 80KDa)
pCp
pCp
+
pCp
Adenovirus DNA Replication: Stage 2
pCp
pCp
+
pCp
single strand DNA forms
base-paired panhandle
structure
pCp
pCp
pCp
pCp
pCp

Adenovirus-host cell interactions

Type of Interaction	Functional Definition	Biologic System
Productive infection	Complete replication of infectious virions	Cultured human cells
Abortive infection	Synthesis of viral gene products without production of infectious virions	Cultured hamster or monkey cells
Semipermissive infection	Complete replication with low yields of infectious virions	Cultured rat cells
Malignant transformation	Associated with integration of viral DNA and differential viral and cellular gene expression	Cultured rodent cells
Tumor induction	Associated with integration of viral DNA and differential viral and cellular gene expression	Newborn hamsters (mice)
Viral latency	Persistence of viral genome	Human tonsils

Adenoviruses: pathogenesis and diseases

Virus

47 adenovirus serotypes that infect humans, classified into six subgroups

Disease

Respiratory diseases
- Febrile upper tract infection
- Pharyngoconjunctival fever
- Acute disease
- Pertussis-like disease
- Pneumonia

Other diseases
- Acute hemorrhagic cystitis
- Epidemic keratoconjunctivitis
- Gastroenteritis

Epidemiology

Transmission
- Respiratory droplets, fecal matter, fomites
- Close contact
- Poorly sanitized swimming pools

Distribution of virus
- Ubiquitous
- No seasonal incidence

At risk or risk factors
- Children aged <14 years
- Day care centers, military camps, swimming clubs

Vaccines or antiviral drugs
- Live, attenuated vaccine, serotypes 4 and 7 for the military

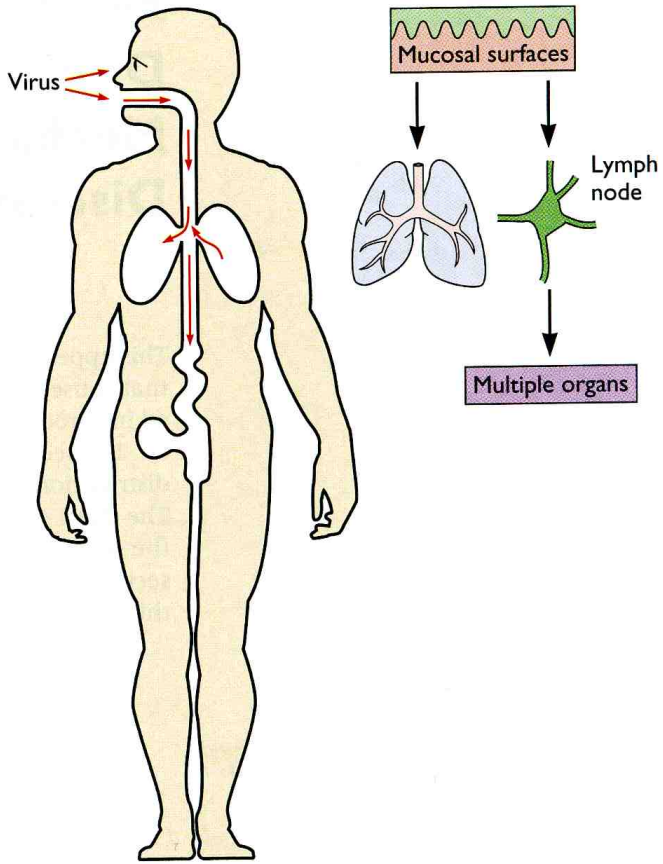
Disease mechanisms

Transmitted by **aerosol**, **close contact**, **fecal-oral route**, or **fingers** and **ophthalmologic instruments** (eye infections)

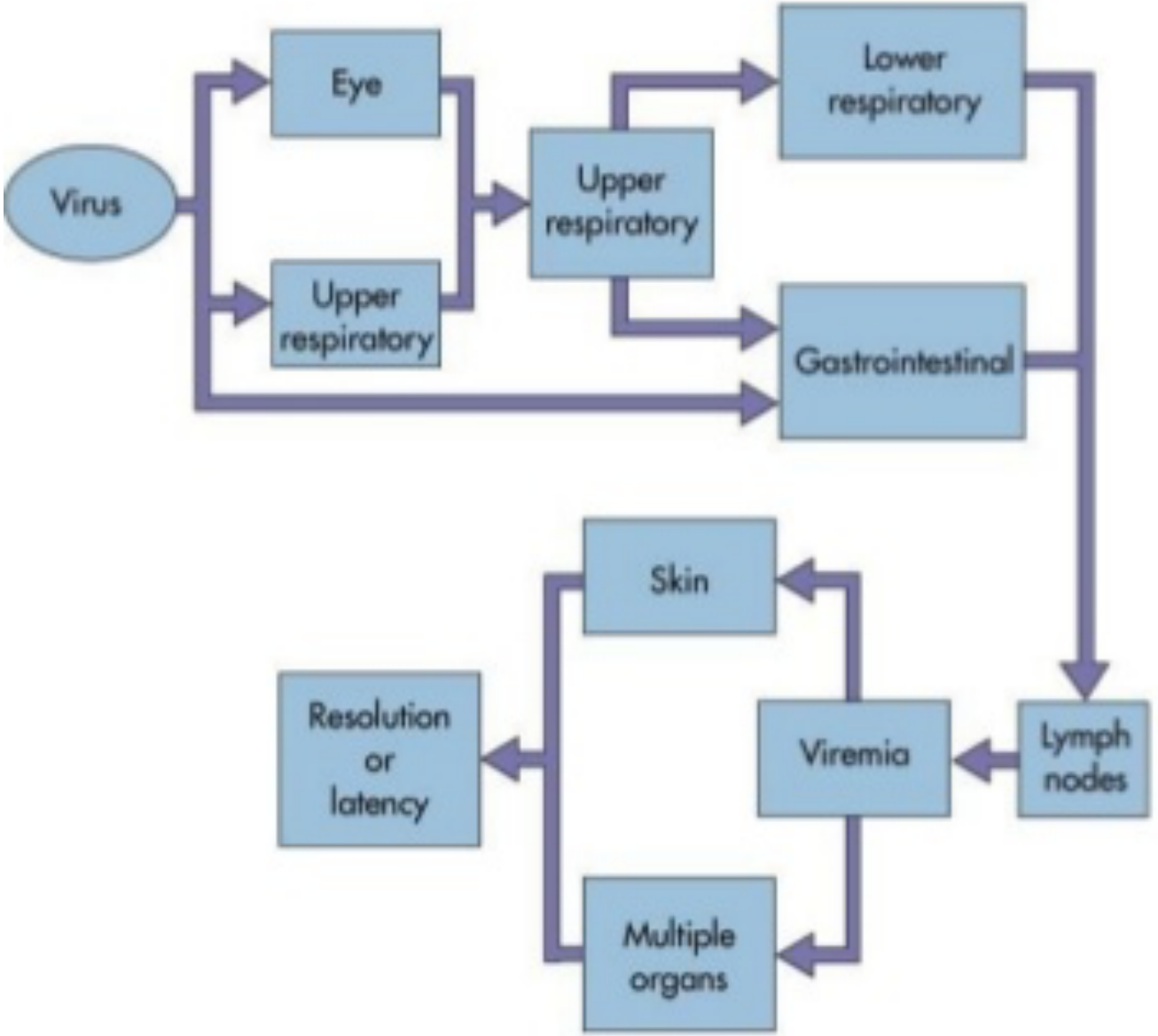
Virus infects mucoepithelial cells of respiratory and gastrointestinal tract, conjunctiva, cornea

Virus persists in lymphoid tissue (tonsils, adenoids, Peyer's patches)

Antibody is essential for recovery from infection



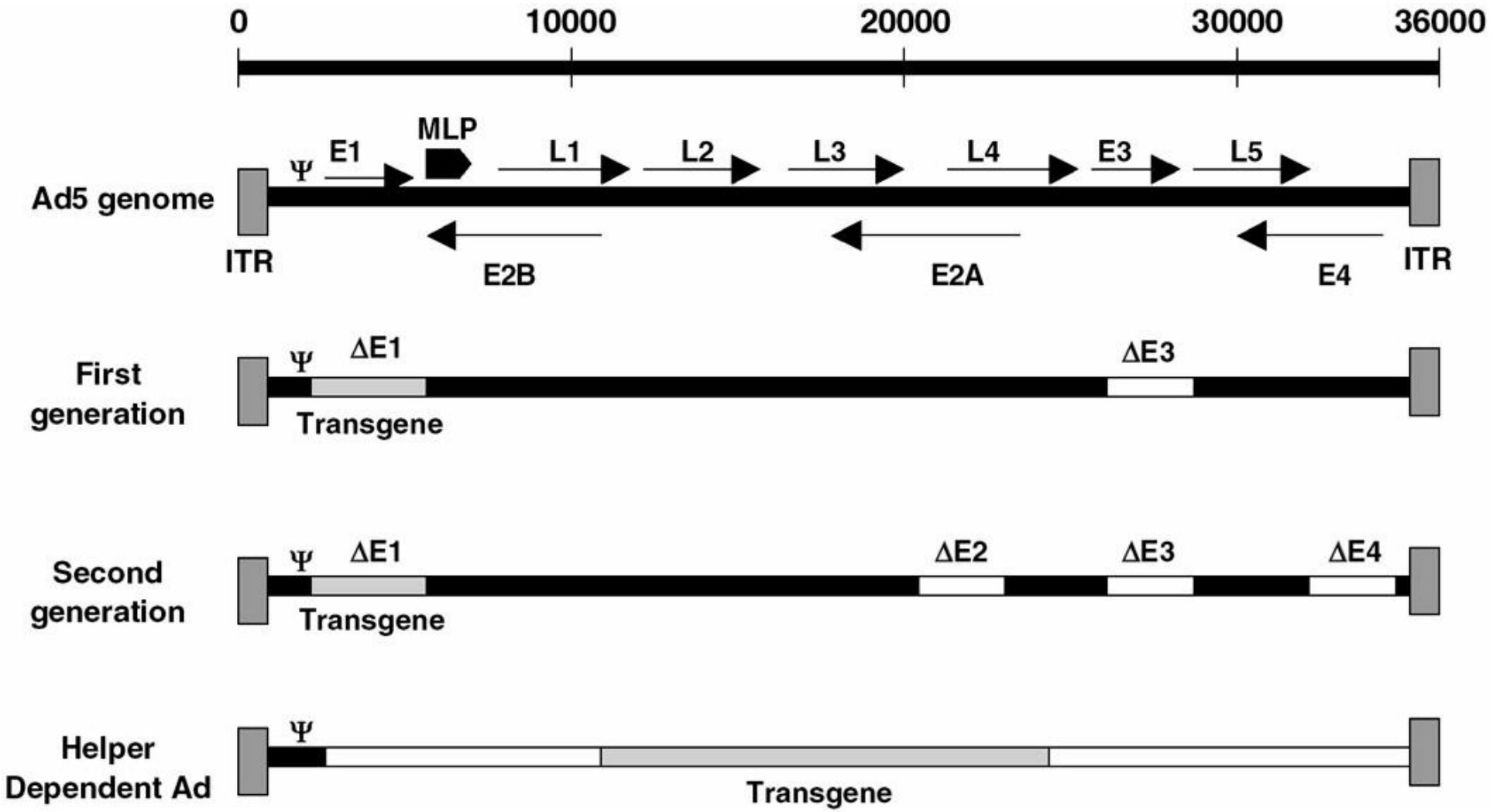
Adenovirus pathogenesis



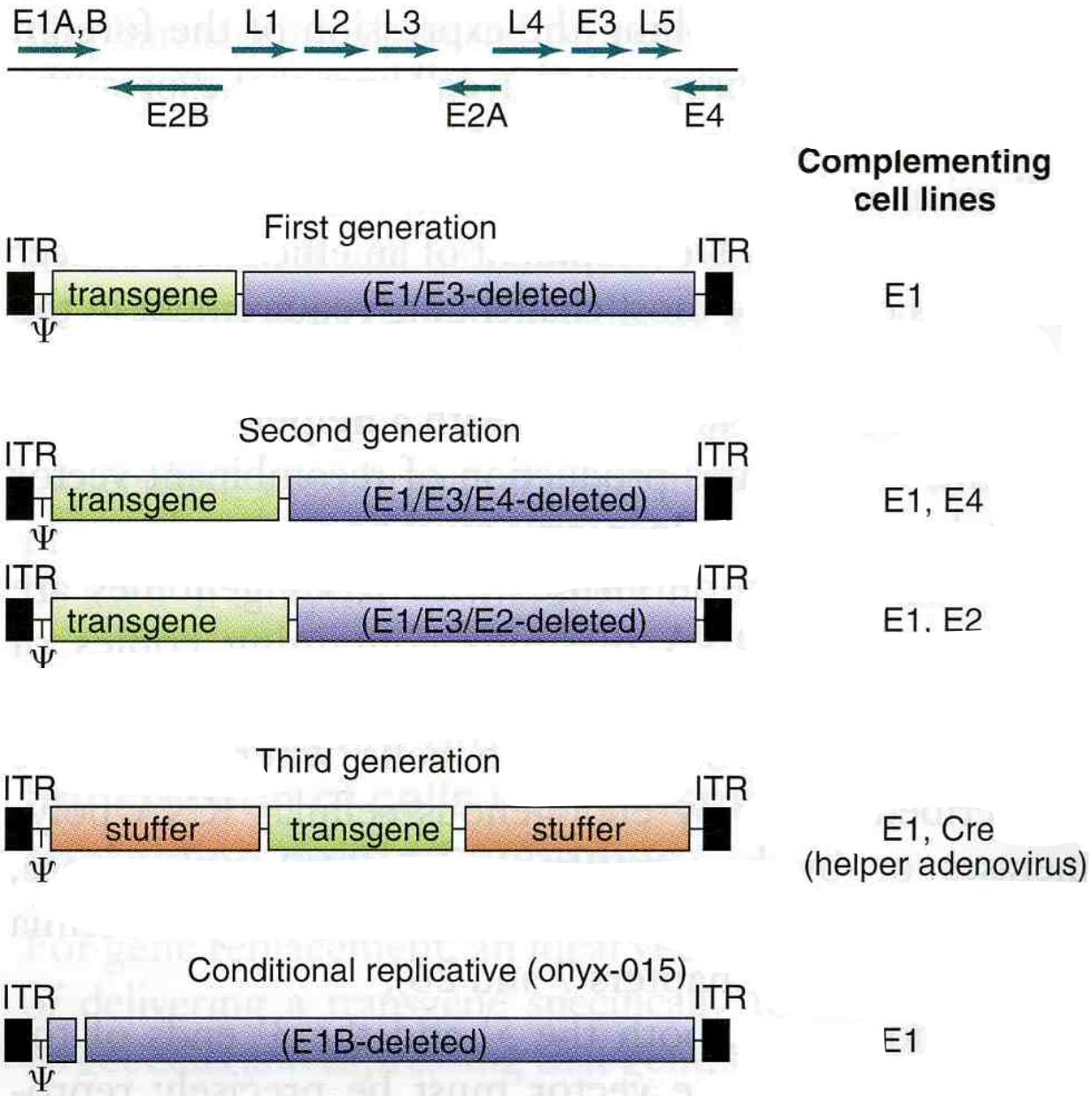
Favorable Features of Adenoviral Vectors

- Causes benign respiratory tract infections
- Safety—lack of association with oncogenicity
- Well characterized and easily manipulated
- Stability and high titers of recombinant vectors
- Ability to infect a broad range of cell types, including dividing and nondividing cells
- High transient expression levels
- High insert capacity (up to 37 kb, gutless ad)
- Little risk of random chromosomal integration

Development of Adenovirus Vectors

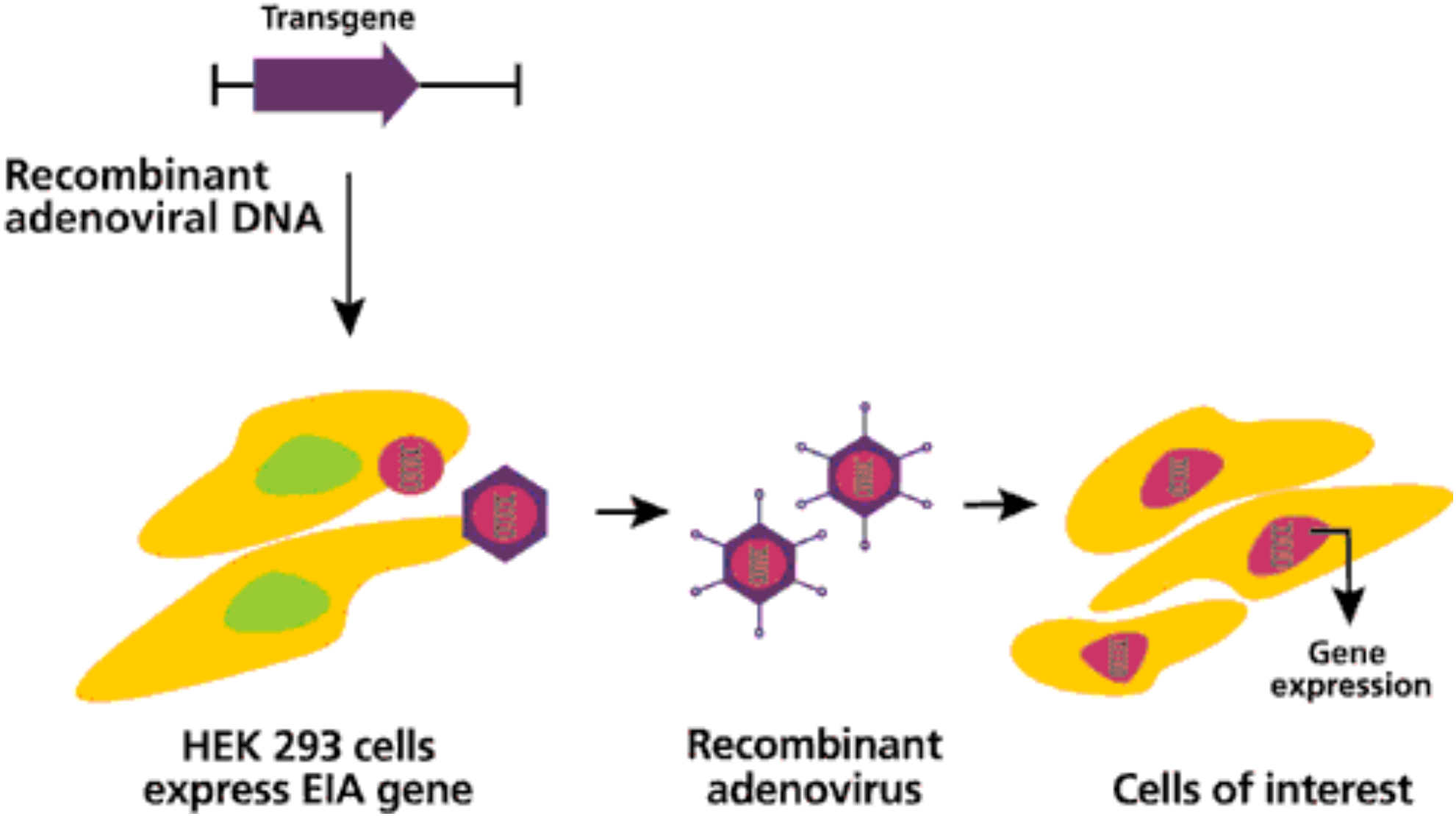


Development of Adenovirus Vectors

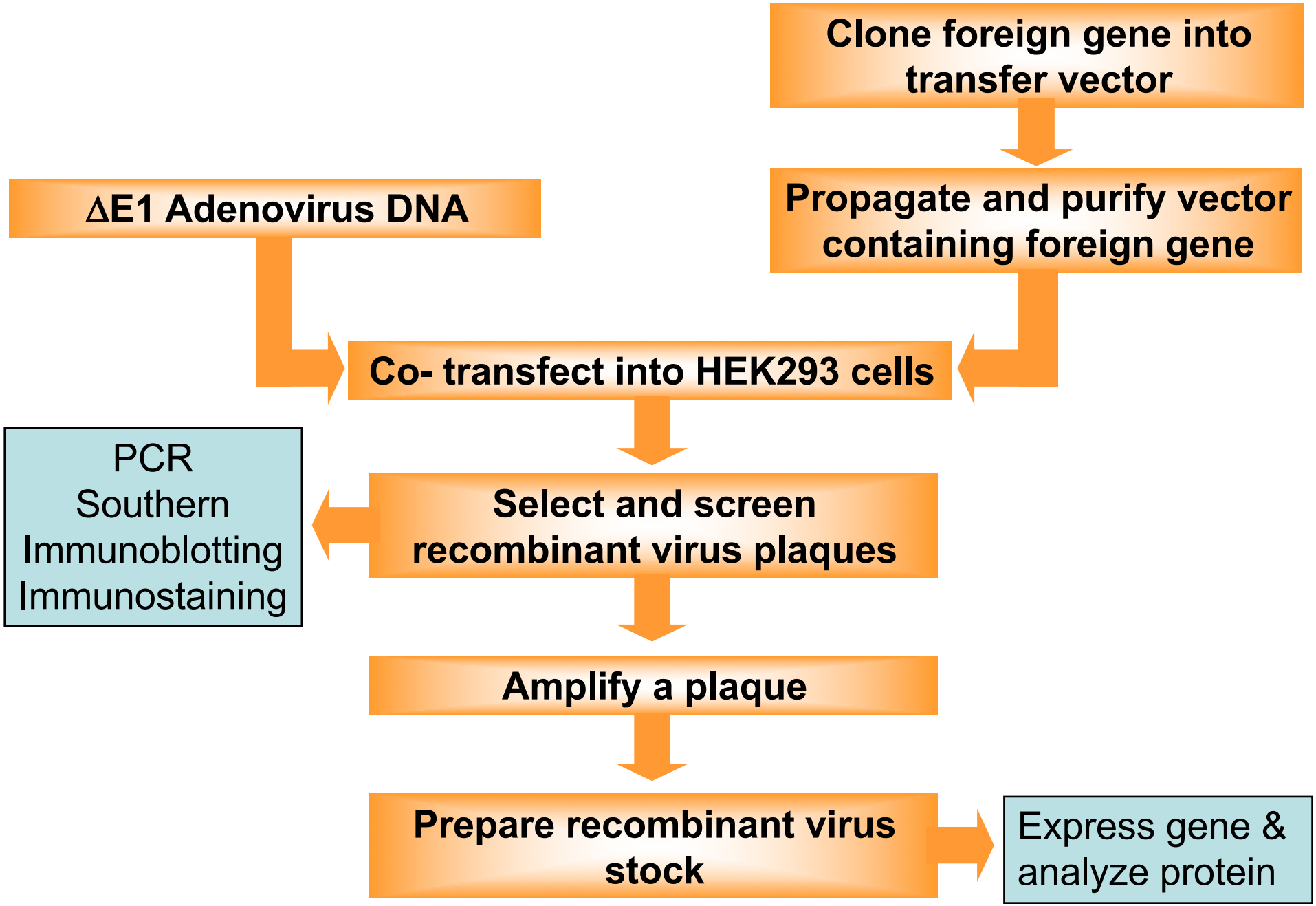


Development of Adenoviral Vectors

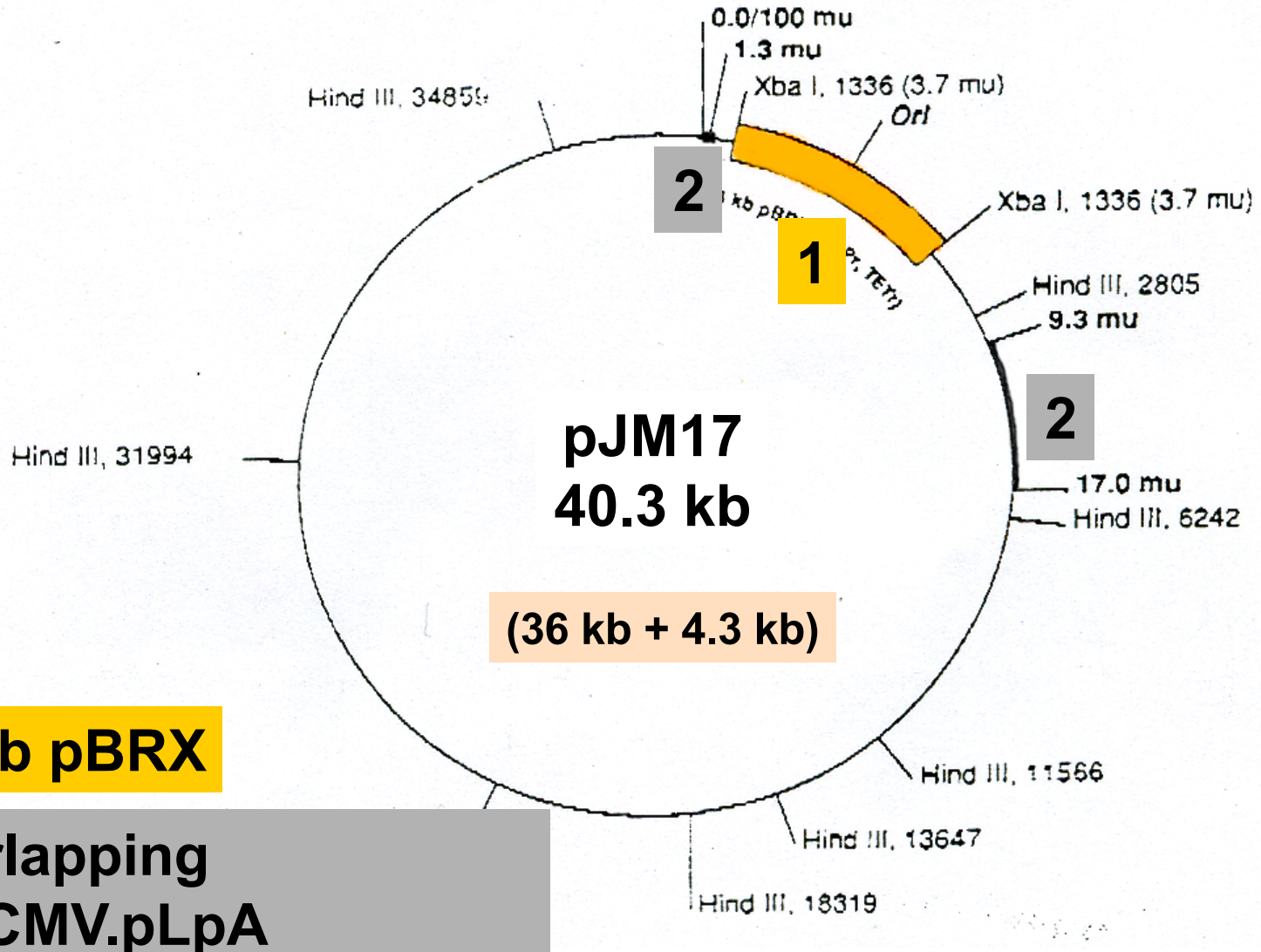
- Generated by replacing E1 and/or E3 with a foreign DNA (up to 6.5 kb, transgene + heterologous promoter-enhancer element)
- The recombinant ΔE1 vectors are replication-defective, and their replication depends on functions provided in trans
- The ΔE1unit vectors can be propagated and amplified to high titers using E1-expressing cell lines
- The vectors can infect cells *in vitro* and *in vivo*
- The expression lasts only 5-10 days due to immune response


Transgene
Recombinant adenoviral DNA
Gene expression
HEK 293 cells express EIA gene
Recombinant adenovirus
Cells of interest

Flow Chart for 1st Generation Ad Expression System



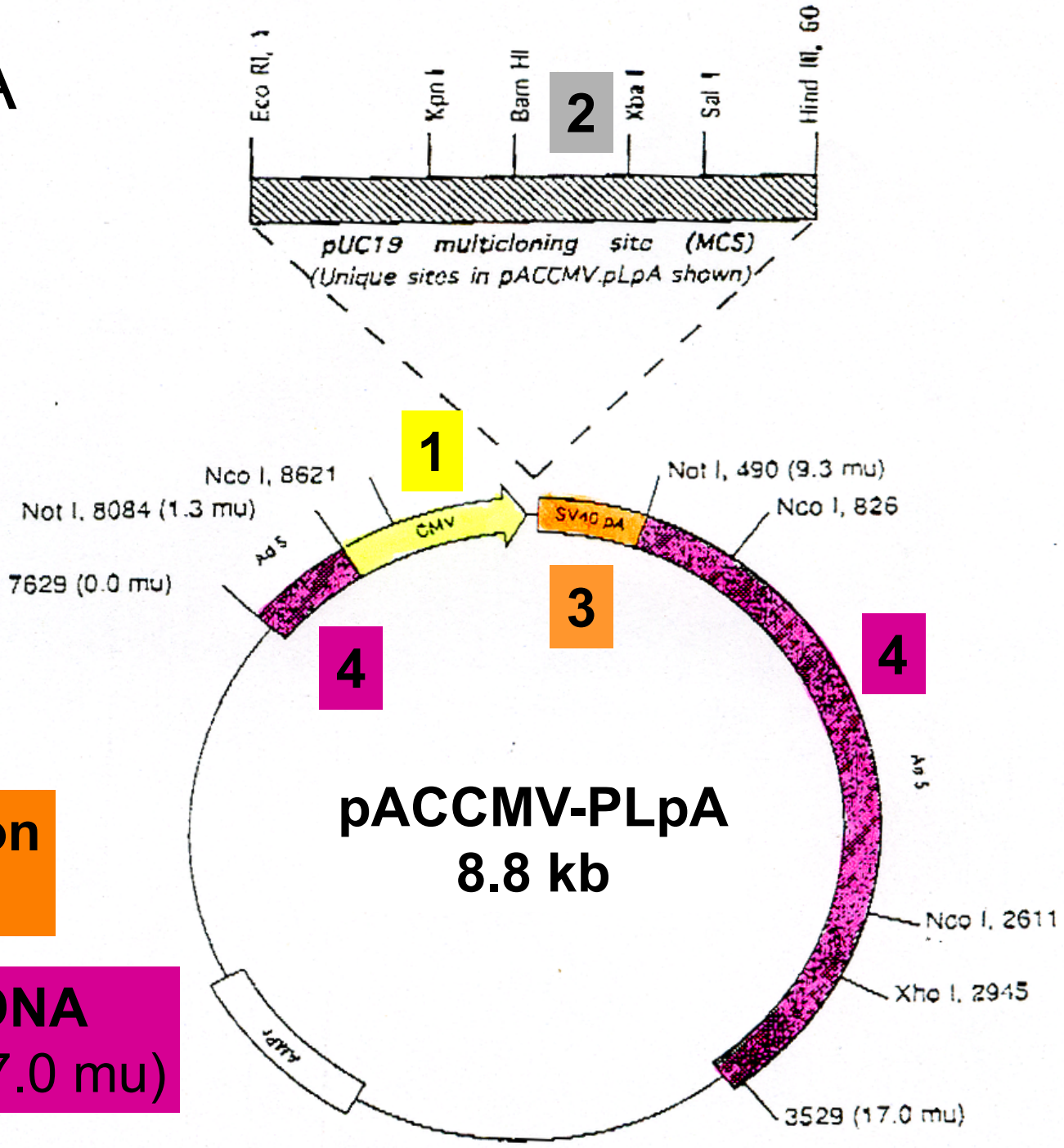
Map of pJM17 plasmid: a modified Ad genome



1. 4.3 kb pBRX

2. Overlapping pAcCMV.pLpA
(0.0-1.3 and 9.3-17.0 mu)

pACCMV-pLpA plasmid



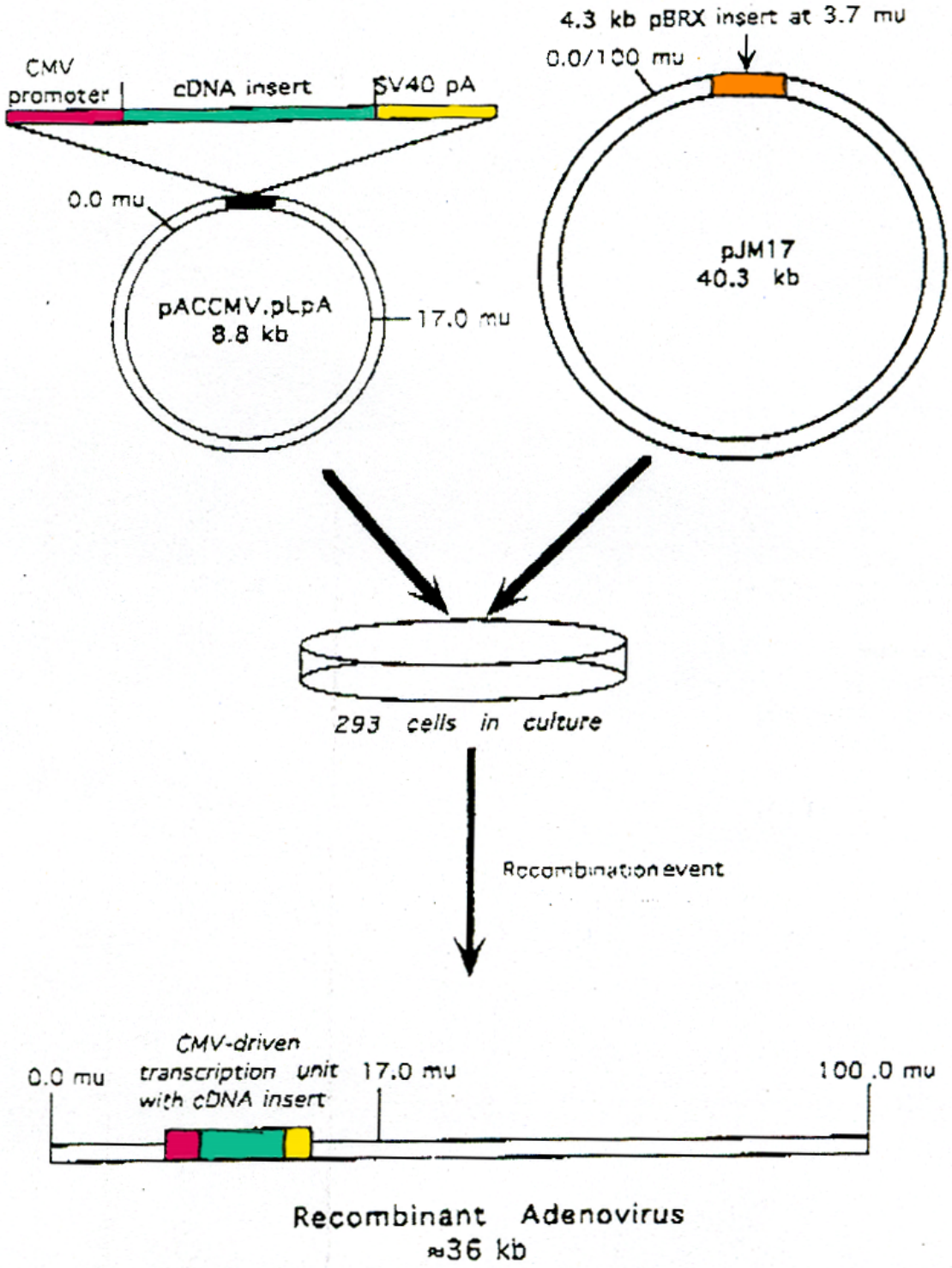
1.CMV IE promoter

2. MCS

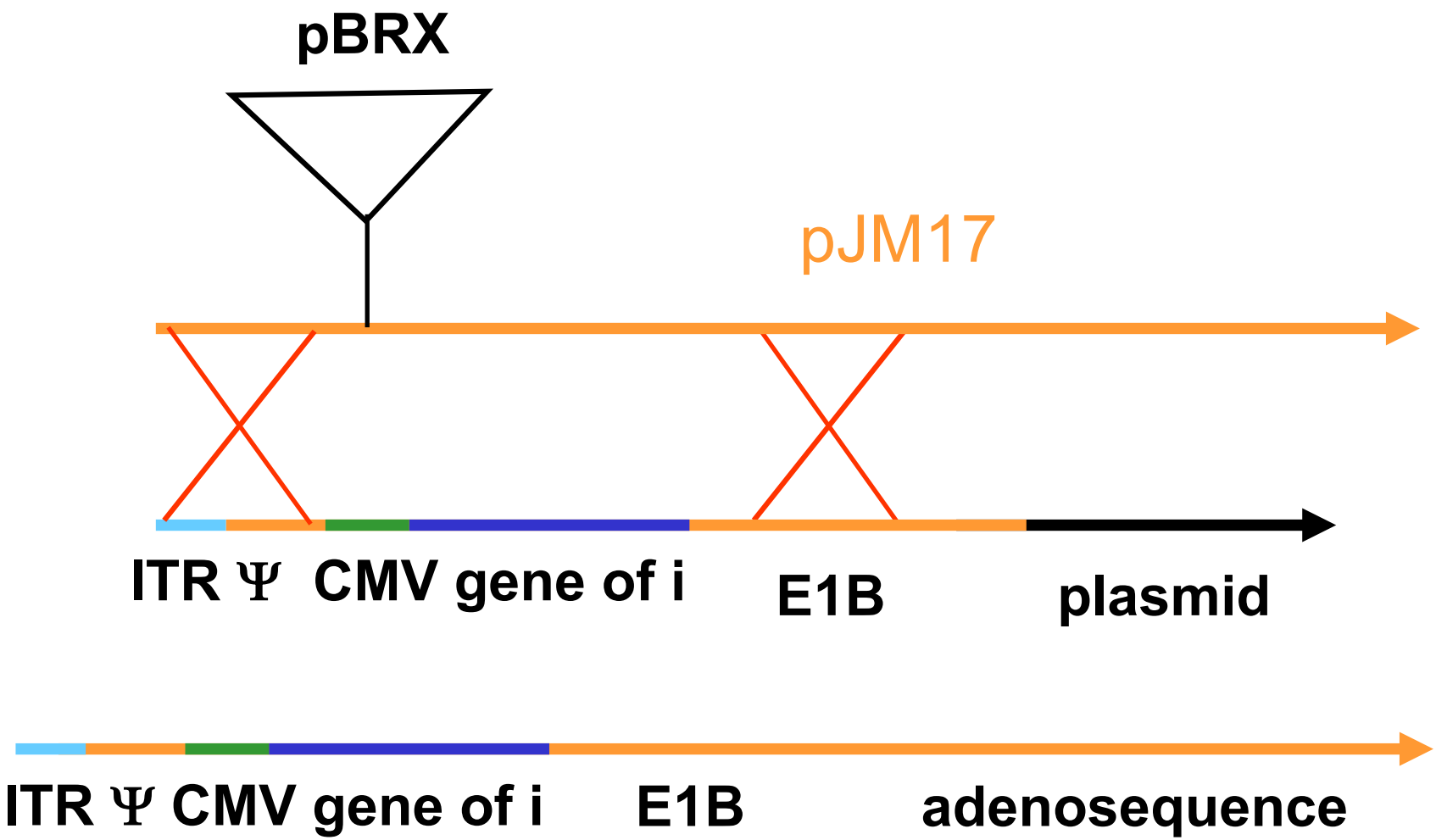
3. SV40 small t intron + polyA

4. Flanking Adeno DNA (0.0-1.3 and 9.3-17.0 mu)

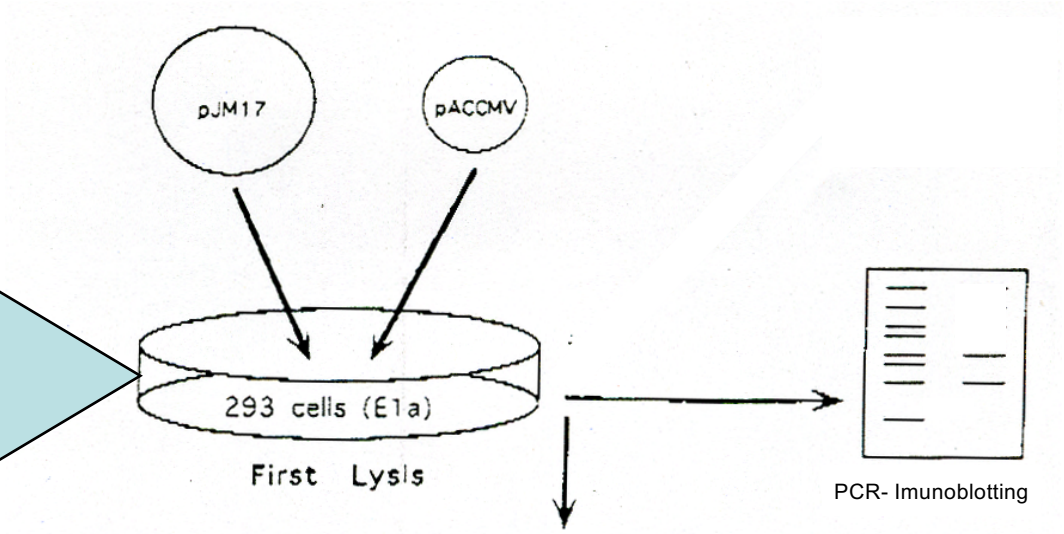
Homologous recombination



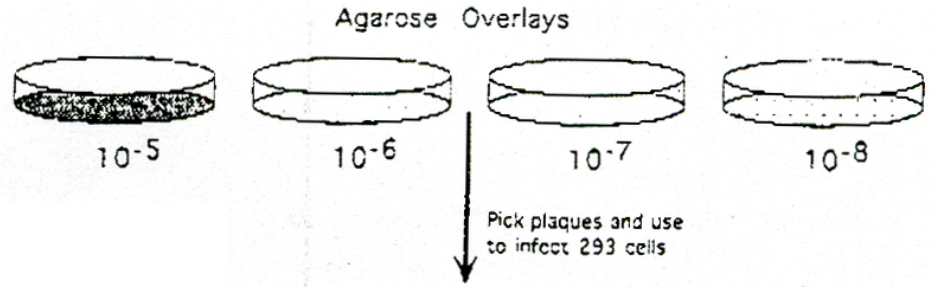
Generation of recombinants



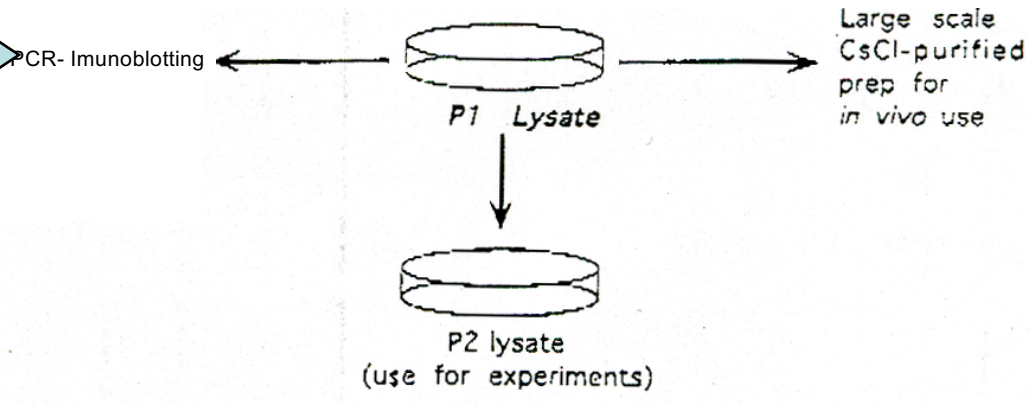
Obtaining recombinant Ad with the gene of interest



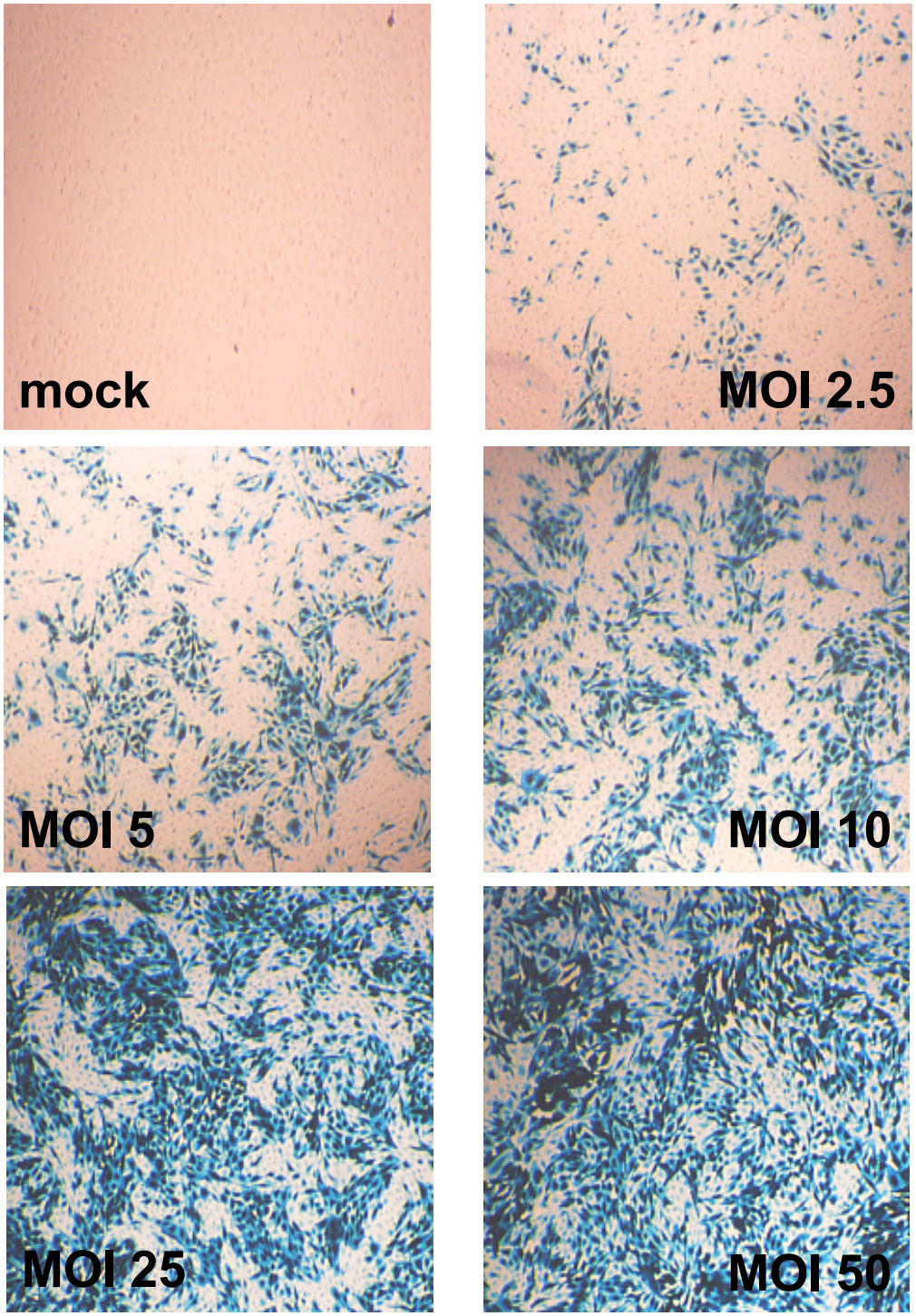
Plaque purify recombinant Ad with the gene of interest



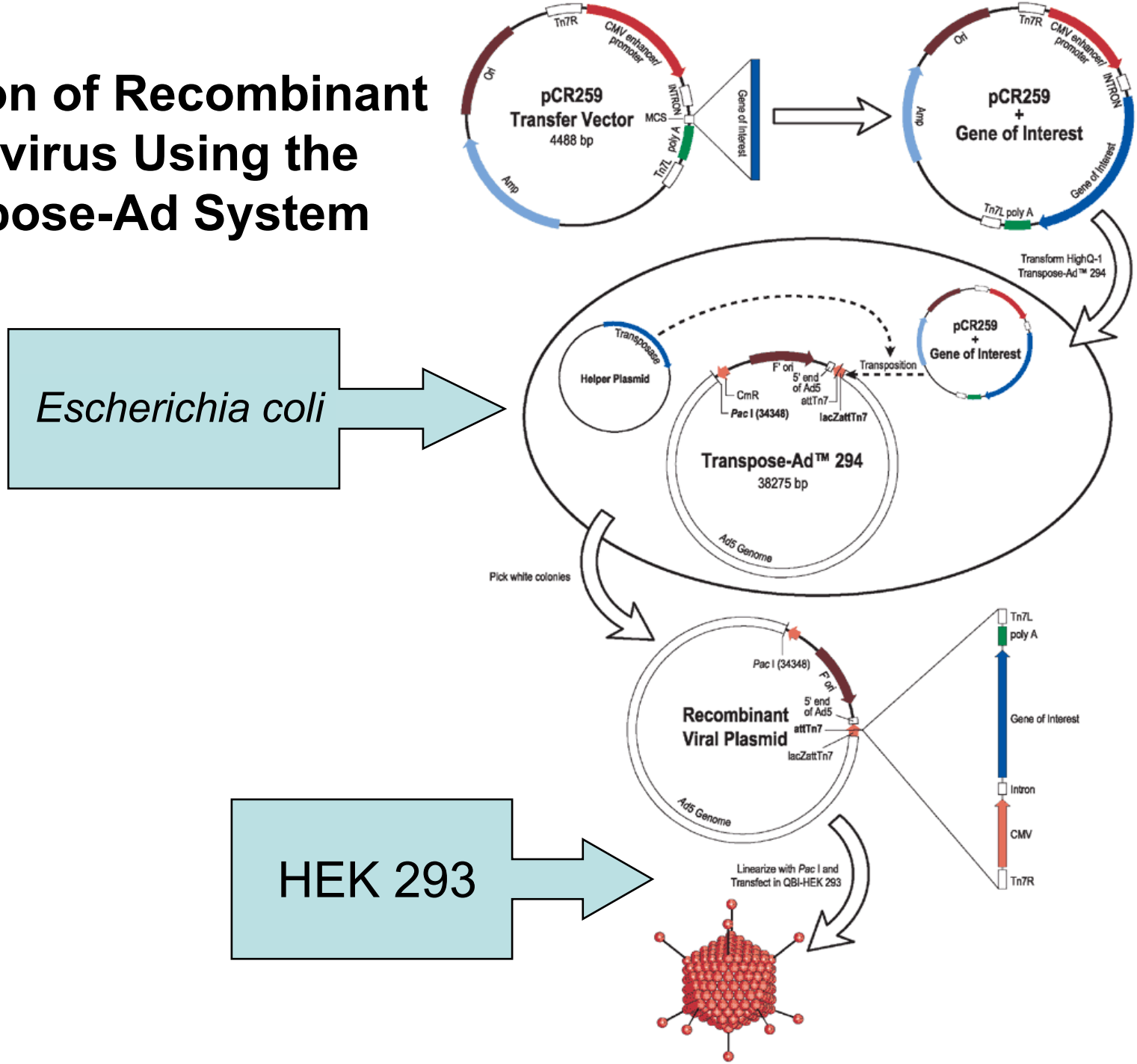
Propagate, titrate and validate recombinant Ad



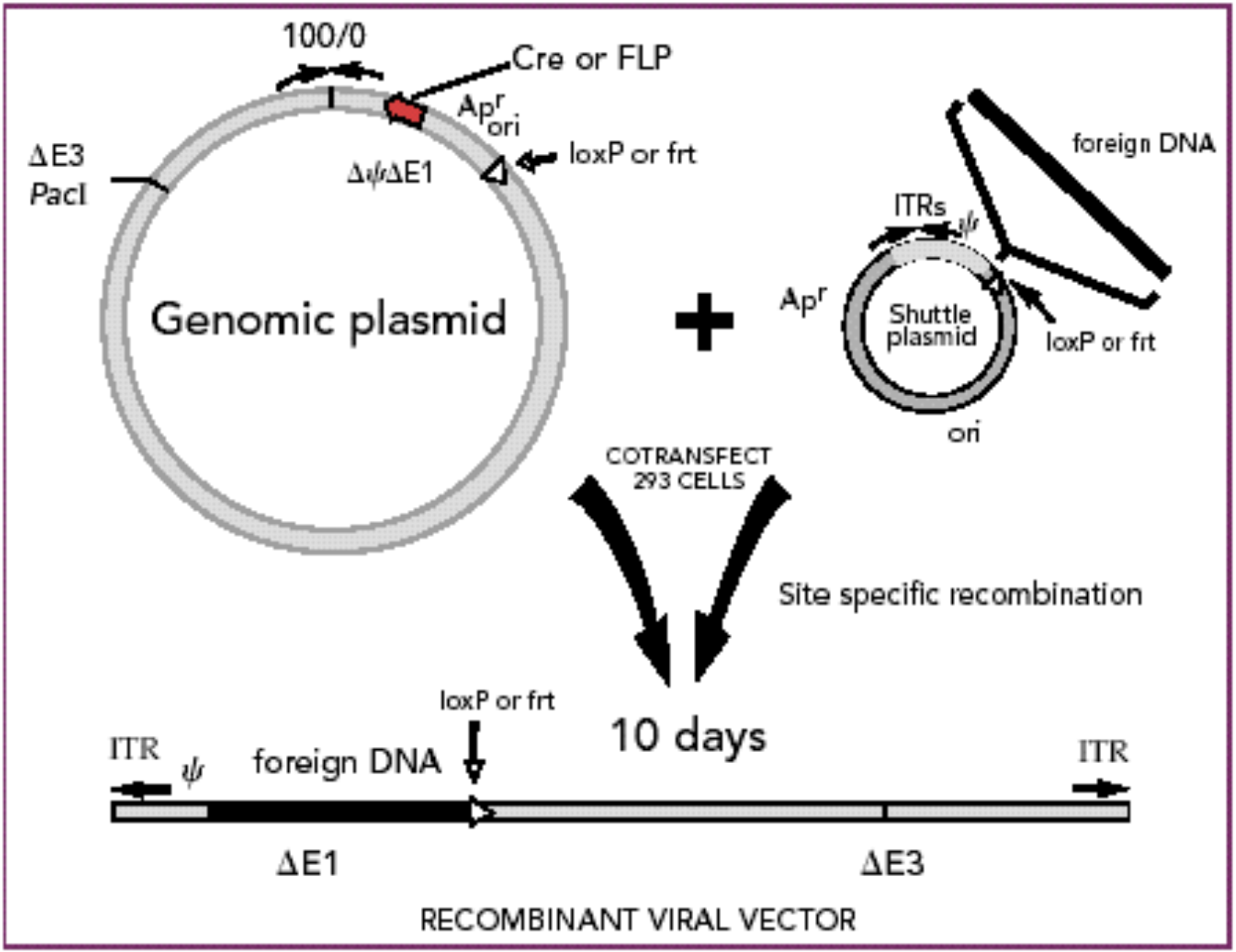
Adv-LacZ transduction in HUVEC (72 hpi)



Generation of Recombinant Adenovirus Using the Transpose-Ad System



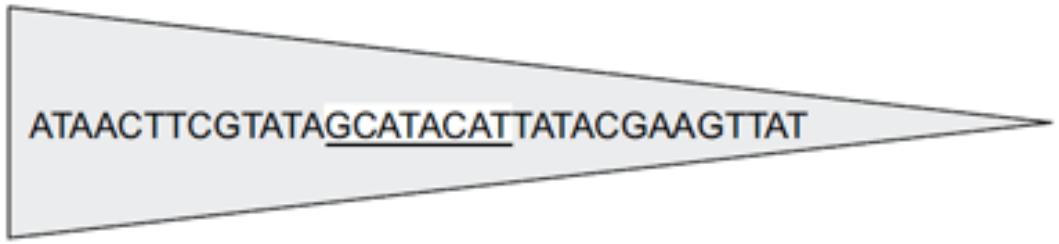
AdMax for Generation of Adenovirus vectors in HEK 293 cells

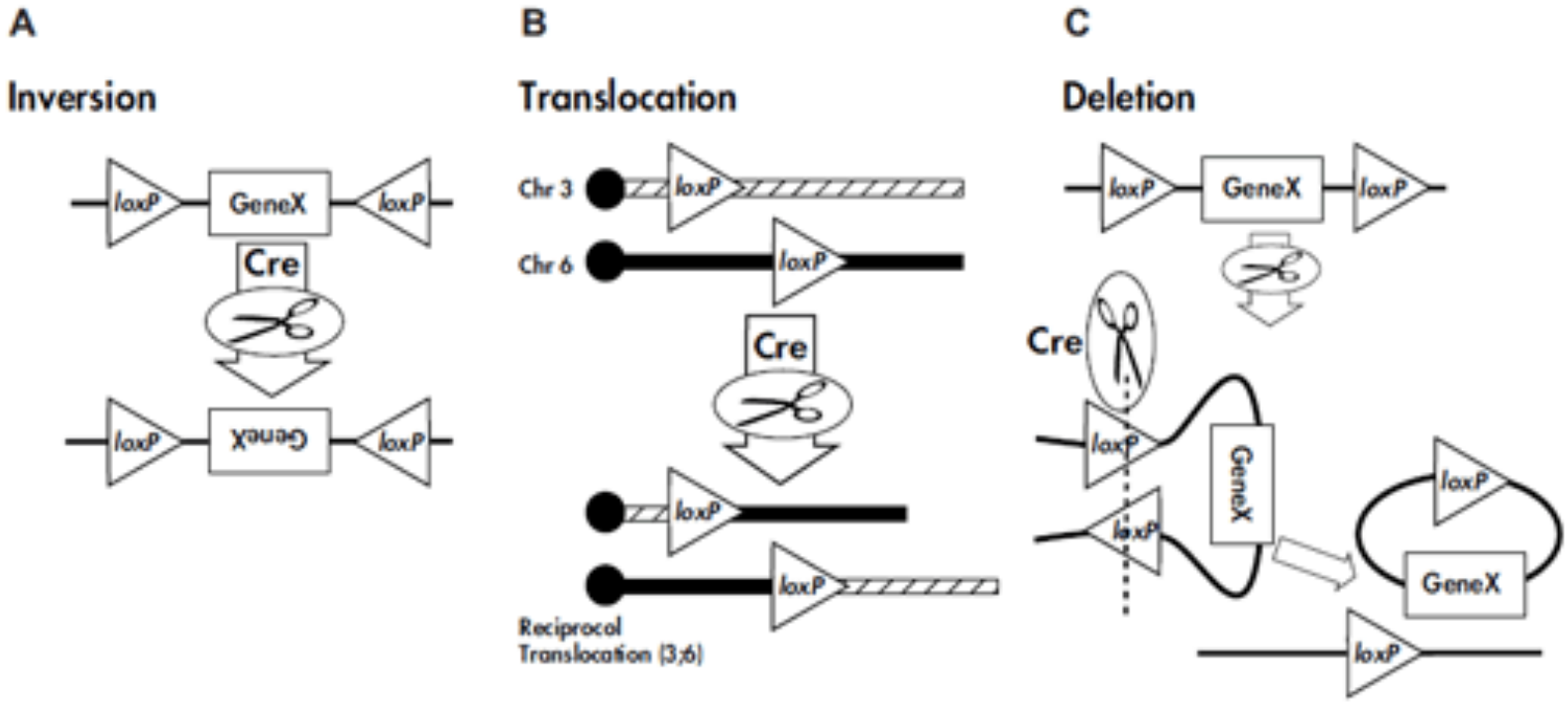


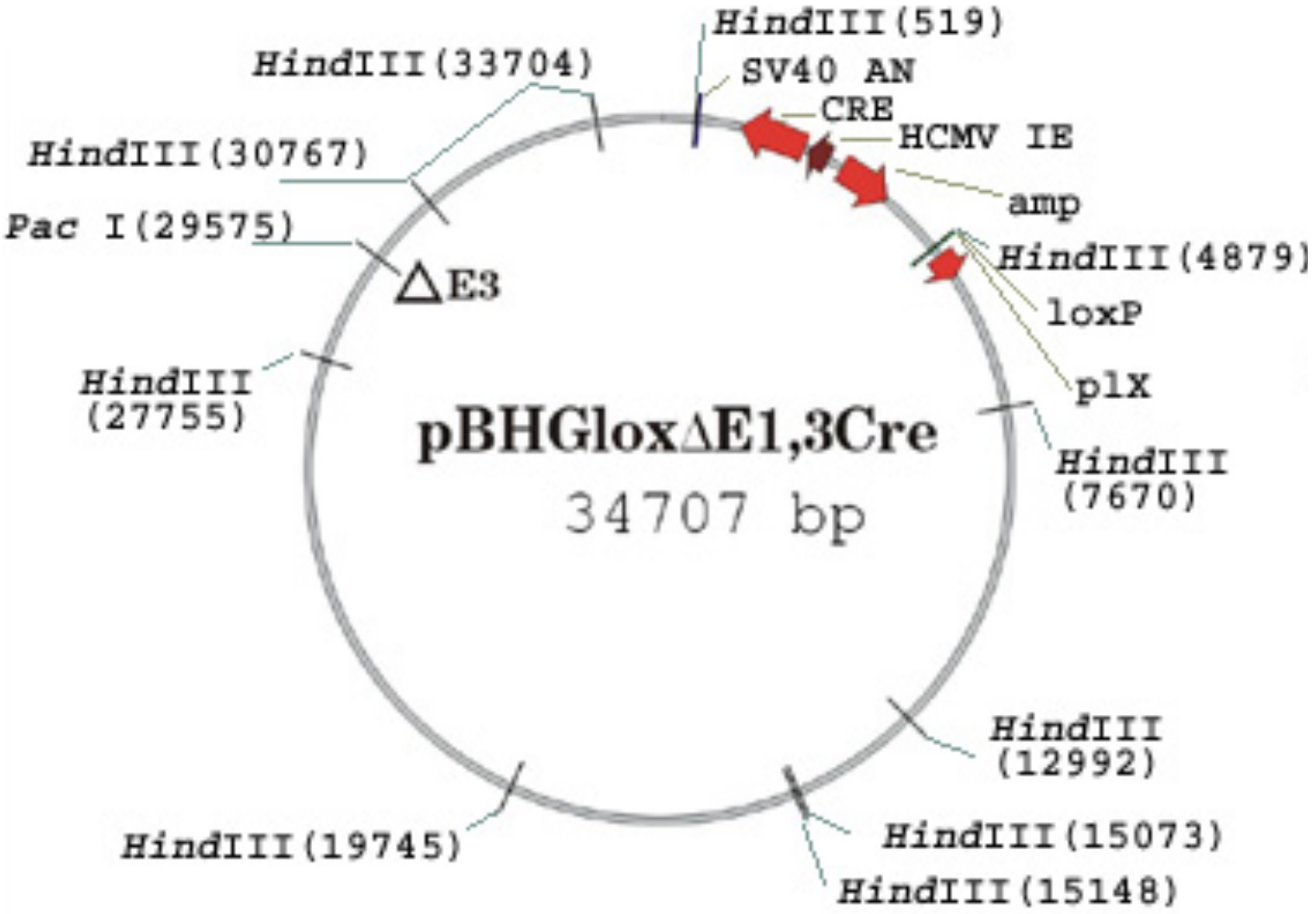
Cre-lox technology

1. **Cre recombinase:** a 38 kDa enzyme from phage P1 that catalyzes recombination between two loxP sites

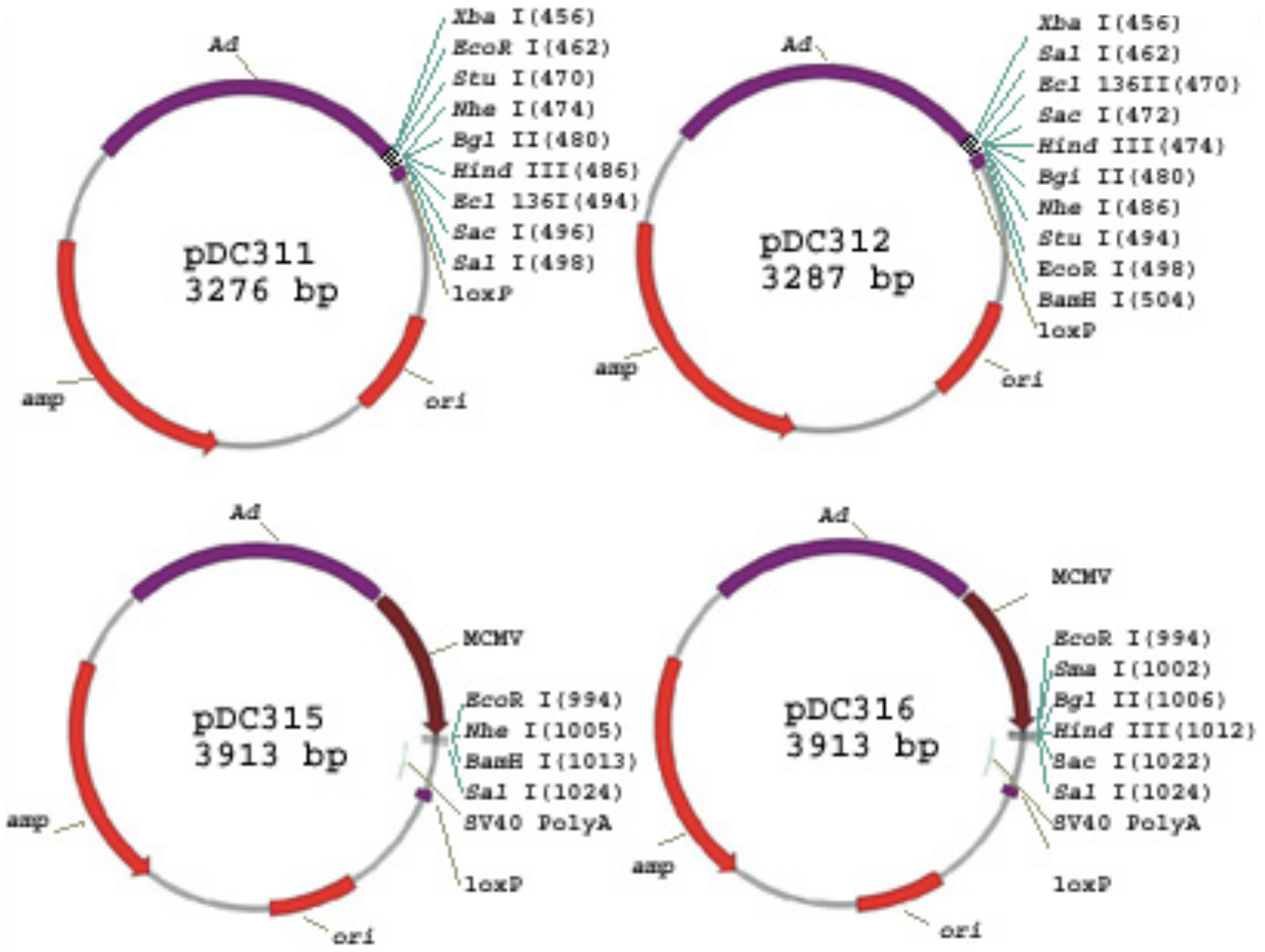
2. **LoxP sites:** a specific 34-base pair sequences consisting of an 8-bp core sequence, where recombination takes place, and two flanking 13-bp inverted repeats





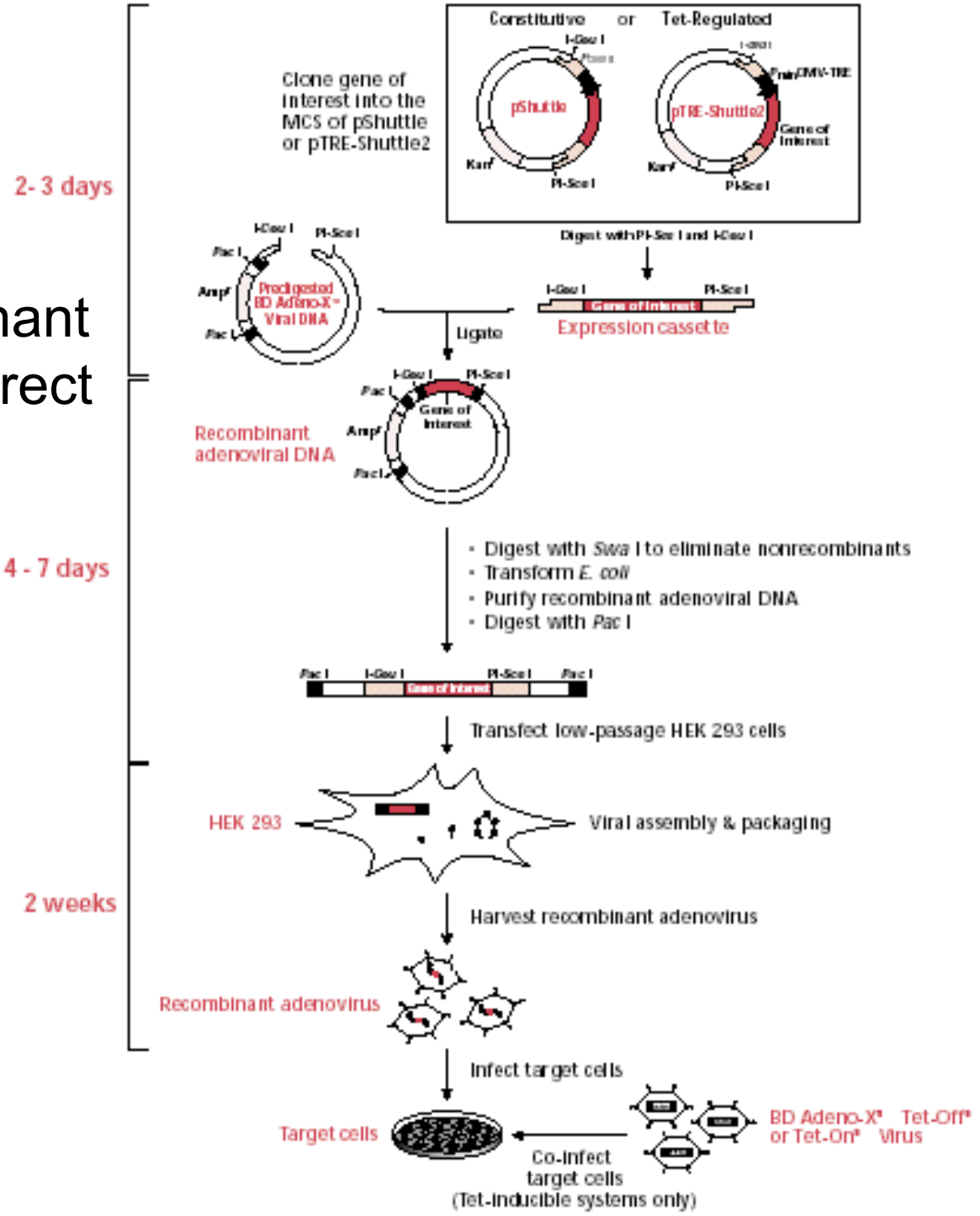


Adenoviral genomic plasmid for construction of Ad vector by *Cre-loxP* recombination

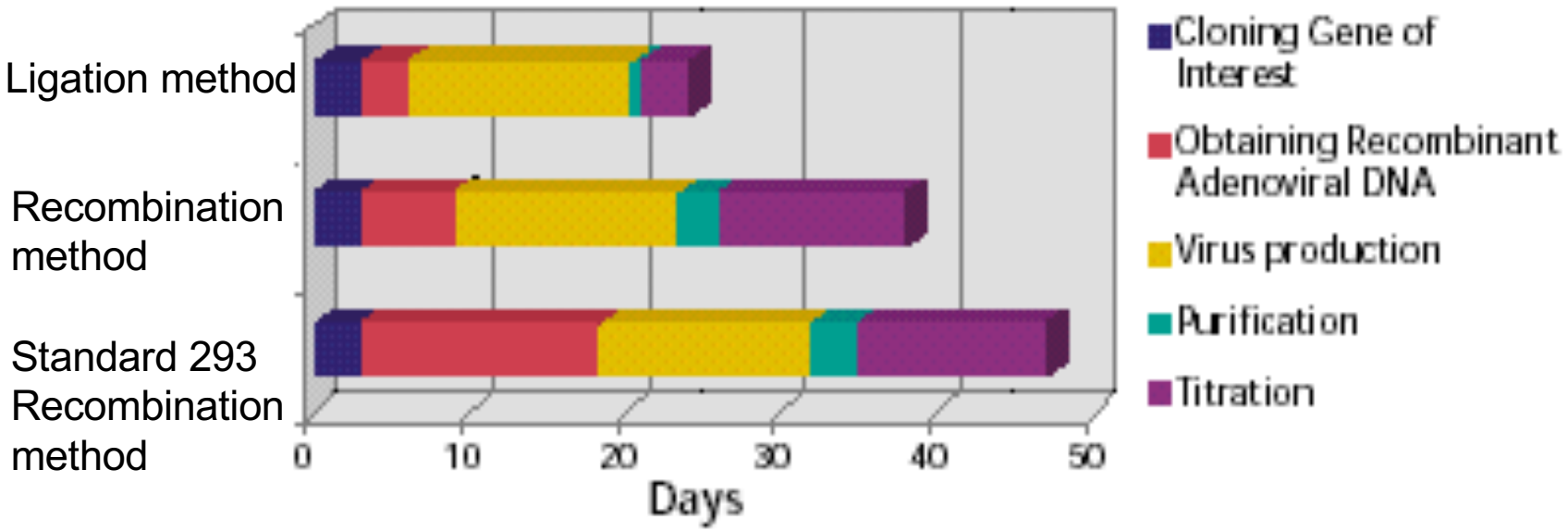


Shuttle plasmids for *Cre-loxP* Ad vector construction

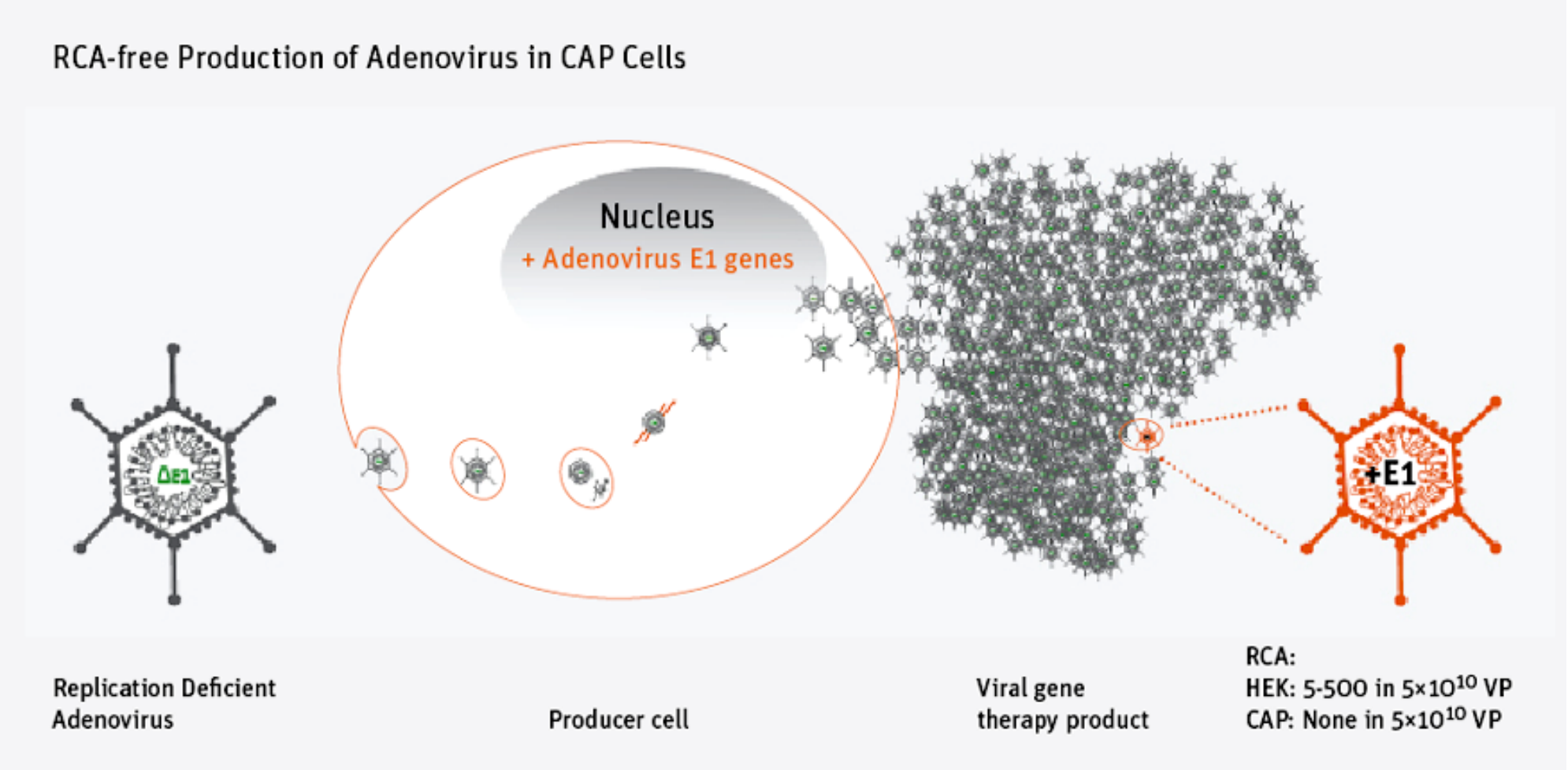
Generation of Recombinant Adenovirus Using by Direct *in vitro* Ligation



Comparison of different Ad systems time requirement



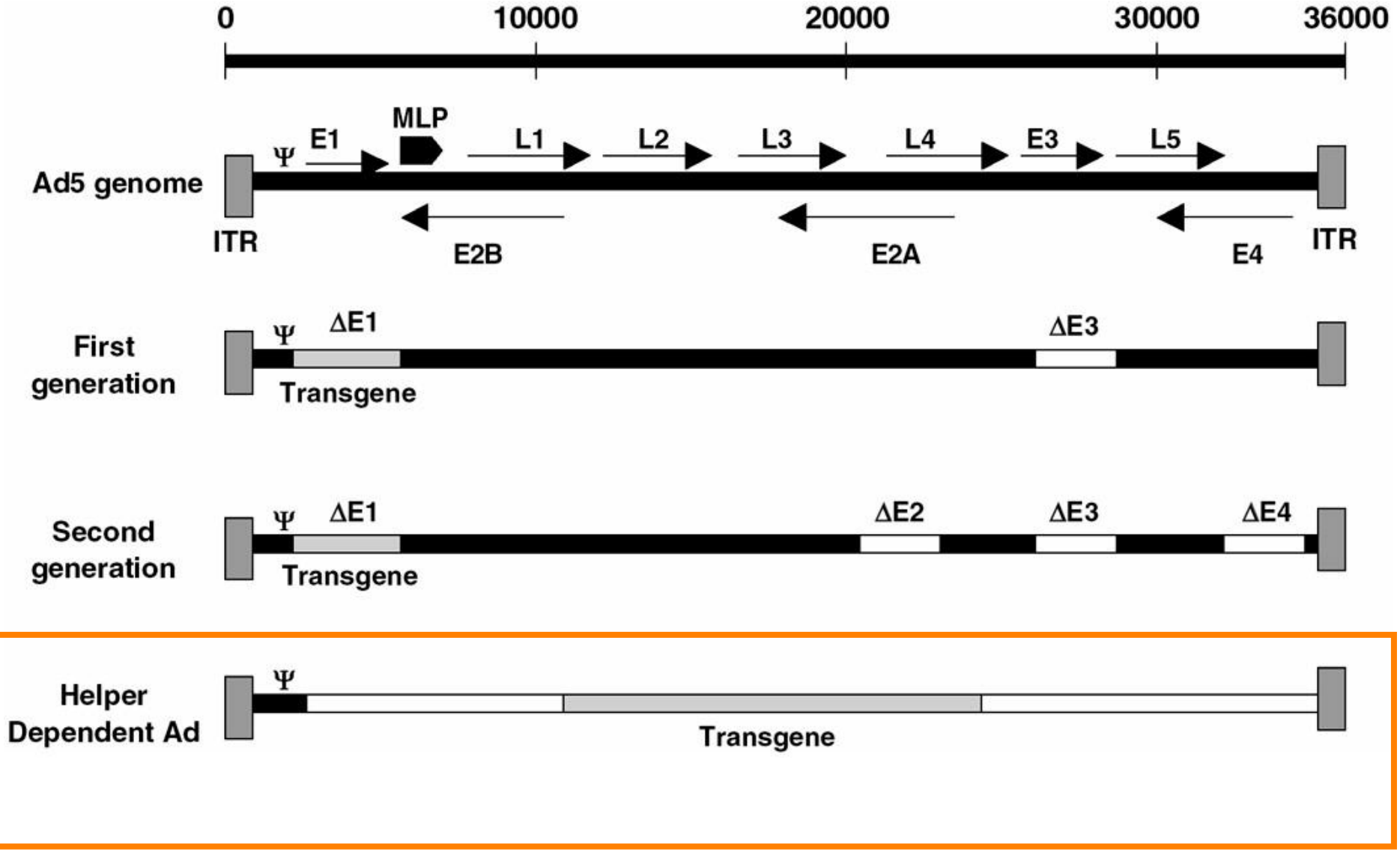
Generation of Gutless Adenovirus Vectors



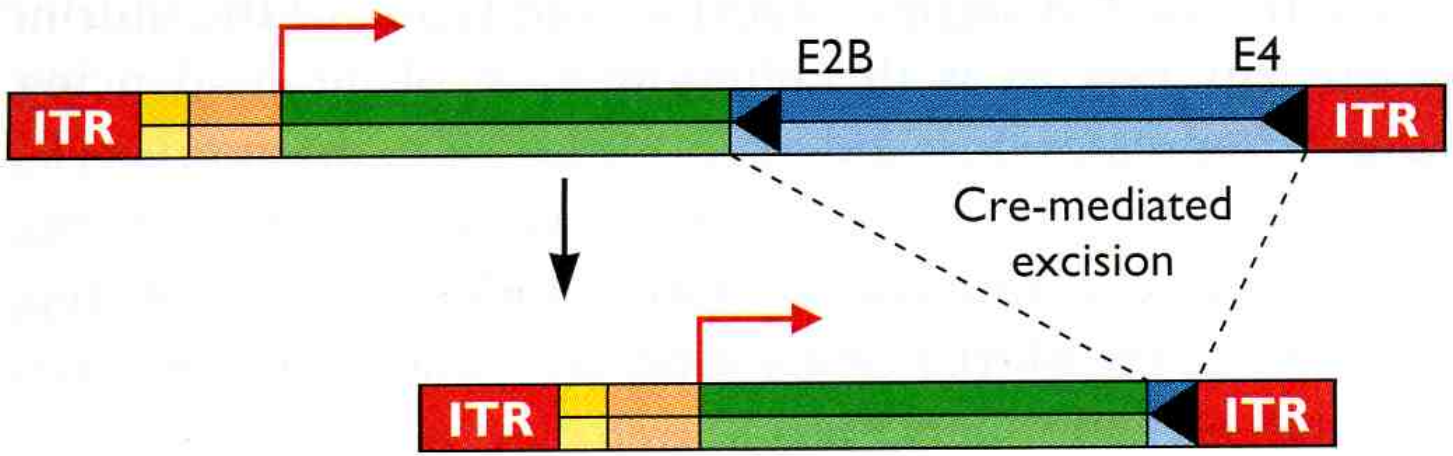
Gutless Adenovirus Vectors

- Based on the finding that all adenoviral proteins can be supplemented *in trans*, thus coding sequences can be eliminated to accomodate a transgene
- The only essential cis elements required for viral propagation and packaging are ITRs and signal (ψ)
- The gutless vector further reduces immunogenicity and enhances insert capacity
- The vector is transfected into 293 cells together with a mutant helper adenoviral vector (ψ-deleted)
- The gutless vector can infect different cells in vitro and in vivo, the expression can last up to 80 days

Gutless Adenovirus Vectors

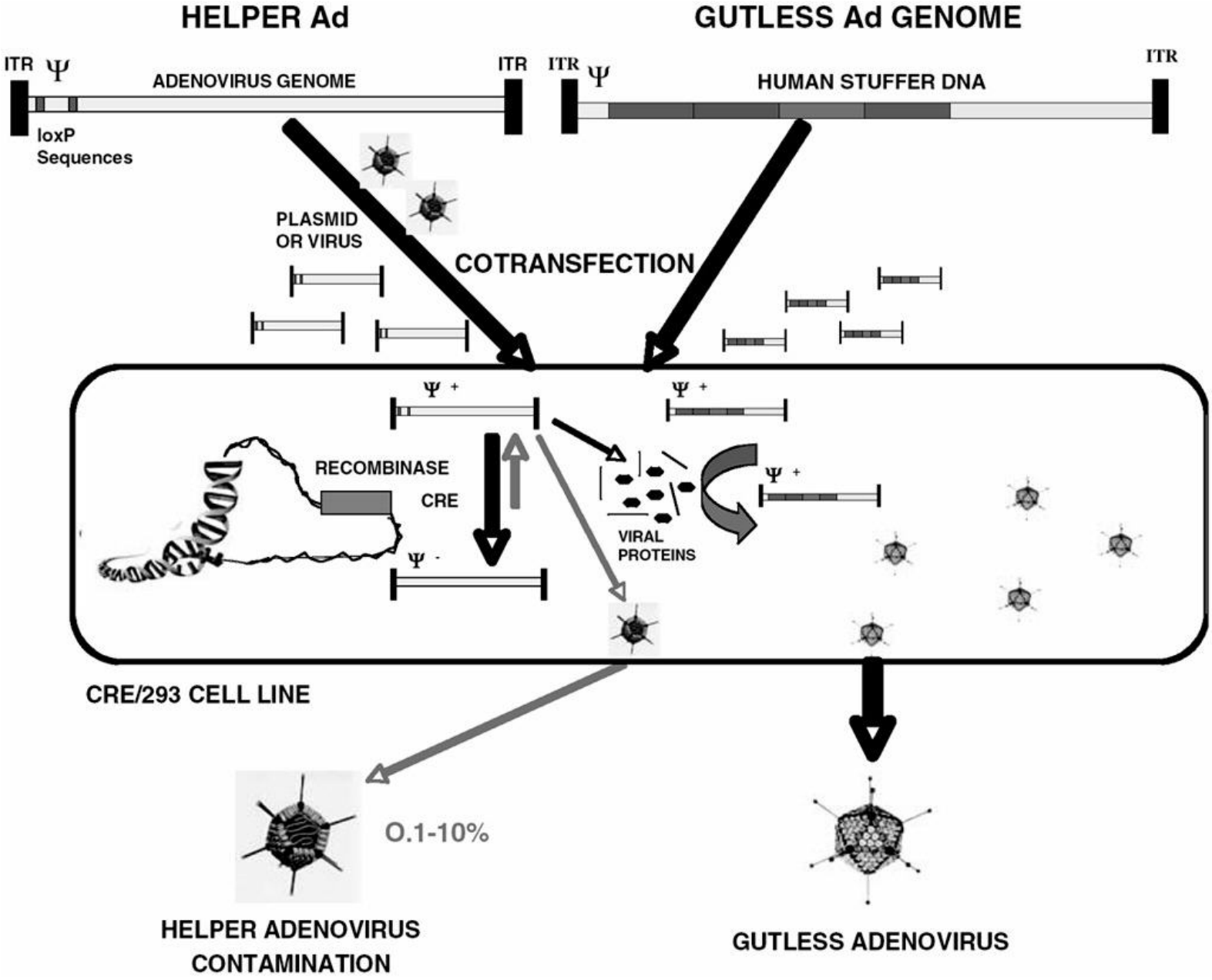


Gutless Adenovirus Vectors

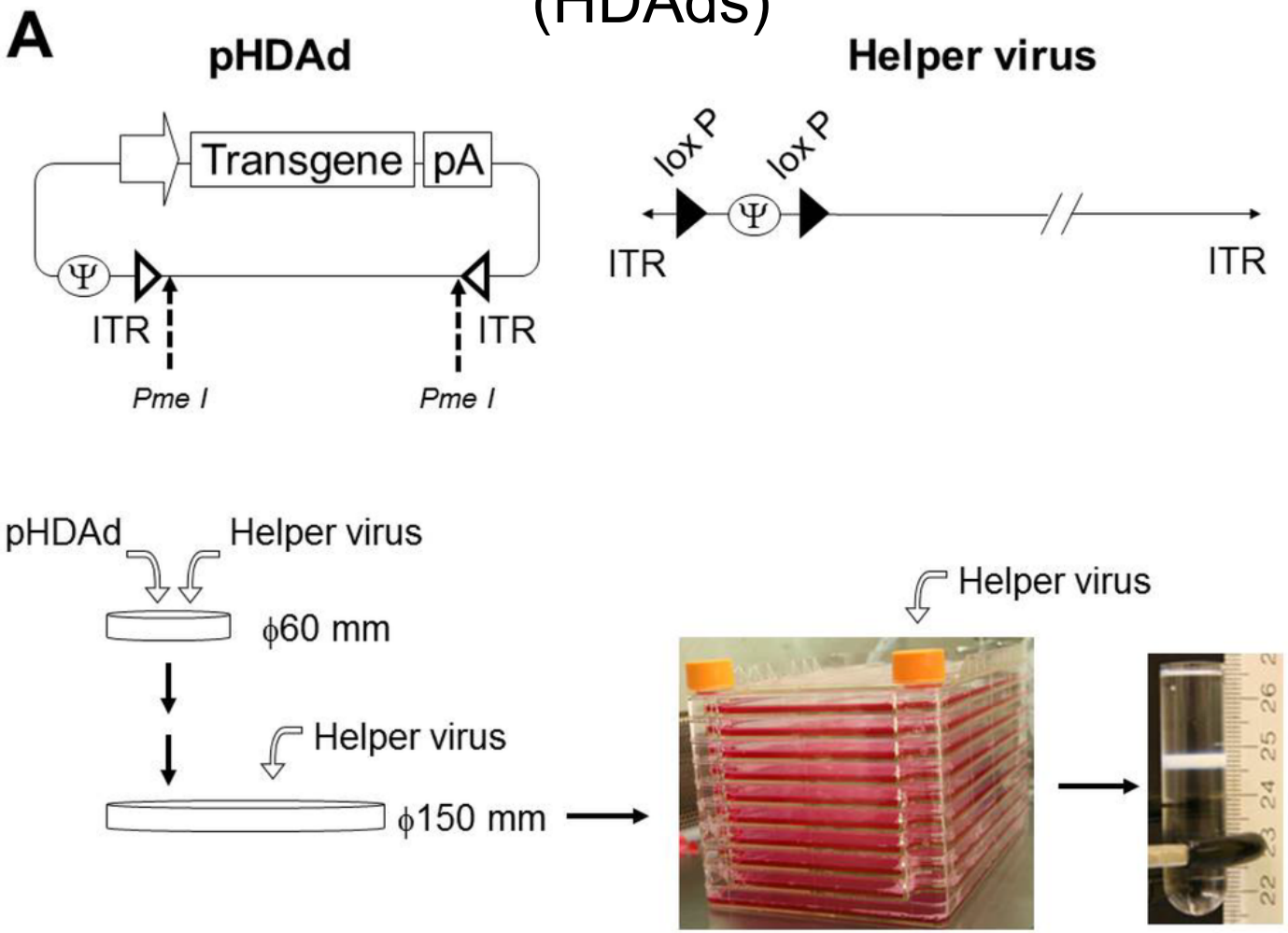


A gutless vector contains only the origin-of-replication-containing inverted terminal repeats (ITR), the packaging signal (ψ), the viral E4 transcription unit and the transgene with its promoter

Generation of Gutless Adenovirus Vectors



Overview of the production of Helper-dependent AdV vectors (HDAds)



Flow chart of the large-scale production of HDAd. The HDAd plasmid DNA (pHDAd) is linearized with the restriction enzyme *Pme*I before transfection to producer cell, 116 cell overexpressing Cre. HDAds are amplified by serial co-infection of helper virus and subjected to a 10-chamber cell factory. HDAd virions are purified from cell lysate by CsCl ultracentrifugation;

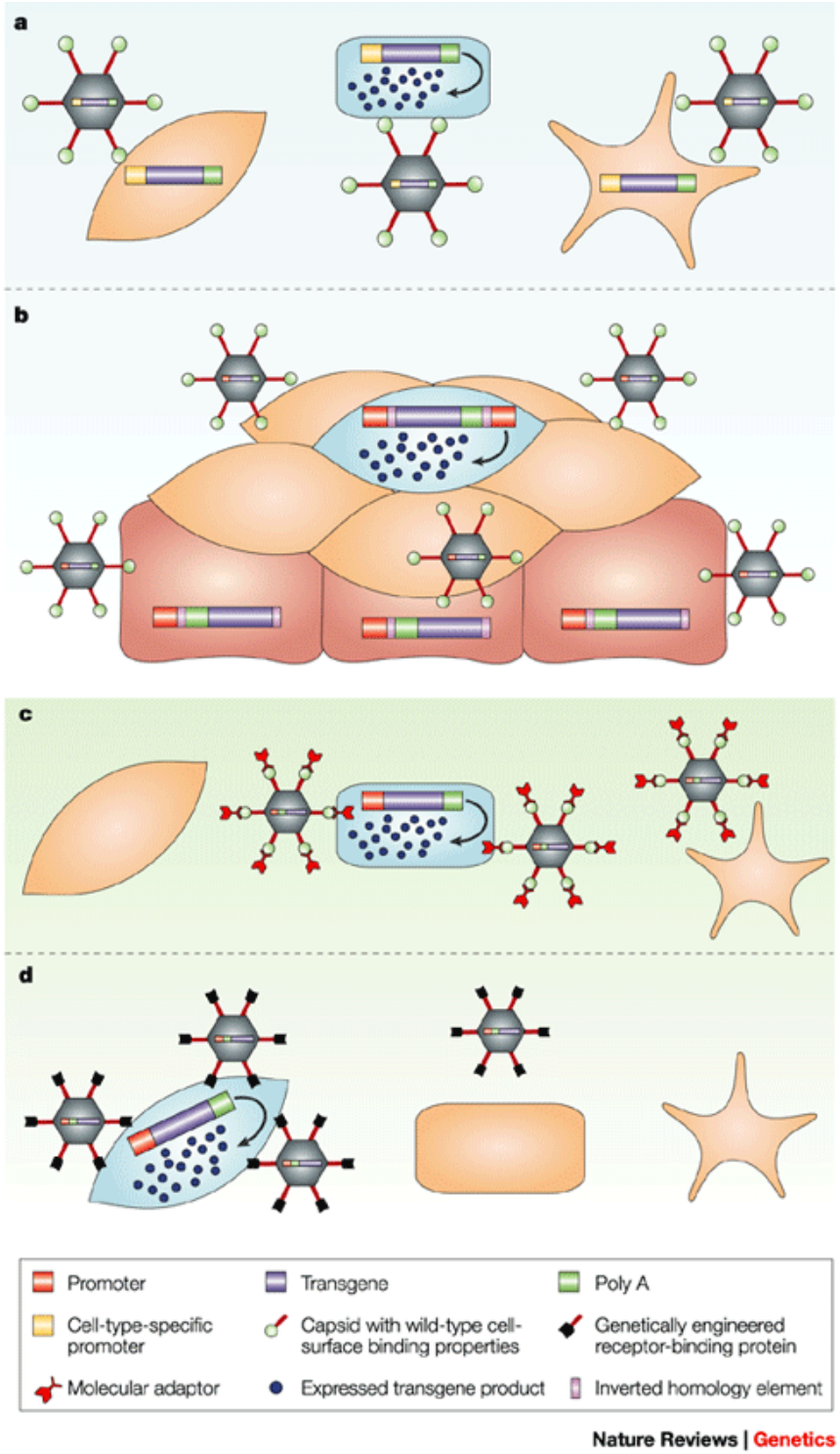
Strategies to achieve targeted gene expression from AdV

a) Transcriptional targeting is generally achieved by placing the transgene under the control of a cell-type-specific promoter.

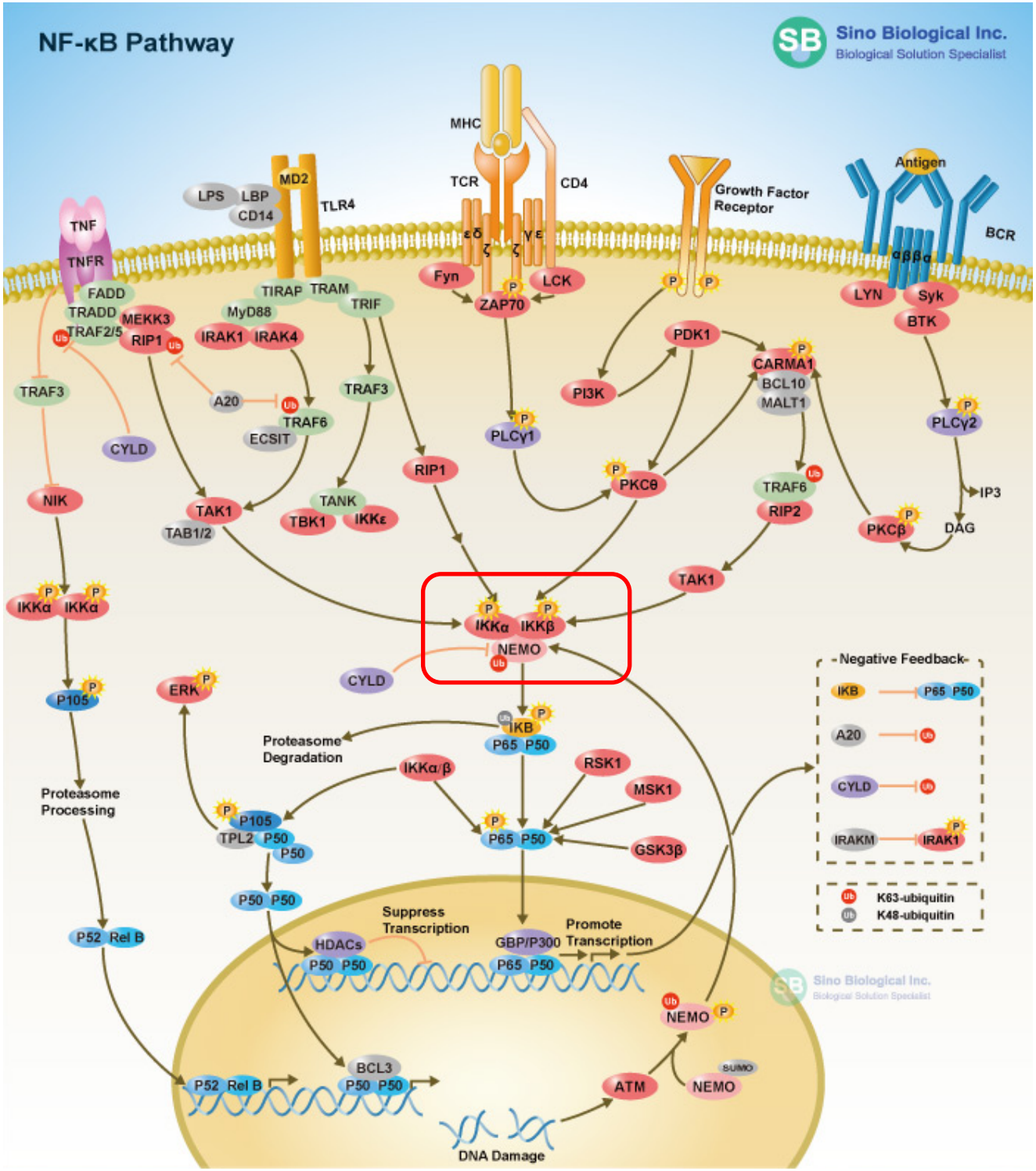
b) Tumour-specific transcriptional targeting from a conditionally replicating adenovirus vector.

c) Transductional targeting by redirecting the vector capsid to new cellular receptors using molecular adaptors (usually bi-specific antibodies), or by genetically altering receptor-binding proteins in the virus capsid so that they recognize and bind to alternative receptors

d) Combining transductional targeting with transcriptional targeting can further increase the efficacy and specificity of viral vector-mediated transduction



AdV vectors - a research lab application:
generation and validation of a dnIKK2-expressing 1st generation AdV vector


NF-κB Pathway
Sino Biological Inc.
Biological Solution Specialist
MHC
TCR
CD4
Antigen
BCR
Growth Factor Receptor
MD2
TLR4
LPS
LBP
CD14
TNF
TNFR
FADD
TRADD
TRAF2/5
MEKK3
RIP1
TIRAP
TRAM
MyD88
TRIF
IRAK1
IRAK4
TRAF3
A20
TRAF6
ECSIT
CYLD
NIK
TAK1
TAB1/2
TANK
TBK1
IKKε
RIP1
Fyn
ZAP70
LCK
PLCγ1
PI3K
PDK1
CARMA1
BCL10
MALT1
PKCθ
LYN
Syk
BTK
PLCγ2
IP3
DAG
PKCβ
TRAF6
RIP2
TAK1
IKKα
IKKα
IKKα
IKKβ
NEMO
CYLD
P105
ERK
IKB
P65 P50
Proteasome Degradation
Proteasome Processing
IKKα/β
RSK1
MSK1
GSK3β
P105
TPL2 P50
P50
P50 P50
P52 Rel B
Suppress Transcription
HDACs
P50 P50
GBP/P300
P65 P50
Promote Transcription
NEMO
ATM
NEMO
SUMO
BCL3
P50 P50
P52 Rel B
DNA Damage
- Negative Feedback -
IKB
P65 P50
A20
CYLD
IRAKM
IRAK1
K63-ubiquitin
K48-ubiquitin

Generation and validation of dnIKK2 adenoviruses: aminoacid sequence of human IKKβ

```
  1 mswspslttq tcgawemker lgtggfgnvi rwhnqetgeq iaikqcrqel sprnrerwcl
 61 eiqimrrlth pnvvaardvp egmqnlapnd lpllameycq ggdlrkylnq fenccglreg
121 ailtllsdia salrylhenr iihrdlkpen ivlqqgeqrl ihkiidlgya keldqgslct
181 sfvgtlqyla pelleqqkyt vtvdywsfgt lafecitgfr pflpnwqpvq whskvrqkse
241 vdivvsedln gtvkfssslp ypnnlnsvla erlekwlqlm lmwhprqrgt dptygpngcf
301 kalddilnlk lvhilnmvtg tihtypvted eslqslkari qqdtgipeed qellqeagla
361 lipdkpatqc isdgklnegh tldmdlvflf dnskityetq isprpqpesv scilqepkrn
421 laffqlrkvw gqvwhsiqtl kedcnrlqqg qraammnllr nnsclskmkn smasmsqqlk
481 akldffktsi qidlekyseq tefgitsdkl llawremeqa velcgrenev kllvermmal
541 qtdivdlqrs pmgrkqggtl ddleeqarel yrrlrekprd qrtegdsqem vrlllqaiqs
601 fekkvrviyt qlsktvvckq kalellpkve evvslmnede ktvvrlqekr qkelwnllki
661 acskvrgpvs gspdsmnasr lsqpgqlmsq pstasnslpe pakkseelva eahnlctlle
721 naiqdtvreq dqsftaldws wlqteeeehs cleqas
```

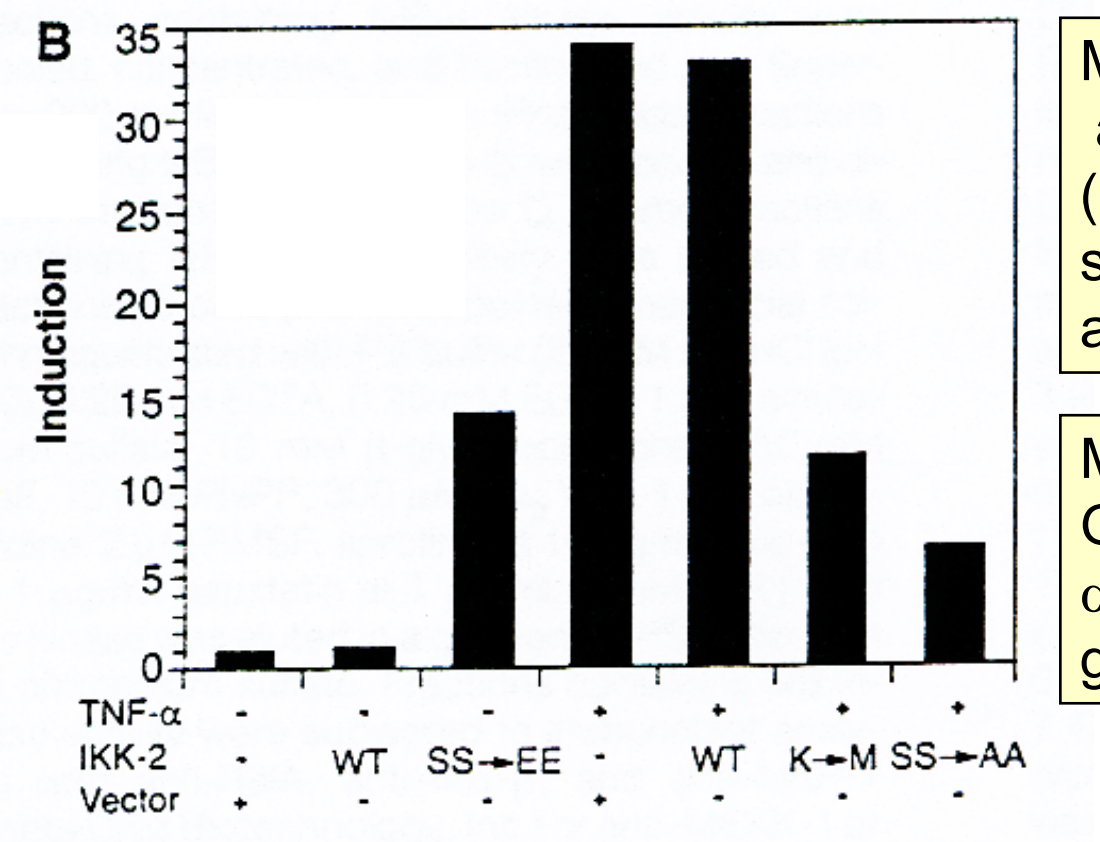
Mercurio,F., Zhu,H., Murray,B.W., Shevchenko,A., Bennett,B.L., Li,J.W., Young,D.B., Barbosa,M., Mann,M., Manning,A. and Rao,A. *IKK-1 and IKK-2: cytokine-activated IkappaB kinases essential for NF-kappaB activation.* Science 278, 860-866 (1997)

Suggested reading: Science, 278, 860- 866, 1997

IKK-1 And IKK-2: Cytokine-Activated IκB Kinases Essential for NF-κB Activation

Frank Mercurio,* Hengyi Zhu, Brion W. Murray, Andrej Shevchenko, Brydon L. Bennett, Jian wu Li, David B. Young, Miguel Barbosa, Matthias Mann,

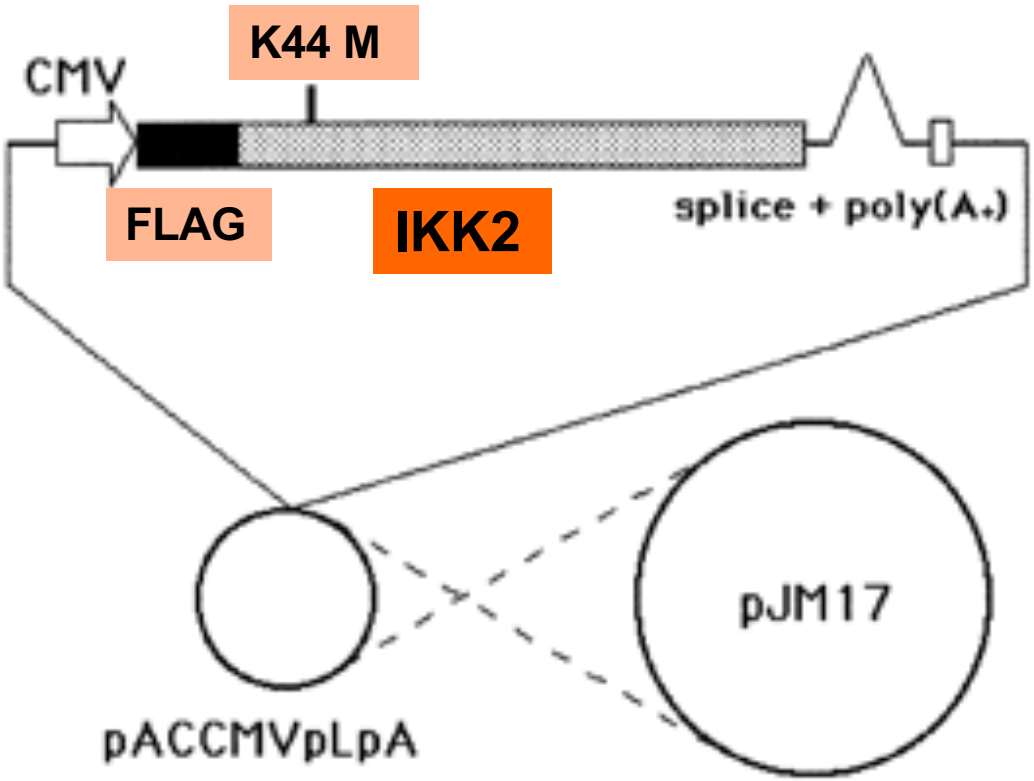
Anthony Manning, Anjana Rao



Mutation of Lys^{44} to Met (K44M) and of Ser^{177} and Ser^{181} to Ala (S177A, S181A) inhibits TNF-α-stimulated NF-κB-mediated gene activation

Mutation of Ser^{177} and Ser^{181} to Glu (S177E, S181E) mimics TNF-α-stimulated NF-κB-mediated gene activation

Generation and validation of dnIKK2 adenoviruses: generation of pACCMVdnIKK2



EcoR I

For: 5'-AAAAGAATTCGCCACCATGGACTACAAGGACGACGATGACAAGAGCTGGTCACCTTCCCTG-3'

Met Asp Tyr Lys Asp Asp Asp Asp Lys Ser Trp Ser Pro Ser Leu

Generation and validation of dnIKK2 adenoviruses: co-transfection of 293 cells

•Plate 5 x10^5 293 cells in 6 cmϕ dishes in DMEM +10% FCS

	A	**B**	**C**
pJM17 (1.6 μg/μl)	6.2 μl	6.2 μl	6.2 μl
pACCMVdnIKK2 (1.5 μg/μl)	-	9.5 μl	-
pACCMVLacZ (2.0 μg/μl)	-	-	5μl
H_20 to 226 μl			
1 M$CaCl_2$	74 μl	74 μl	74 μl
2 xHBS	300 μl	300 μl	300 μl

•Glycerol shock –15% for 1 min after 6 h.
•Wash and incubate in growth medium for 6 days
•Collect supernatant and scrape off cells. Lysis by freezing and thawing. Save supernatants and store at –80° C.

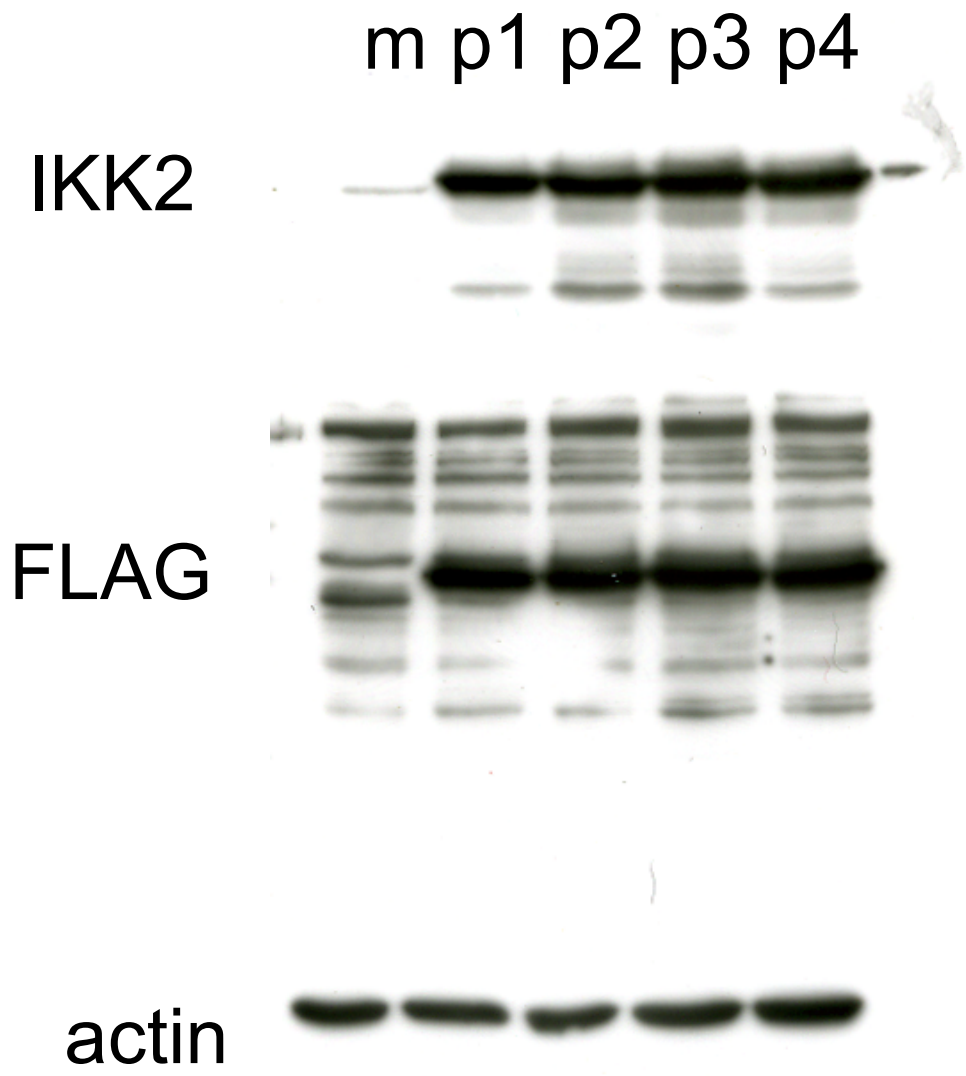
Generation and validation of dnIKK2 adenoviruses: isolation and screening of adeno plaque isolates from vector rescues

•Infect subconfluent 293 cell monolayers with 1 ml containing viral stock dilutions between 10^{-3} and 10^{-9}. Agarose overlay.

•After 4-6 d pick well isolated plaques and amplify on 293 monolayers. Titrate viral stocks P1 on 293 monolayers.

•Infect target cells with P1 stocks and screen for dnIKK2 expression by immunoblotting (FLAG and pIKK2)

Generation and validation of dnIKK2 adenoviruses:
screening plaques for dnIKK2 expression



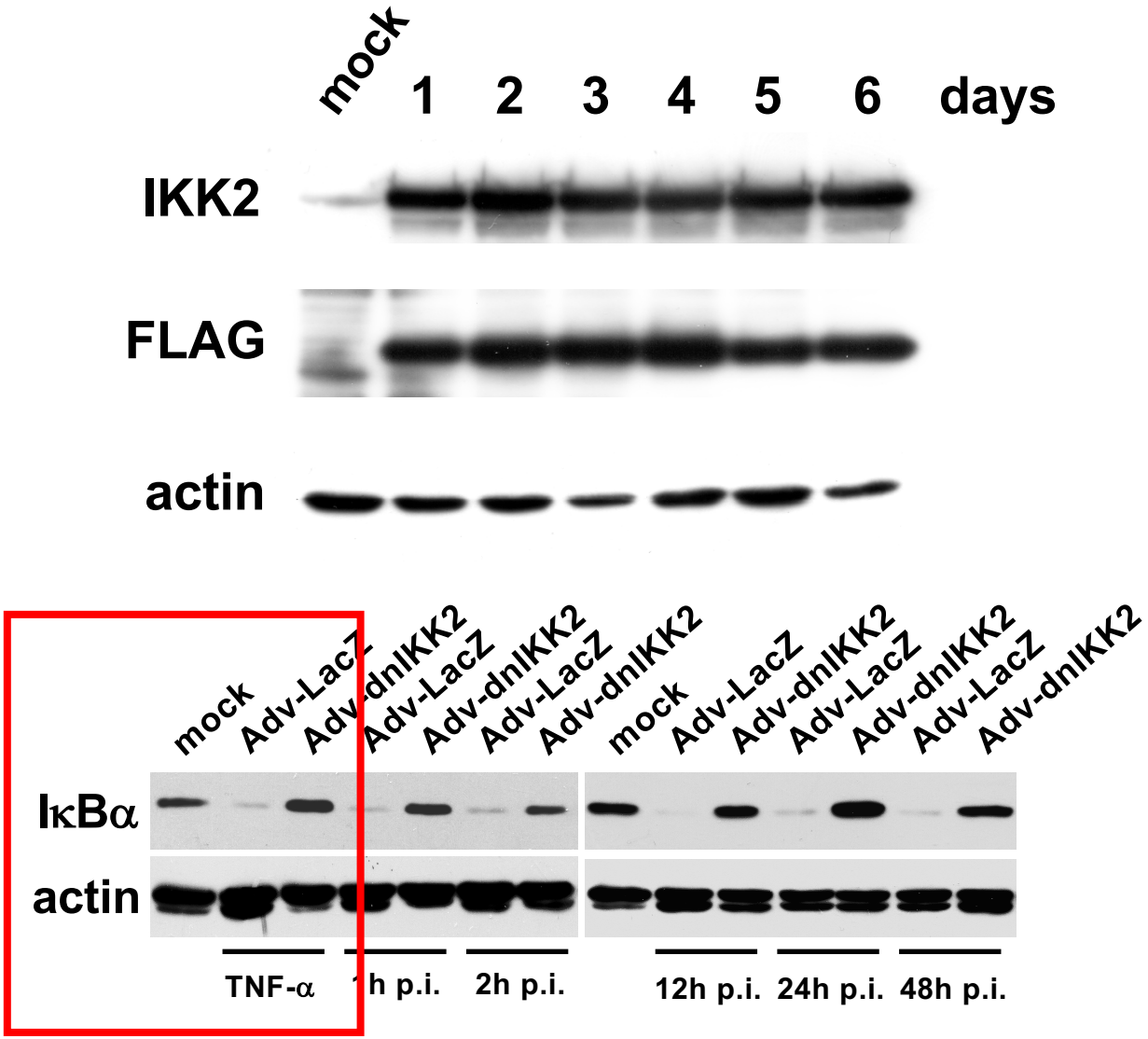
Generation and validation of dnIKK2 adenoviruses: amplification of dnIKK2 adenoviral clones

- Infect subconfluent 293 cell monolayers (4.5 x10^6 cells/175 cm^2 flask) at a MOI of 1 PFU/cell.

- Recovery supernatants and scrape off cells. Lysis by freezing and thawing. Titrate viral stocks P2 on 293 cells.

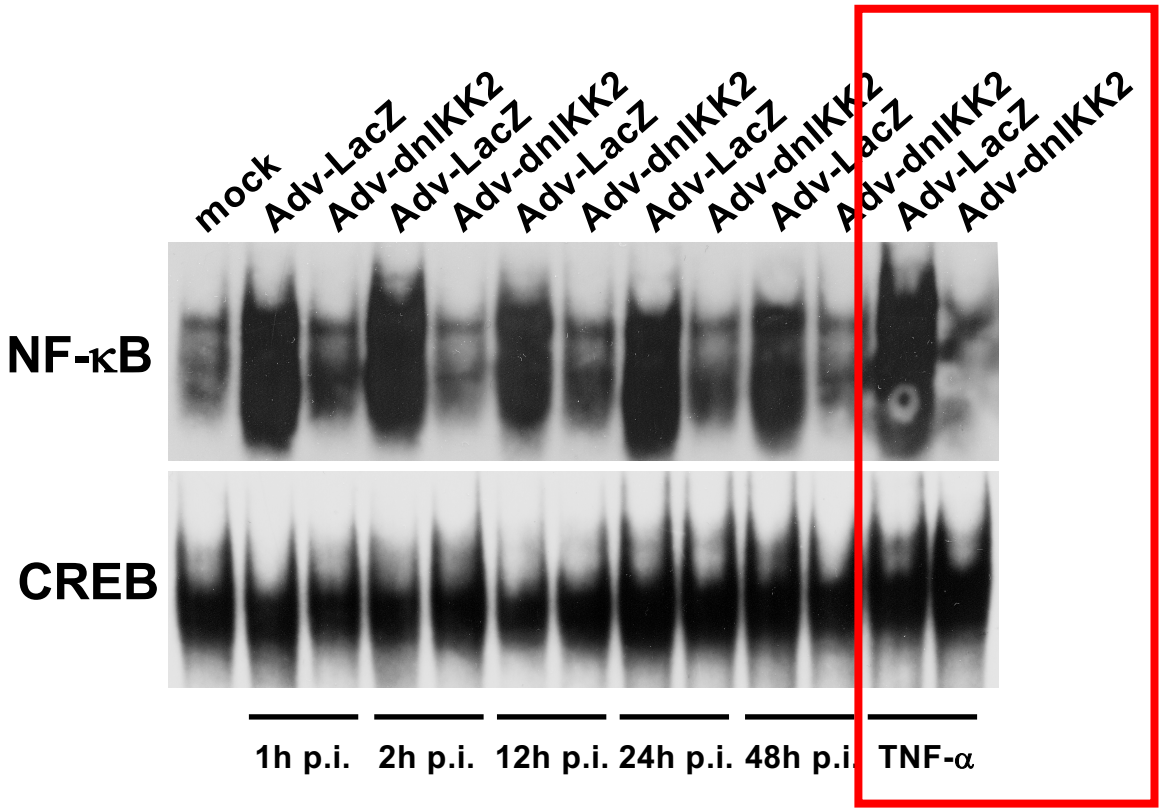
- Infect target cells with P2 stocks (MOI 5 to 500) and characterize dnIKK2 expression and the impairment of endogenous IKK2 functions (NF-κB activation and viral gene expression)

Expression and activity of the dnIKK2 protein in HUVEC



Caposio *et al.*, 2007

Effects of dnIKK2 expression on NF-κB activation in HUVEC



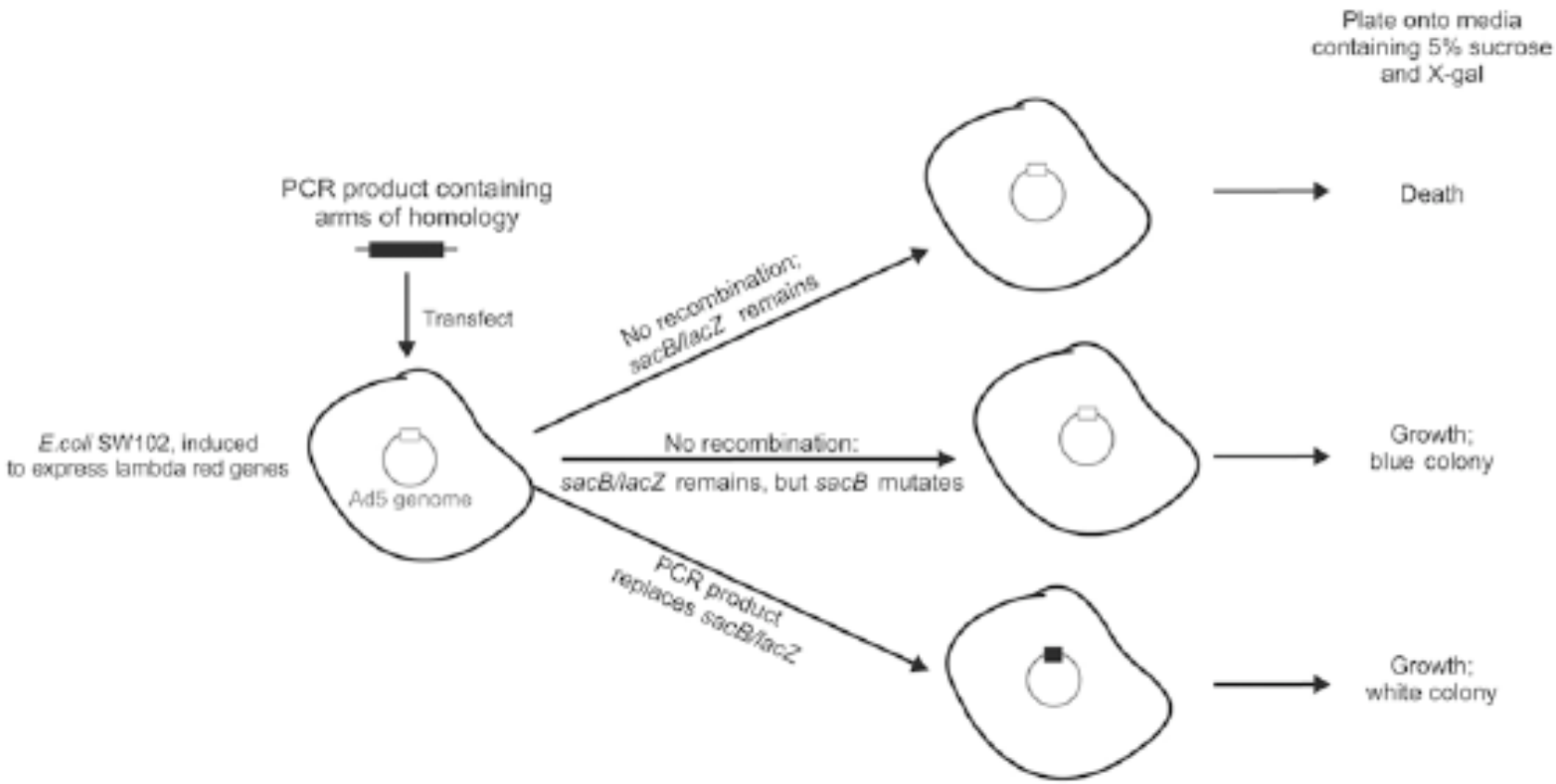
Caposio *et al.*, 2007

AdV vectors - a research lab application: generation of a GFP-expressing AdZ vector by the recombineering technology

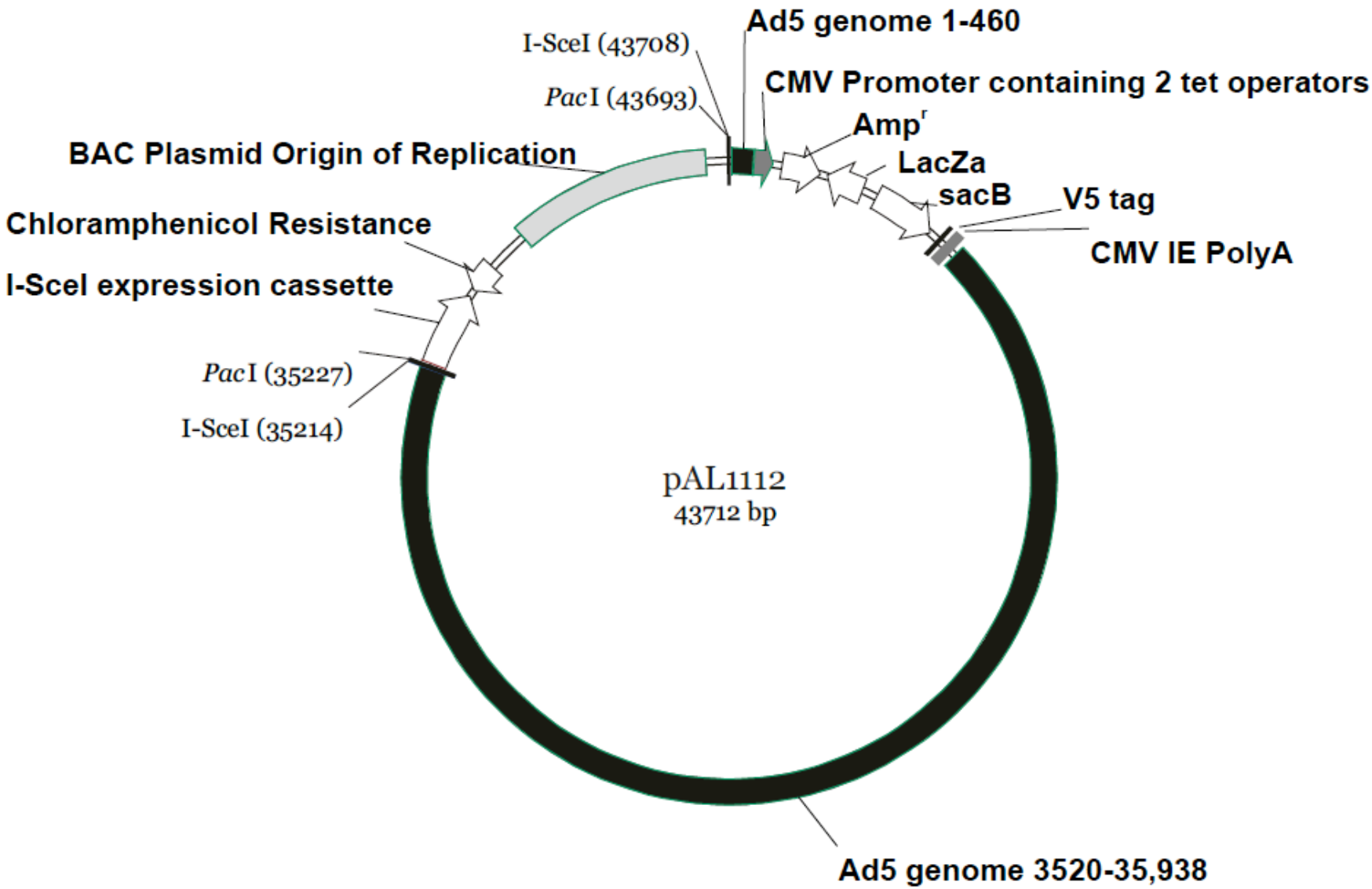
Re-engineering adenovirus vector systems to enable high-throughput analyses of gene function

Richard J. Stanton, Brian P. McSharry, Melanie Armstrong, Peter Tomasec, and Gavin W.G. Wilkinson

BioTechniques 45:659-668 (December 2008)
doi 10.2144/000112993



The AdZ adenovirus cloning system



The AdZ adenovirus cloning system

AdZ-5 vectors

These vectors are based on wildtype adenovirus type 5 virus kindly provided by Vivien Mautner from Birmingham University.

All vectors are Ad5 ΔE1 (461-3519bp), ΔE3 (28131-30,800bp) (deletion numbering based on the prototype Ad-5 sequence (AC000008)).

Vector	*Tet-operators in promoter?*	*Self Excising?*	*Tag*
pAdZ5-CV5	Yes	Yes	C terminal V5
pAdZ5-NV5	Yes	Yes	N terminal V5
pAdZ5-NGFP	Yes	Yes	N terminal eGFP
pAdZ5-CGFP	Yes	Yes	C terminal eGFP
pAdZ5-CCherry	Yes	Yes	C terminal mCherry
pAdZ5-mIR155	Yes	Yes	miR-155 arms of homology (for cloning shRNAs)
pAdZ5-CStrep2	Yes	Yes	C terminal StrepII tag
pAdZ5-CV5-NT	No	Yes	C terminal V5
pAdZ5-CGFP-NT	No	Yes	C terminal eGFP

The AdZ adenovirus cloning system: PCR your gene

Primer design: 100 bp primers with 20bp homology to the sequence to be inserted at the 3’ end and 80 bp arms of homology to target insertion site on the BAC

If cloning your PCR product with no tag, use the following primers, and any of the vectors:

To your forward primer (this does not include a Kozak-optimized sequence, you may want to add your own):

5′AACCGTCAGATCGCCTGGAGACGCCATCCACGCTGTTTTGACCTCCATAGAAGAC
ACCGGGACCGATCCAGCCTGGATCC-**YOUR-PRIMER-HERE**-3′

To your downstream primer:

5′GGCGTGACACGTTTATTGAGTAGGATTACAGAGTATAACATAGAGTATAATATAG
AGTATACAATAGTGACGTGGGATCC-**YOUR-PRIMER-HERE**-3′

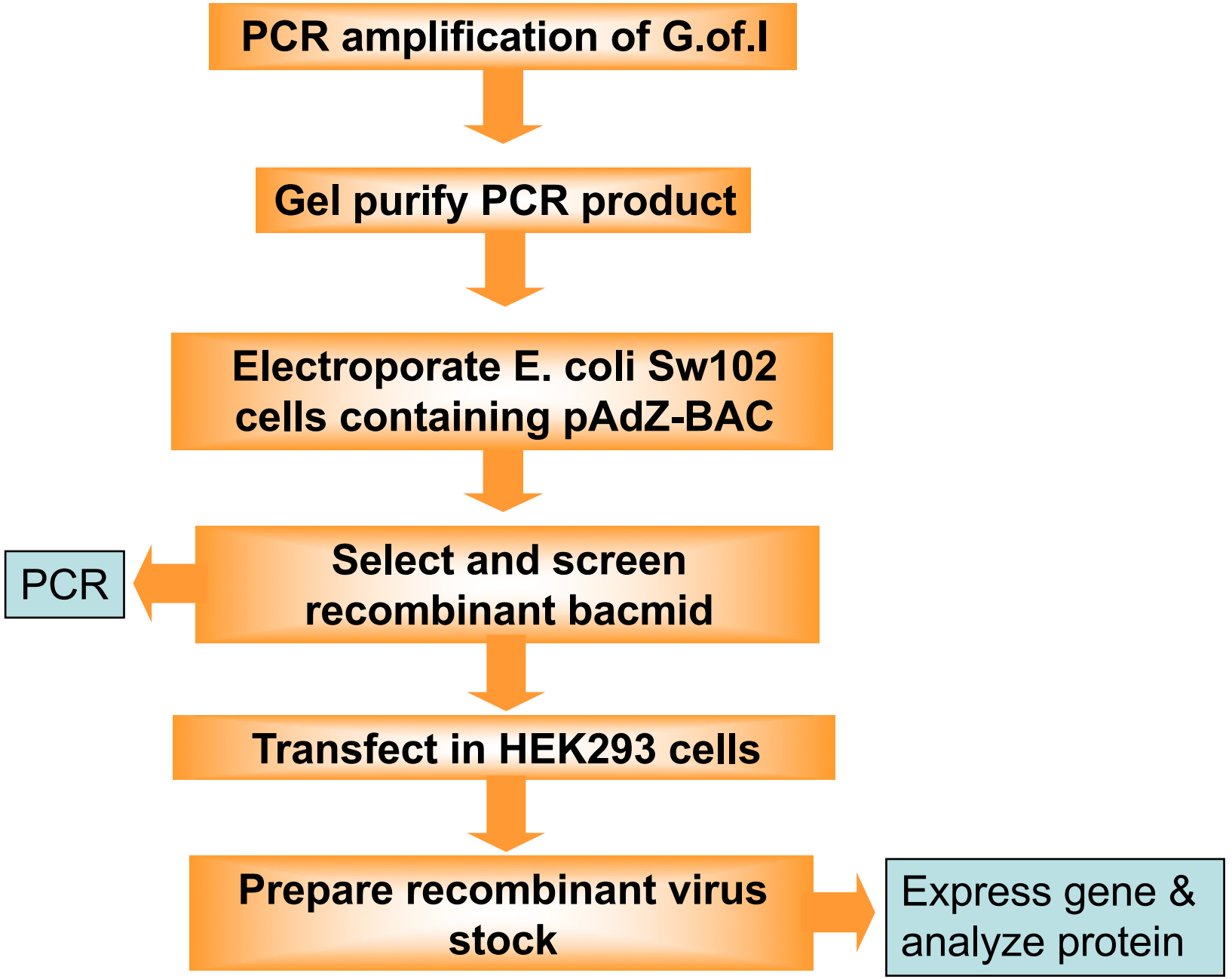
Cloning with a C terminal V5 tag

pAdZ5-CV5 and pAdZ5-CV5-NT contain a C-terminal V5 tag. If you want to clone a gene with this tag, use the same arm of homology as for untagged genes for the forward primer, and the following arm of homology for the reverse primer (tag is in bold, linker in italics):

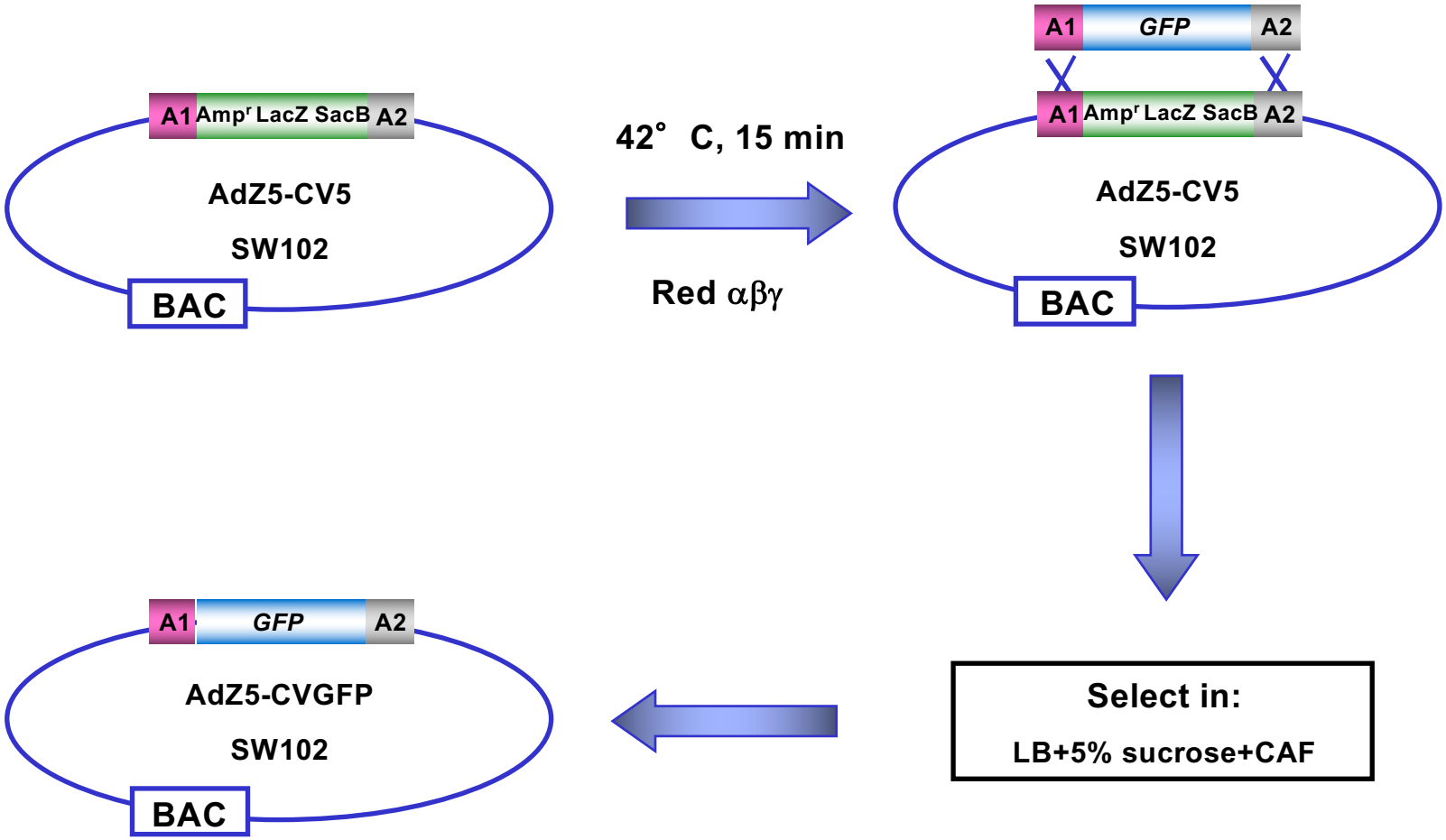
5′ - TATAGAGTATACAATAGTGACGTGGGATCC**CTACGTAGAATCAAGACCTAGGAGCGGGTTA**
***ThrSerAspLeuGlyLeuLeuProAsn

GGGATTGGCTTACC*AGCGCT*-**YOUR-PRIMER-HERE**-3′
ProIleProLysGlyAlaSer

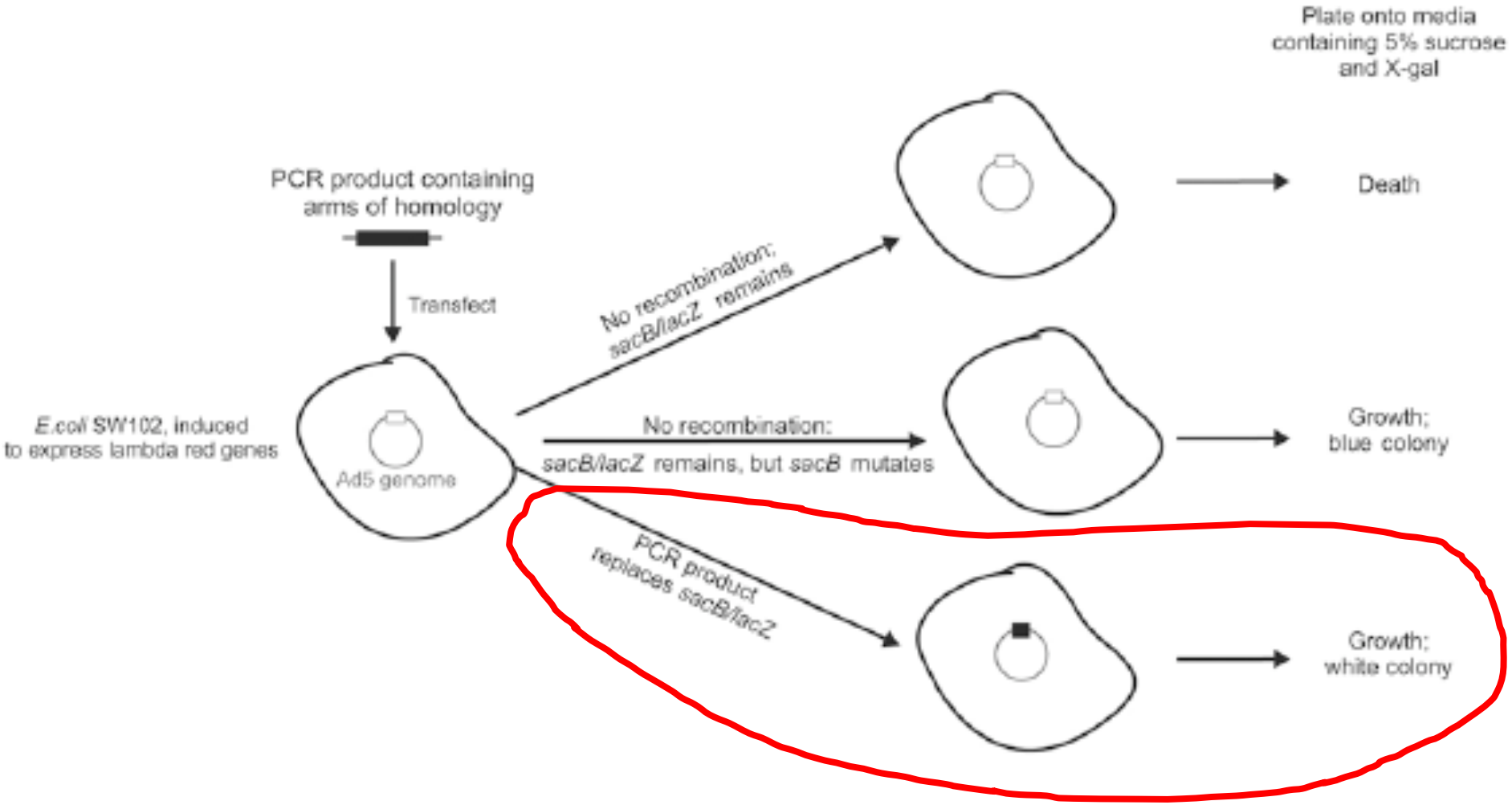
Flow Chart for the AdZ Expression System



Cloning strategy in AdZ5

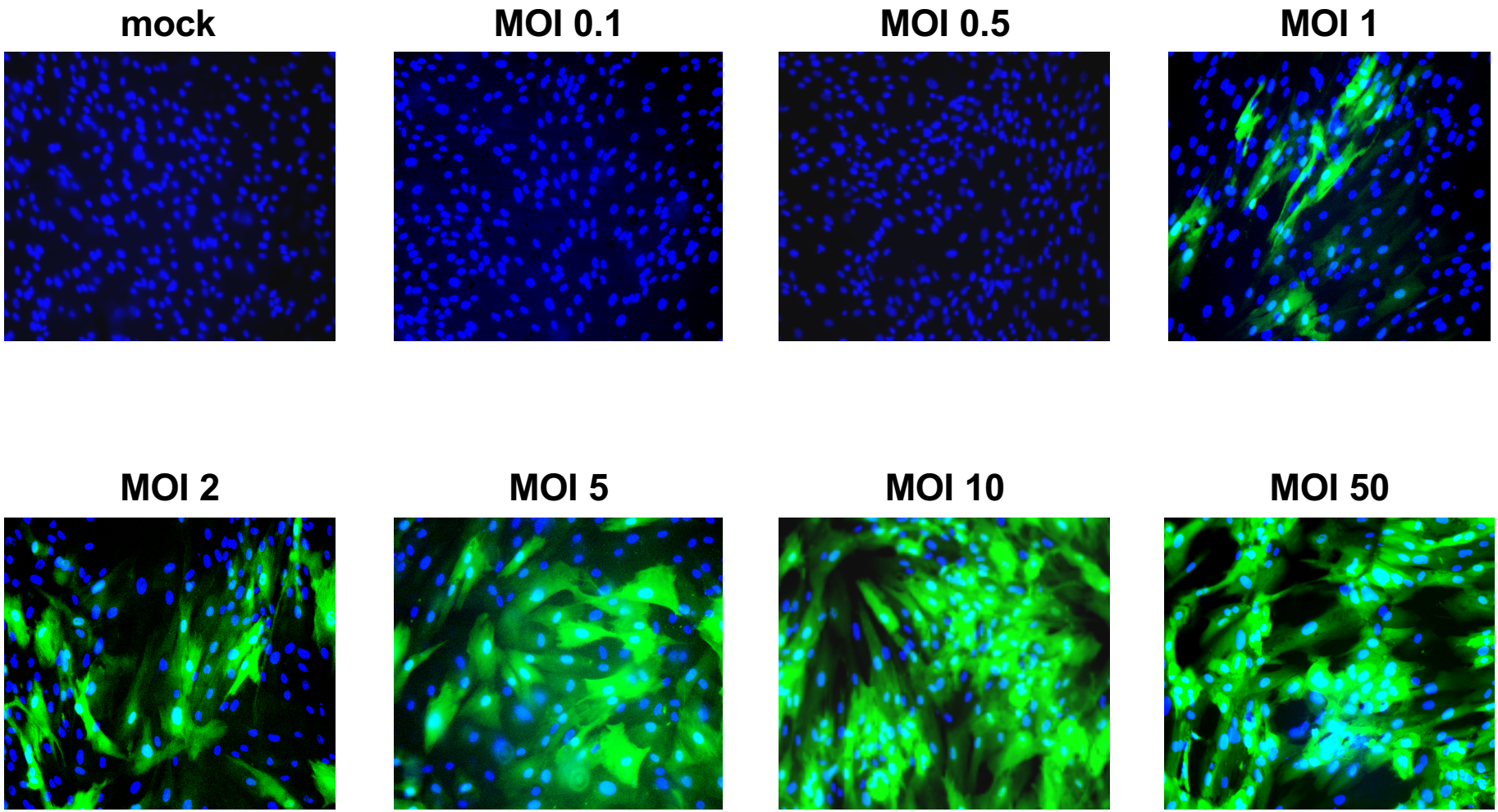


The AdZ adenovirus cloning system: selection of recombinants



The *sacB* gene encodes the secreted enzyme levansucrase. The enzyme catalyzes the formation of high molecular weight fructose polymers. If this gene is expressed in a Gram-negative cell it will accumulate in the periplasm and catalyze the formation of large polymers. It is thought that the accumulation of these polymers in the periplasm interferes with metabolism of these strains. Thus, the *sacB* gene is lethal to a Gram-negative cell growing on a medium containing 5% sucrose

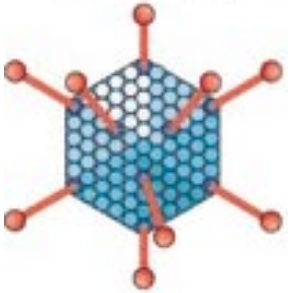
Expression of GFP (48 h p.i.) in HELFs infected with AdZ-GFP

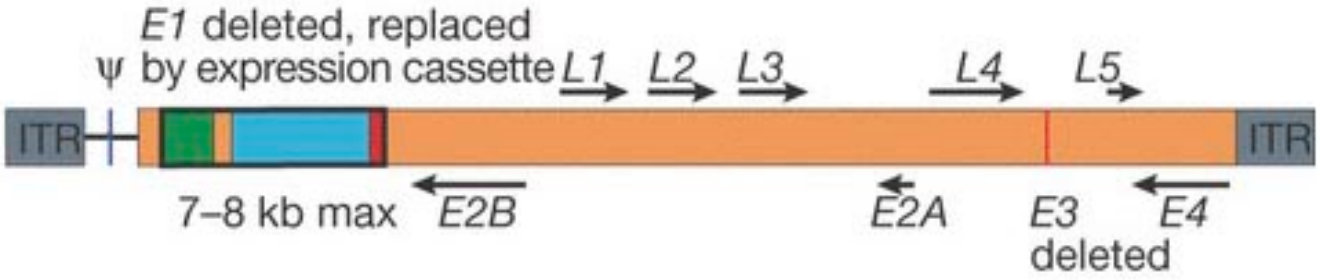


#F140

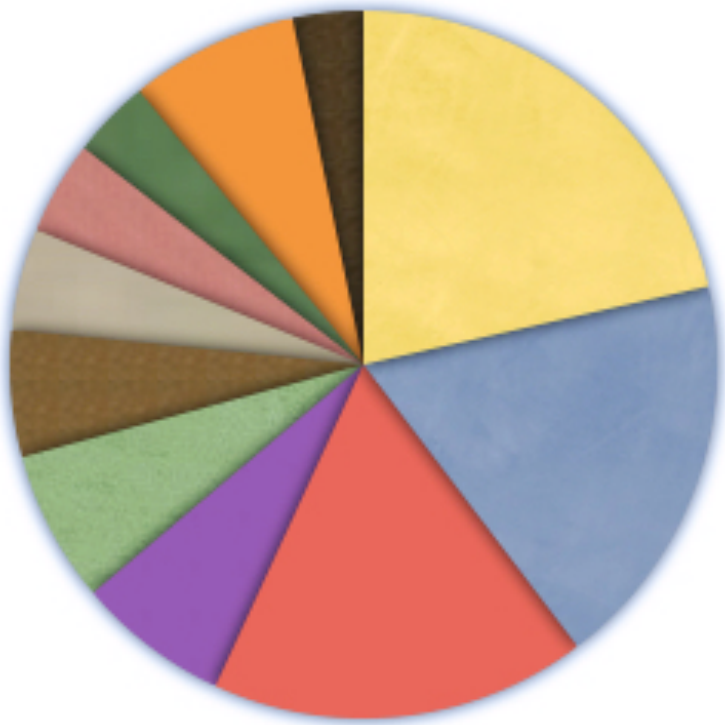
Adenovirus Vectors and Gene Therapy

Adenovirus (~36 kb genome)





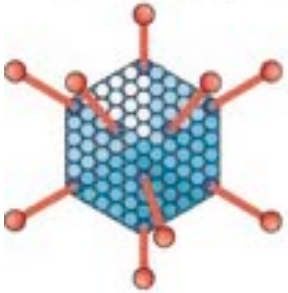
Vectors Used in Gene Therapy Clinical Trials

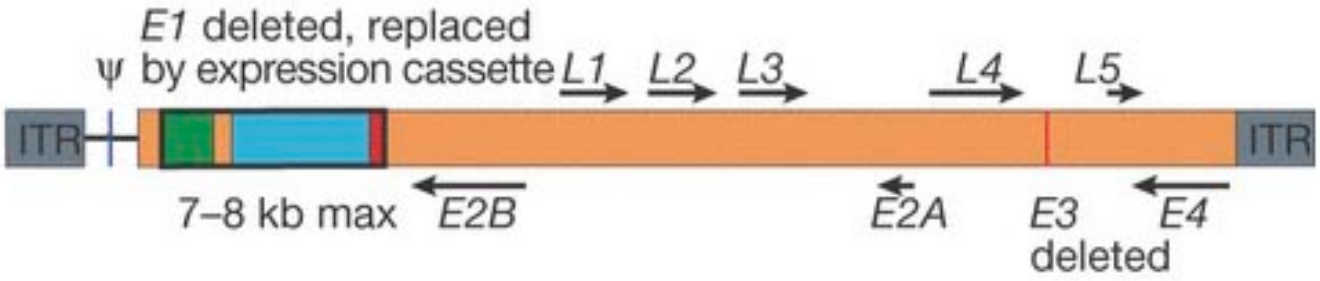


- Adenovirus 21.4% (n=532)
- Retrovirus 18.2% (n=452)
- Naked/Plasmid DNA 17.2% (n=427)
- Adeno-associated virus 7.0% (n=173)
- Vaccinia virus 6.9% (n=172)
- Lentivirus 5.8% (n=144)
- Lipofection 4.6% (n=115)
- Poxvirus 4.3% (n=106)
- Herpes simplex virus 3.6% (n=89)
- Other vectors 7.7% (n=191)
- Unknown 3.2% (n=80)

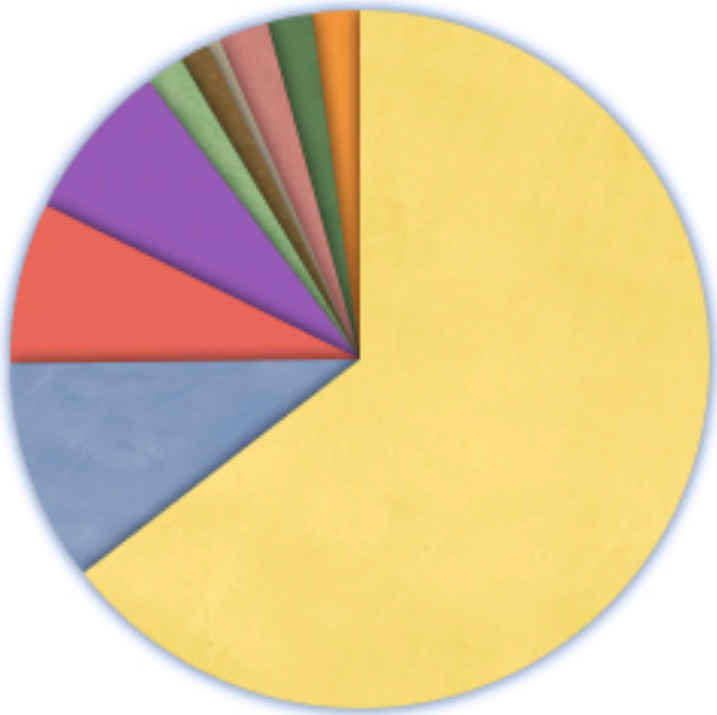
www.wiley.co.uk/genmed/clinical

Adenovirus (~36 kb genome)





Indications Addressed by Gene Therapy Clinical Trials



- Cancer diseases 64.5% (n=1554)
- Monogenic diseases 10.3% (n=248)
- Infectious diseases 7.5% (n=180)
- Cardiovascular diseases 7.4% (n=178)
- Neurological diseases 1.8% (n=43)
- Ocular diseases 1.4% (n=33)
- Inflammatory diseases 0.6% (n=14)
- Other diseases 2.3% (n=56)
- Gene marking 2.1% (n=50)
- Healthy volunteers 2.2% (n=53)

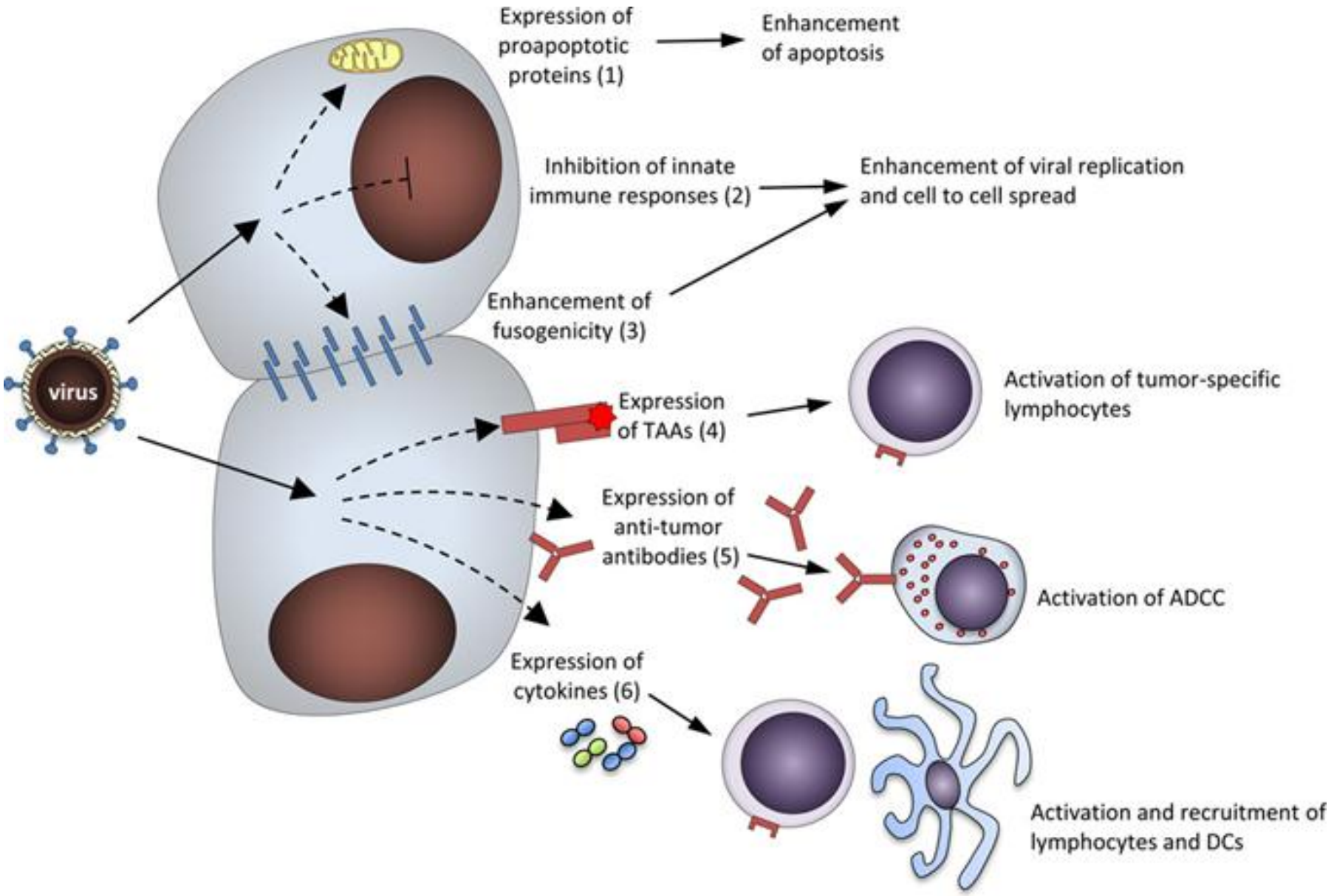
www.wiley.co.uk/genmed/clinical

Table 1. List of functional molecules used with Ad-based vectors in clinical trials in the USA.

Function	Gene	Cancer type	Clinical trial Code
Cytokine	*IFNβ*	Pleural Mesothelioma, Colorectal Carcinoma	NCT00299962, NCT00107861
	IFNα2b	Mesothelioma	NCT01212367
	IFNγ	B-Cell Lymphoma	NCT00394693
	IL-12	Breast Cancer, Colorectal Cancer, Prostate Cancer, Melanoma, Neoplasms	NCT00849459, NCT00072098, NCT00406939, NCT01397708, NCT00110526
	IL-2	Neuroblastoma	NCT00048386
	MDA-7 (IL-24)	Malignant Melanoma	NCT00116363
	TNFα	Esophageal Cancer, Pancreatic Cancer	NCT00051480, NCT00051467
	GM-CSF	Malignant Solid Tumor	NCT01598129
	FLt3L	Malignant Glioma	NCT01811992
Tumor suppressor	*p53*	Squamous Carcinoma, Lip and Oral Cavity Cancer, Head and Neck Carcinoma, Brain Tumors, Liver Cancer, Ovarian Cancer, Lung Cancer, Bladder Cancer, Breast Cancer	NCT00041613, NCT00064103, NCT00004041, NCT00003147, NCT00003880, NCT00003649, NCT00003167
	REIC/Dkk-3	Prostate cancer	NCT01197209
	RTVP-1	Prostatic Neoplasms	NCT00403221
Suicide molecule	*TK*	Malignant Glioma, Brain Tumors, Hepatocellular Carcinoma, Ovarian Cancer, Melanoma, Pancreatic Cancer	NCT01811992, NCT00002824, NCT00844623, NCT00638612, NCT00005057
Costimulatory molecule	*CD40L*	Malignant Melanoma, Bladder Cancer, Breast Cancer, Neoplasms, Leukemia, Lymphoma	NCT01455259, NCT00706615, NCT00504322, NCT00942409
Anti-angiogenic molecule	*Endostatin*	Head and Neck Squamous Carcinoma, Advanced solid tumors	NCT00634595, NCT00262327
Antigen	*PSA*	Prostate cancer	NCT00583752

Adenovirus Vectors and Cancer Virotherapy

Adenoviral Vectors and Cancer Therapy



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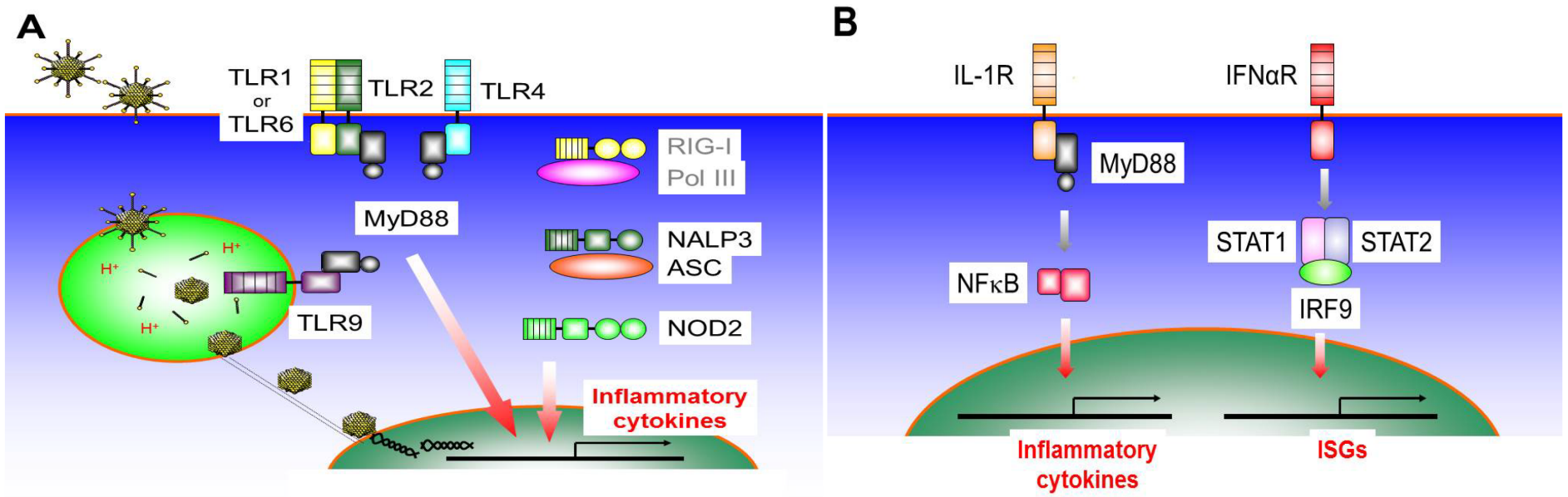
Anti-angiogenic molecule	*Endostatin*	Head and Neck Squamous Carcinoma, Advanced solid tumors	NCT00634595, NCT00262327
Antigen	*PSA*	Prostate cancer	NCT00583752

The Innate Inflammatory Response to AdV Vectors may Contribute to Cancer Immunotherapy



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	REIC/Dkk-3	Prostate cancer	NCT01197209
	RTVP-1	Prostatic Neoplasms	NCT00403221
Suicide molecule	*TK*	Malignant Glioma, Brain Tumors, Hepatocellular Carcinoma, Ovarian Cancer, Melanoma, Pancreatic Cancer	NCT01811992, NCT00002824, NCT00844623, NCT00638612, NCT00005057
Costimulatory molecule	*CD40L*	Malignant Melanoma, Bladder Cancer, Breast Cancer, Neoplasms, Leukemia, Lymphoma	NCT01455259, NCT00706615, NCT00504322, NCT00942409
Anti-angiogenic molecule	*Endostatin*	Head and Neck Squamous Carcinoma, Advanced solid tumors	NCT00634595, NCT00262327
Antigen	*PSA*	Prostate cancer	NCT00583752

An example of cancer gene therapy with AdV vectors

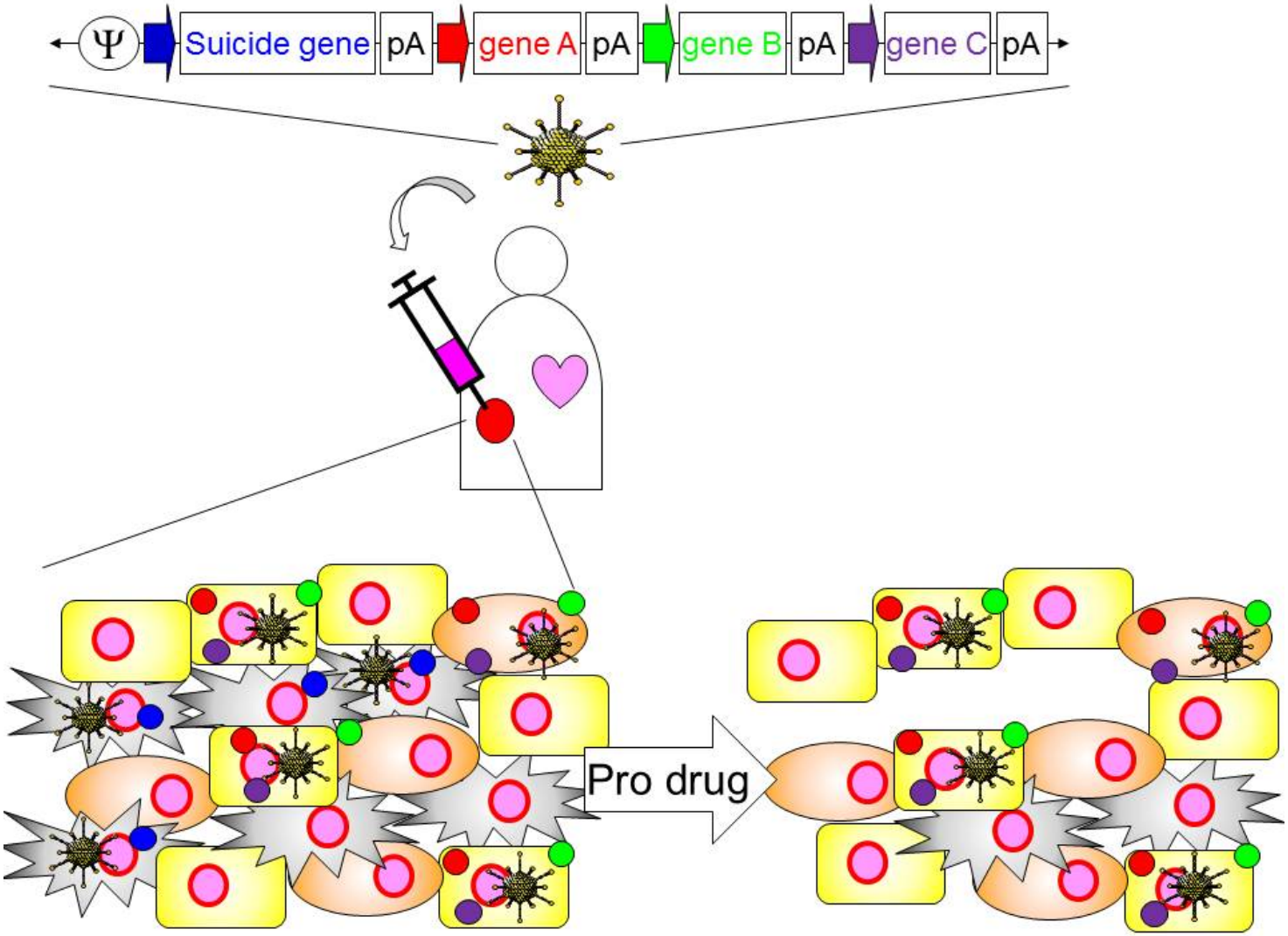

Table 1. List of functional molecules used with Ad-based vectors in clinical trials in the USA.

Function	Gene	Cancer type	Clinical trial Code
Cytokine	*IFNβ*	Pleural Mesothelioma, Colorectal Carcinoma	NCT00299962, NCT00107861
	IFNα2b	Mesothelioma	NCT01212367
	IFNγ	B-Cell Lymphoma	NCT00394693
	IL-12	Breast Cancer, Colorectal Cancer, Prostate Cancer, Melanoma, Neoplasms	NCT00849459, NCT00072098, NCT00406939, NCT01397708, NCT00110526
	IL-2	Neuroblastoma	NCT00048386
	MDA-7 (IL-24)	Malignant Melanoma	NCT00116363
	TNFα	Esophageal Cancer, Pancreatic Cancer	NCT00051480, NCT00051467
	GM-CSF	Malignant Solid Tumor	NCT01598129
	FLt3L	Malignant Glioma	NCT01811992
Tumor suppressor	*p53*	Squamous Carcinoma, Lip and Oral Cavity Cancer, Head and Neck Carcinoma, Brain Tumors, Liver Cancer, Ovarian Cancer, Lung Cancer, Bladder Cancer, Breast Cancer	NCT00041613, NCT00064103, NCT00004041, NCT00003147, NCT00003880, NCT00003649, NCT00003167
	REIC/Dkk-3	Prostate cancer	NCT01197209
	RTVP-1	Prostatic Neoplasms	NCT00403221
Suicide molecule	*TK*	Malignant Glioma, Brain Tumors, Hepatocellular Carcinoma, Ovarian Cancer, Melanoma, Pancreatic Cancer	NCT01811992, NCT00002824, NCT00844623, NCT00638612, NCT00005057
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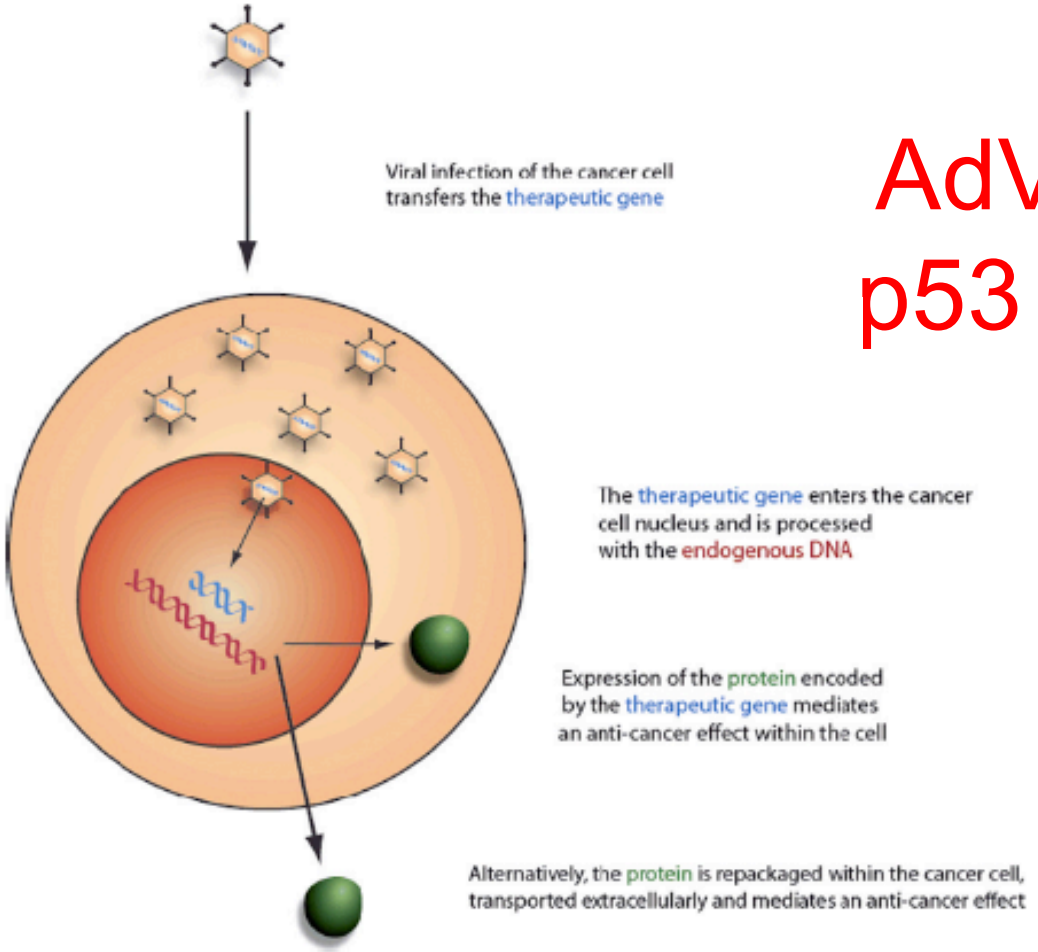
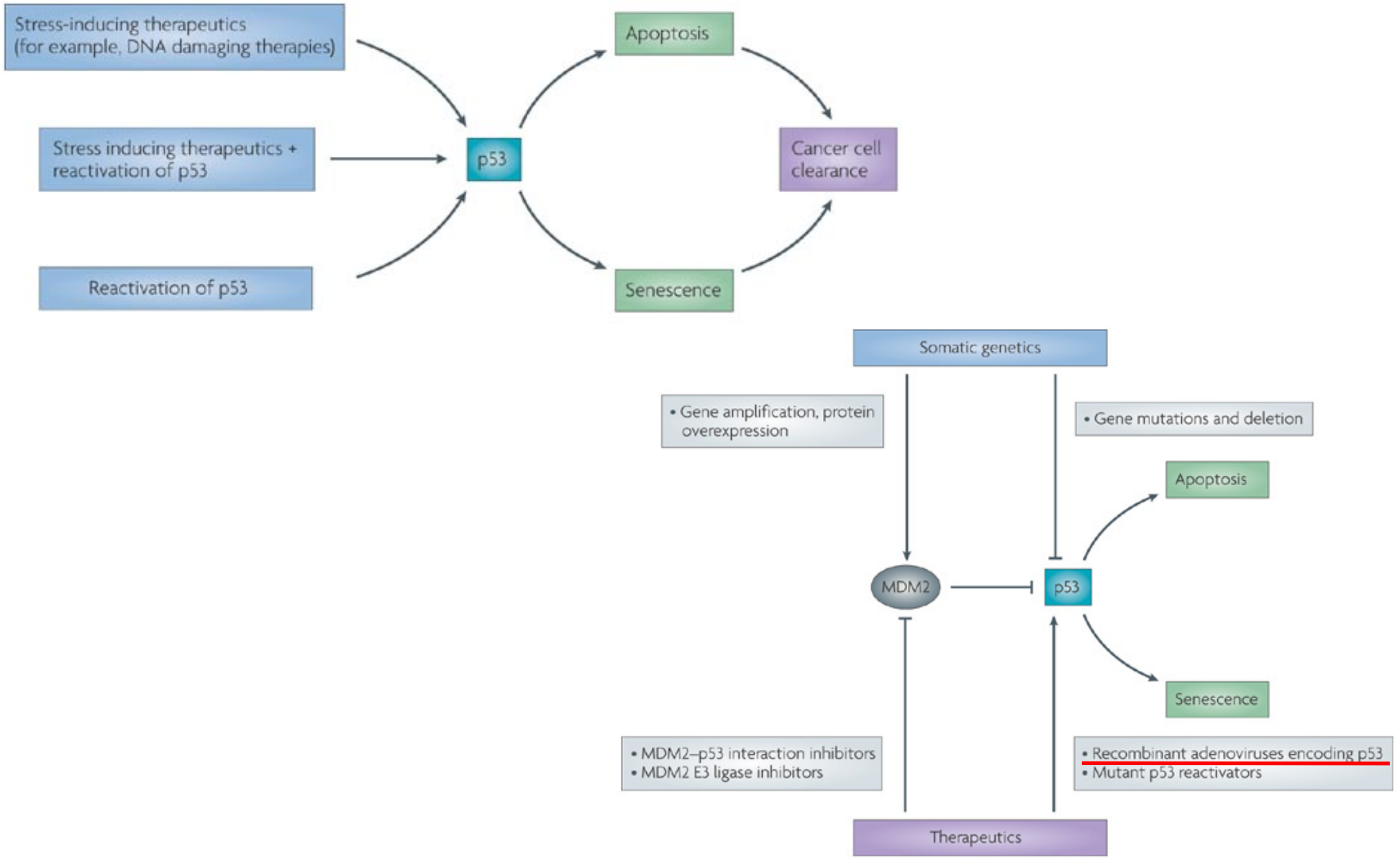


Fig. 1b. Combined viral gene therapy and oncolysis. In addition to viral oncolysis, viral vectors of gene therapy are capable of introducing a gene whose protein product mediates a cytotoxic effect.

AdV-based p53 therapy

Adenovirus-based p53 therapy



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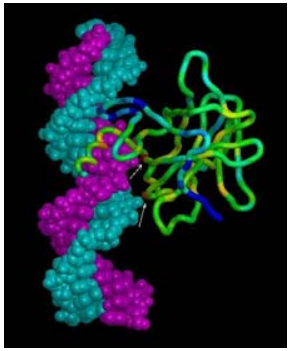
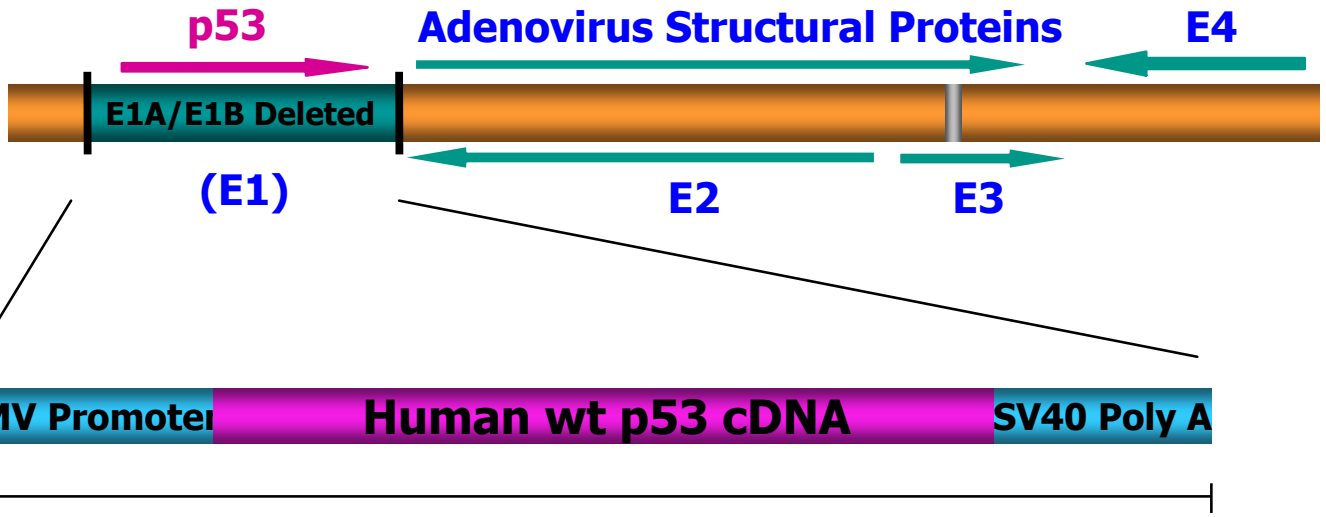
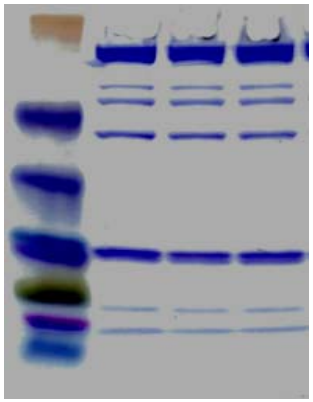
ADVEXIN® Construct



35.4 kb Adenovirus genome



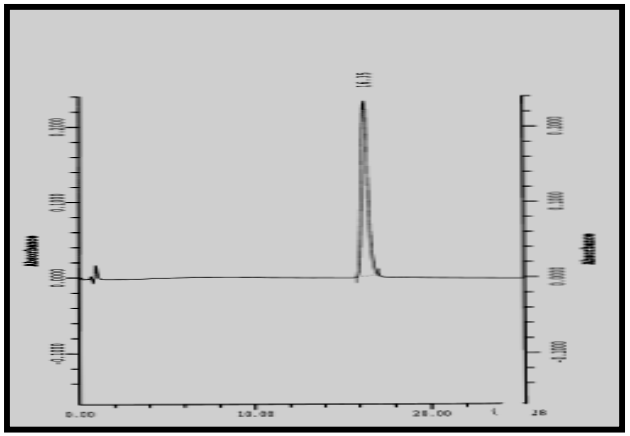
2.3 kb Expression cassette insert

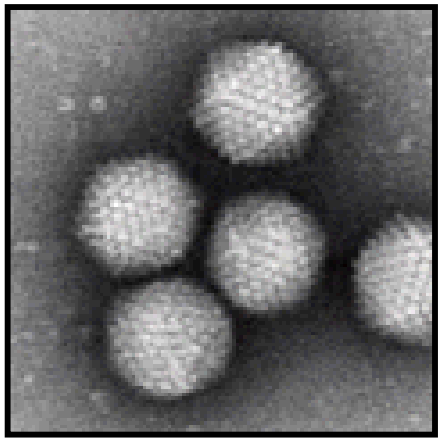


Hexon
Penton
Fiber
Core

Core

Hexon associated





AdV-based p53 therapy

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Ad5CMV-p53 gene
A replication-defective adenoviral-CMV vector that encodes a wild-type p53 gene. Ad5CMV-p53 induces tumor cells that have been transfected with the vector to produce wild-type p53, a tumor suppressor gene that is deleted or mutated in a significant number of cancers. In transfected tumor cells, the wild-type p-53 gene product exerts an antitumor effect by blocking cell cycle progression at the G1/S regulation point, activating DNA repair proteins in the presence of DNA damage, and initiating apoptosis when DNA damage is irreparable. Check for active clinical trials or closed clinical trials using this agent. (NCI Thesaurus)

Synonyms:	Adeno-p53 Adenovirus p53
US brand name:	ADVEXIN
Abbreviations:	Ad5CMV-p53 Ad-p53
Code names:	INGN-201 RPR/INGN-201

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Therapeutics, Inc.

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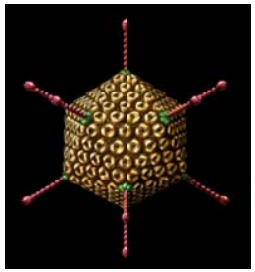
- INGN 225
- INGN 401
- INGN 234
- INGN 402
- INGN 403
- INGN 007

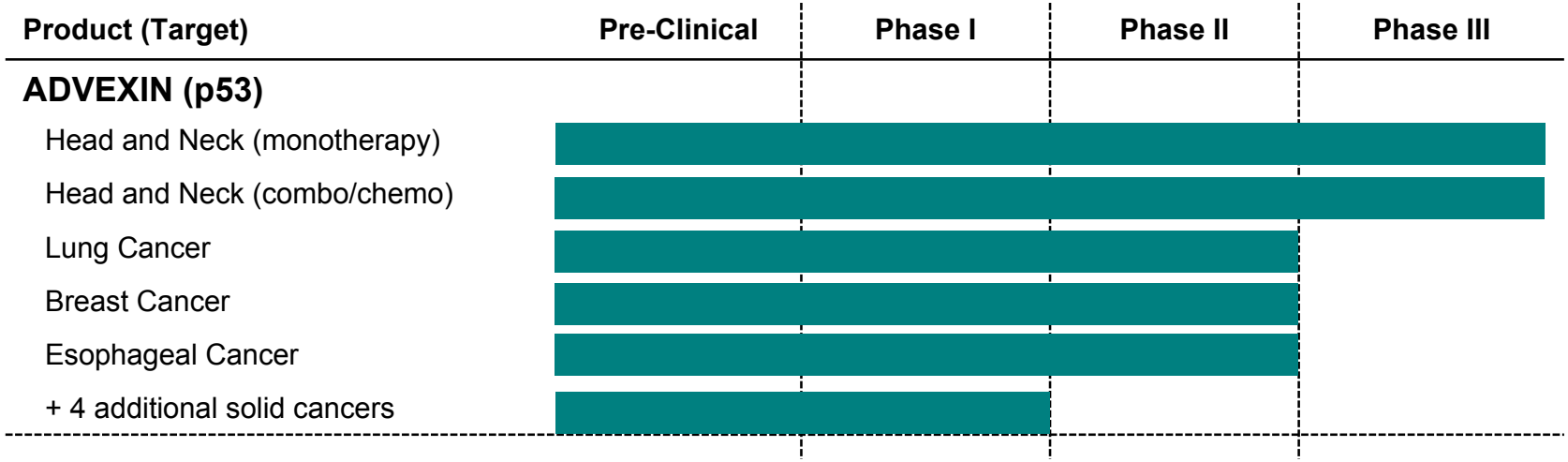
p53 tumor suppressor therapy

ADVEXIN® therapy combines the p53 tumor suppressor with a non-replicating, non-integrating adenoviral delivery system we have developed and extensively tested. The p53 gene is one of the most potent members of a group of naturally-occurring tumor suppressors, which act to kill cancer cells, arrest cancer cell growth and protect cells from becoming cancerous. Introgen's clinical trial strategy for ADVEXIN® is to test it in a variety of life-threatening cancers for which there are no effective treatments. Introgen is seeking to register ADVEXIN® for the treatment of head and neck cancer and Li-Fraumeni Syndrome. Additional late stage clinical trials in breast and lung cancers will enable Introgen to add follow-on indications.

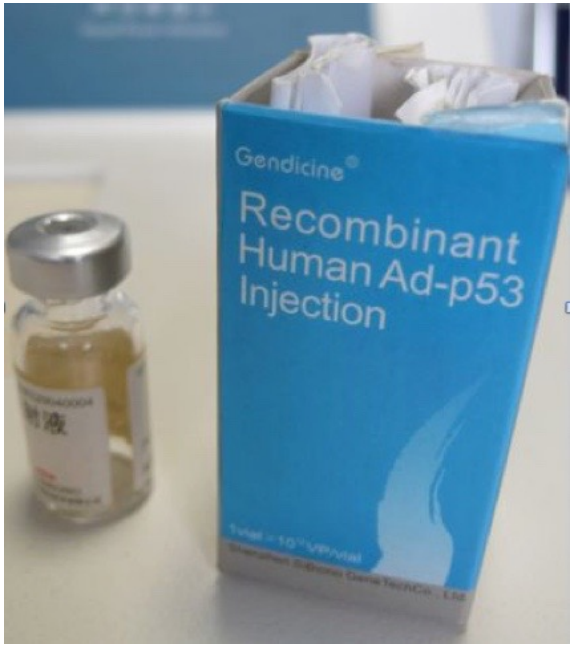
- ADVEXIN® -- Clinically advanced, late-stage oncology product development program. Phase I through Phase 3 trials currently ongoing.
- FDA designated Fast Track Drug Product Development program
- # FDA and EMEA designated Orphan Drug status for ADVEXIN® in head and neck cancer.
- ADVEXIN® therapy well tolerated and clinically active.

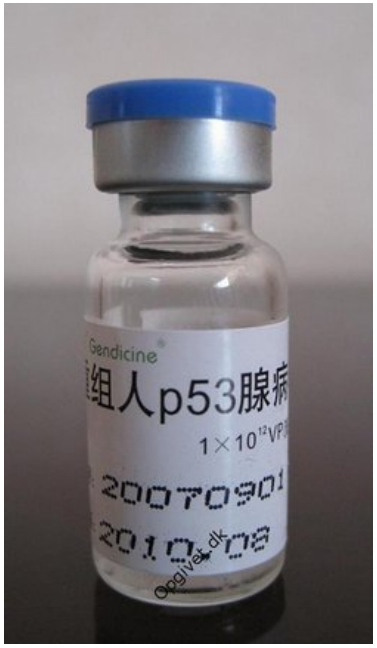


Clinical Pipeline



Gendicine®





- Gendicine® consists of the human wild-type p53 tumor suppressor gene and an Adv vector.
- It is the first approved commercial gene therapy product in the world.
- Gendicine® has acquired all licenses and approvals issued by SFDA (State Federal Drug and Food Administration of China), including the new drug license, manufacturing approval, and GMP license.
- Gendicine® is considered a wide spectrum anti-cancer product since it targets a variety of human tumors.
- Safety of Gendicine® until now,about 15,000 patients with a variety of more than 40 cancers from China and abroad have been treated by Gendicine®. It indicates that Gendicine® is safe.

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Oncolytic ViroTherapy of Cancer

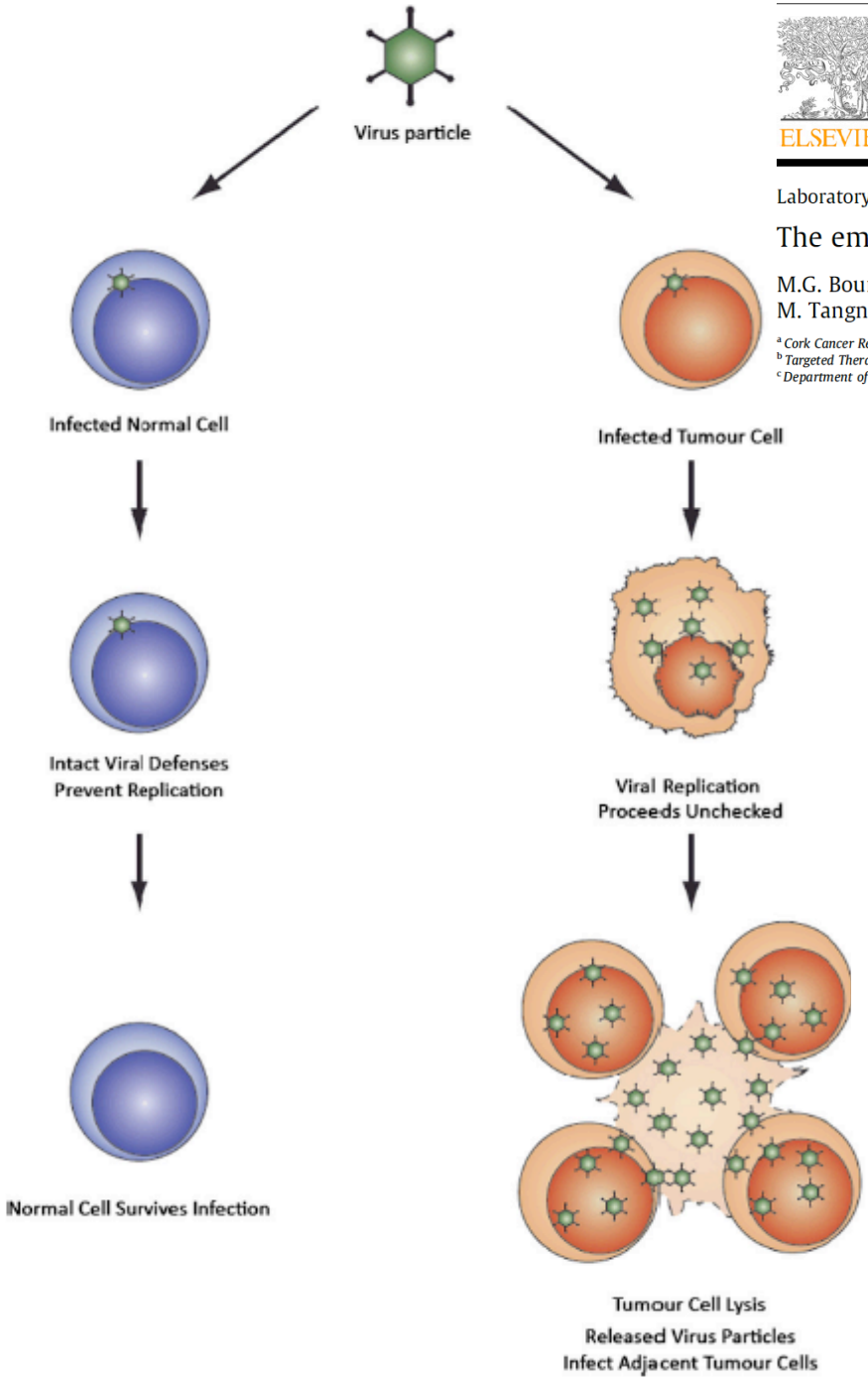
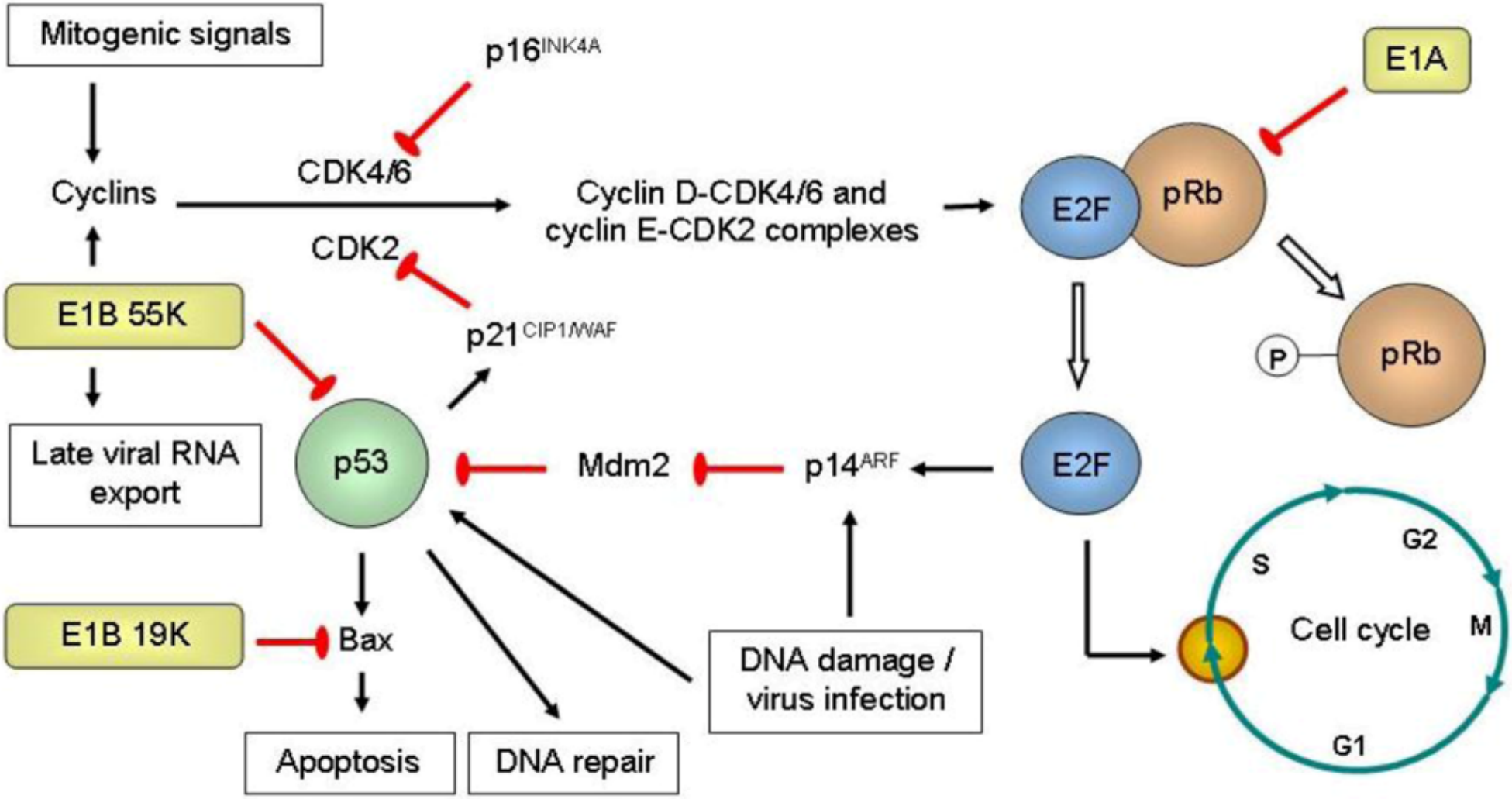


Table 4
Features of the ideal virotherapy agent.

Preferentially enters cancer cells
Efficiently kills cancer cells
Capable of replication preferentially within or exclusively within neoplastic cells
Causes only mild, self-limited or no human disease
Treatment available to control or eliminate viral particles
Viral agent has a large degree of genetic stability
Recombination events unlikely or recombinant agents capable of being eliminated

Fig. 1a. Oncolytic virotherapy. Following viral infection a normal, non-neoplastic cell is capable of evading viral infection due to its intact viral defences. By contrast, the defective viral defences associated with neoplasia result in unchecked viral replication occurring within the tumour cell, with consequent oncolysis.

Adenoviral Vectors and Cancer Therapy



Cancer-selective killing efficacy of oncolytic Adenovirus.

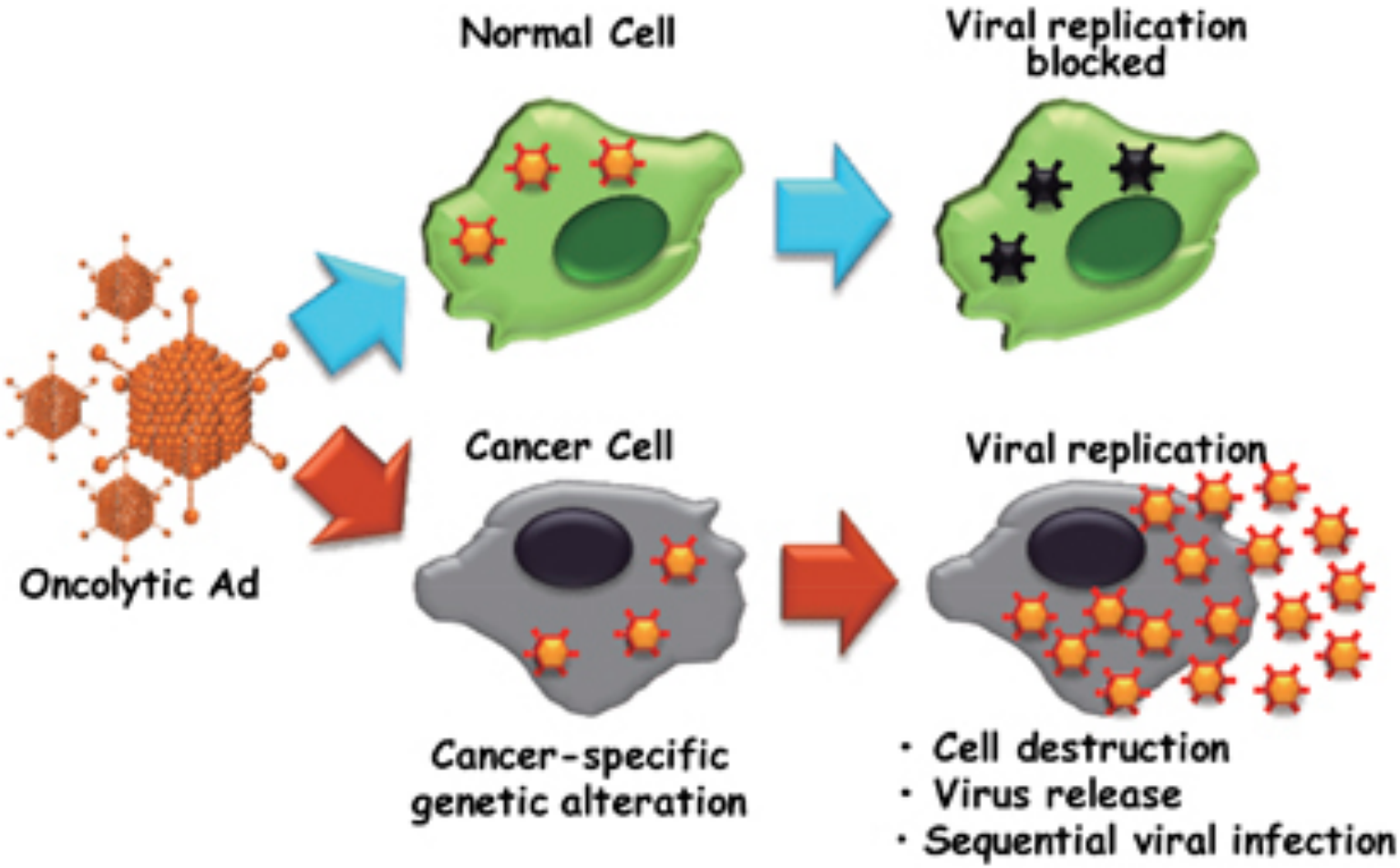


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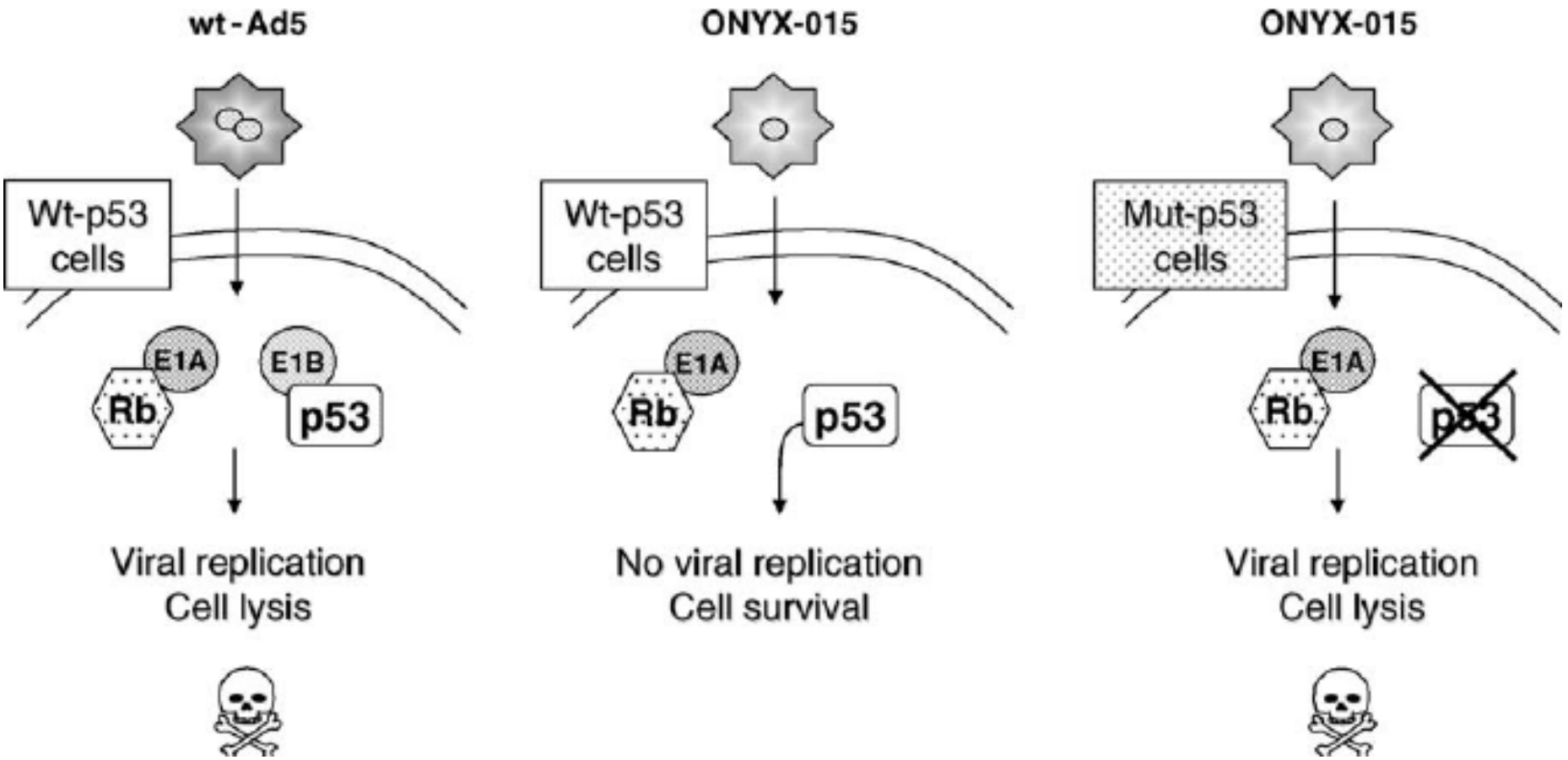
Table 1
Features of Viral Agents that offer an advantage for their use as anti-cancer treatments.

Features	Advantages
Induce cell death by mechanisms other than apoptosis Can also be engineered to carry a wide variety of transgenes that induce cell death by a variety of mechanisms	Decreased risk of resistance developing to viral therapy and of cross-resistance developing to current anti-cancer treatments
Replicate within tumour cells to produce multiples of the original viral dose	Amplification leads to cytolysis in cells beyond that initially infected Increases therapeutic index of viral treatments
Naturally replicate, or can be engineered to replicate, in a tumour-selective manner	Minimises toxicity to normal tissues
Capable of specifically targeting and eliminating cancer stem cells	Elimination of the cell population thought to confer chemoradiotherapy resistance
Robust evidence emerging that they may be used safely with other treatment modalities and have a synergistic anti-cancer effect	Increased potency of multi-modality treatment regimes
Can be armed to induce tumour-specific immunological reactions	Induction of additional specific anti-tumour effects Potential to target metastases by immunological strategies

Table 2
Targeting Viral Agents to Tumours.

Naturally oncotropic viruses	
Oncotropic due to Genetic changes associated with Neoplasia	
Reovirus	Growth restricted to cells with *ras* mutations
Parvovirus	Growth restricted to cells expressing proteins associated with S phase
Vesicular Stomatitis Virus	Replicates in cells with defective interferon response
Newcastle Disease Virus	Evolves through serial passage to become dependent on a defective interferon response to allow replication
Sindbis Virus	Infection mediated by laminin receptor-known to be overexpressed in neoplastic cells
Oncotropic dependent on expression of cell surface receptors	
Poliovirus	Infects cells expressing the membrane receptor CD155
Adenovirus	Infection mediated by the Coxsackie adenovirus receptor
Oncotropic due to changes associated with Neoplasia	
Hepres Simplex Virus type 1	Altered extracellular matrix rendering it more susceptible to infection
Engineered mechanisms of viral tumour targeting	
Deletion of genes necessary for viral replication in normal tissue (deleted genes)	
Adenovirus	(*E1A, E1B*)
Herpes Simplex Virus	(*TK, RR, UL6, gamma34.5*)
Vaccinia	(*TK, vgf*)
Introduction of tissue-specific transcriptional promoters (promoters-tissue)	
Adenovirus	
Herpes Simplex Virus	(*PSA-Prostate*)
	(*aFP-Hepatocellular tissue*)
	(*muc1-Breast*)
	(*CEA-Colonic tissue*)
Viral surface protein modification	
Measles Virus	Alteration of H protein such that viral attachment is to tumour specific ligands
	Modification of F protein such that activation, and subsequent viral attachment, is dependent on MMPs
Poliovirus	Swapping of IRES elements

Cancer-selective killing by ONYX-105 oncolytic Adenovirus.

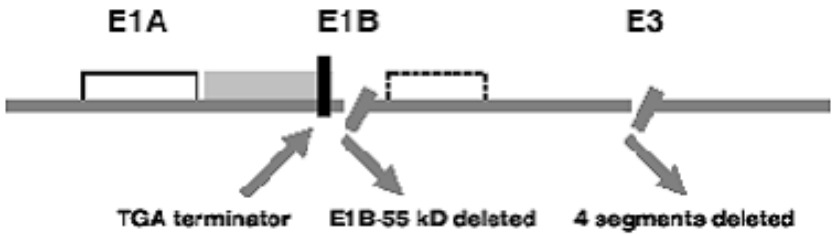


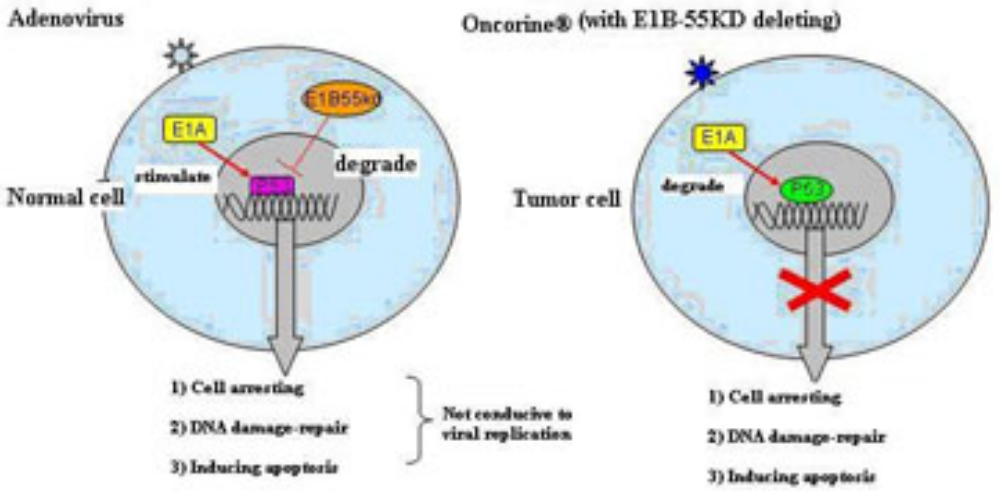
The 55-kDa E1B protein of Adenovirus, which binds to and inactivates the tumor suppressor protein p53, is not expressed in ONYX-105. The mutant virus due to a deletion in E1B is able to replicate only in cells deficient for wild-type p53.

Recombinant AdV as Oncolytic Viruses

上海三维生物技术有限公司
Shanghai Sunway Biotech Co.,LTD.

H101 (Recombinant Human Adenovirus Type 5 Injection; Brand name: **Oncorine®**) is on market since September, 2006.
Oncorine is the first oncolytic virus drug which was approved in the world





The 55-kDa E1B protein of Adenovirus, which binds to and inactivates the tumor suppressor protein p53, is not expressed in this adenoviral mutant. The mutant virus due to a deletion in E1B is able to replicate only in cells deficient for wild-type p53.

Mechanism of H101 oncolytic action

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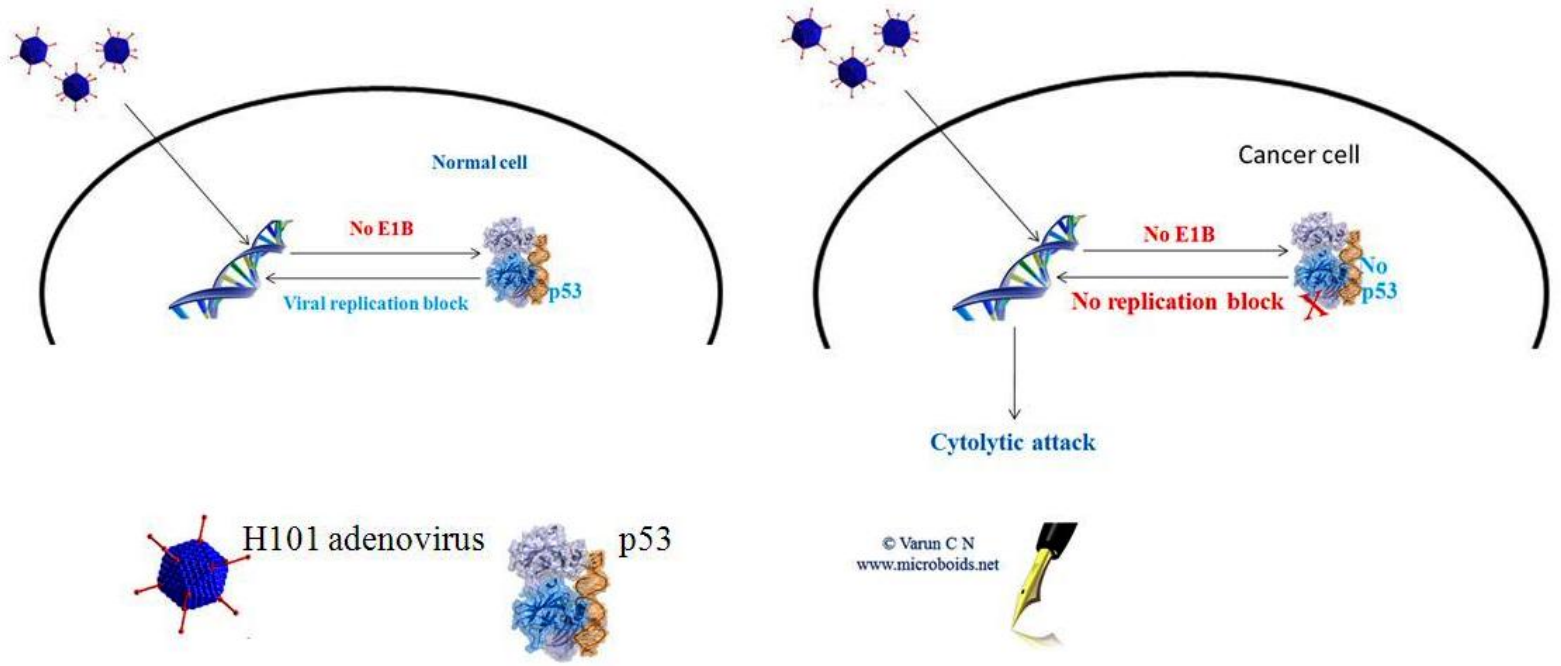

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