adenovirus

From Crystal 2014 “In the late 1980s, when we began to think about strategies of transferring genes in vivo, the adenovirus was known to be trophic for the respiratory epithelium. The virus had been sequenced in its entirety, many human serotypes were known, and the detailed biology of how the virus replicated and assembled was well described (Ginsberg, 1984; Russell, 2009). **The stage was set, partly by serendipity, to adapt the adenovirus to be an effective means of transferring genes in vivo**”.

Adenovirus and Adenoviral vectors

Wild-type adenovirus contains a single, 36-kb, double- stranded DNA genome flanked by inverted terminal repeats.

There are over 50 serotypes, from which serotypes 2 and 5 have been most developed for use as gene-therapy vectors.

This virus infects the upper respiratory tract, producing symptoms similar to those associated with colds and influenza, but as far as is known, it does not normally cause more serious disorders.

Adenovirus was known to be trophic for respiratory epithelium.

Human adenovirus

serotypes are based on the ability of human sera to neutralize viral infection

Table 1 Classification of human Ads

The classification of the different human Ad serotypes and the major diseases associated with each species is summarized. The major attachment protein found to mediate interactions with host cells is shown for each species, along with the typical number of shaft repeats found within the fibre protein [17]. The Table is adapted from [20,32].

Species	Serotypes	Commonly associated disease	Primary attachment molecule	Oncogenic potential (in rodents)	GC content of genome (%)	Number of shaft repeats in fibre*
A	12, 18, 31	Gastroenteritis	CAR	High	48–49	23
B	3, 7, 16, 21, 50 (B:1) 11, 14, 34, 35 (B:2)	Respiratory (B:1) or urinary tract disease (B:2)	CD46/CD80/CD86	Moderate	50–52	6
C	1, 2, 5, 6	Respiratory disease	CAR	Low/none	57–59	22
D	8, 9, 10, 13, 15, 17, 19, 20, 22–30, 32, 33, 36–39, 42–49, 51	Keratoconjunctivitis	CAR (sialic acid for 8, 19, 37 and CD46 for 37)	Low/none	57–61	8
E	4	Respiratory disease/conjunctivitis	CAR	Low/none	57–59	12
F	40, 41	Gastroenteritis	CAR (for long fibres; short fibres unknown)	Unknown	Unknown	22
G	52	Gastroenteritis	Unknown	Unknown	Unknown	Unknown

*Number of fibre shaft repeats found for individual serotypes within each species, not necessarily representative of all serotypes within a particular species.

struttural components

The Ad capsid is icosaedric (20 factes, 12 vertices) without envelop

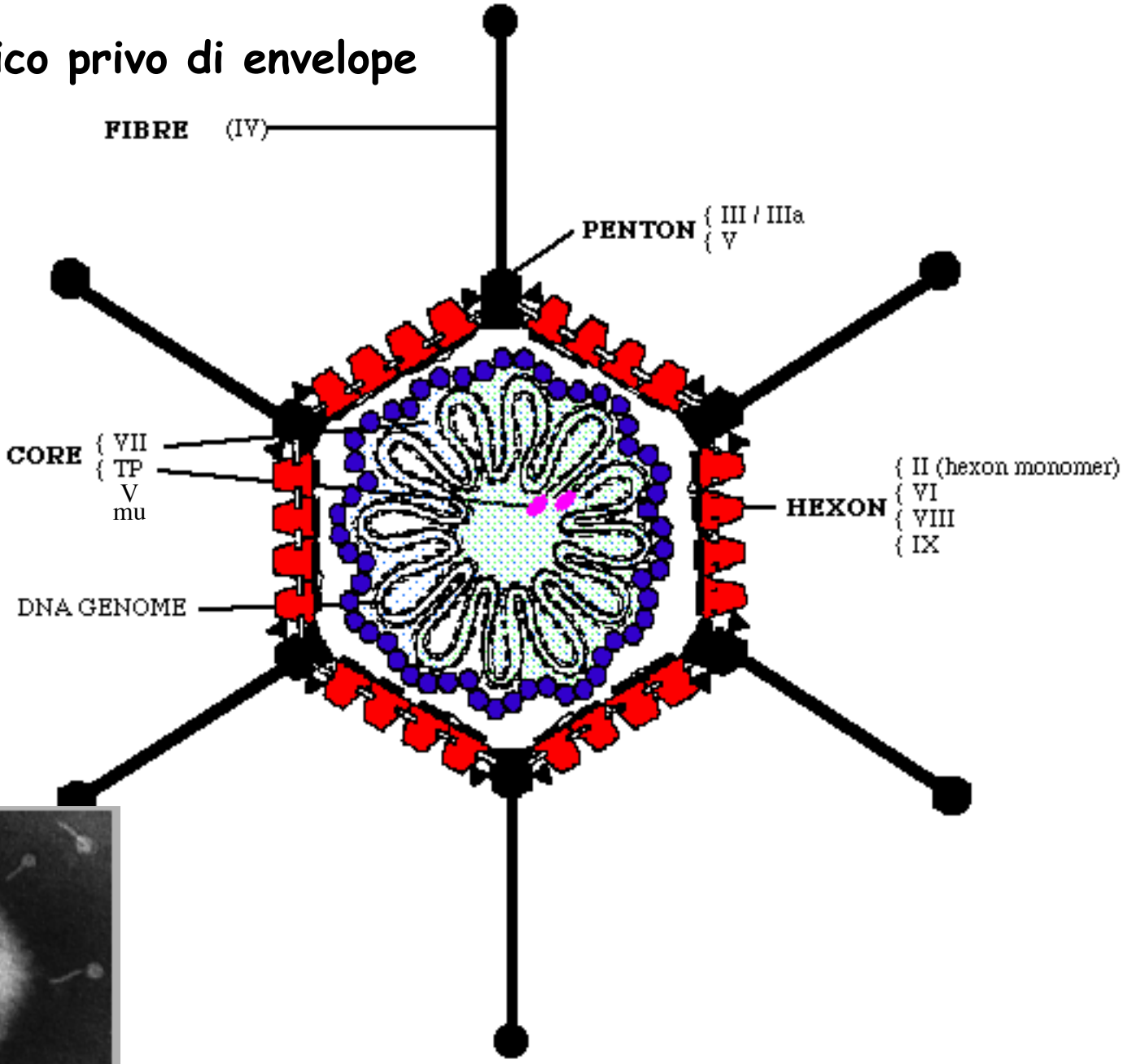
Table 2 The structural components of the Ad capsid

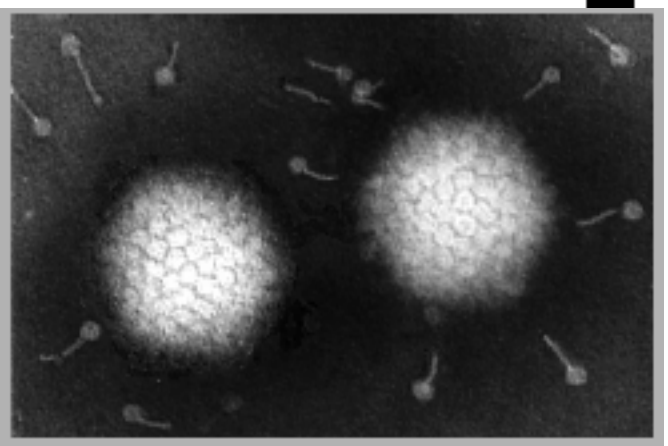
The 13 structural proteins which constitute the Ad capsid are described in terms of their location and major functional role. Major capsid proteins are highlighted in bold, with minor capsid proteins in italics. The additional core proteins which are associated with the viral genome are shown in normal type.

Polypeptide/name	Location	Function
II (hexon)	**Facets of icosahedron**	**Major structural component, forms facets of the capsid**
III (penton base)	**Capsid vertices**	**Contains an RGD motif which facilitates interaction with cellular integrins***
IIIa	*Underside of penton base*	*Stabilizes the vertices*
IV (Fibre)	**Projecting from the penton base**	**Mediates the initial attachment to host cells**
V	Core	Links core to capsid, possibly aids nuclear localization
VI	*Inner hexon cavity*	*Protease cofactor, assembly, endosome disruption and nuclear import of hexon*
VII	Core	Targets viral genome to the nucleus and condenses DNA
VIII	*Between hexons*	*Stabilization of peripentonal hexon–hexon interactions*
IX	*External faces of the capsid*	*Stabilization of virion. Transcriptional activator*
TP	5′-End of the genome	Primes DNA replication
Mu	Core	DNA condensation
IVa2	Core	DNA packaging
Ad protease	Core	Cleaves precursor proteins

*Exceptions include the species F Ads

Capside icosaedrico privo di envelope



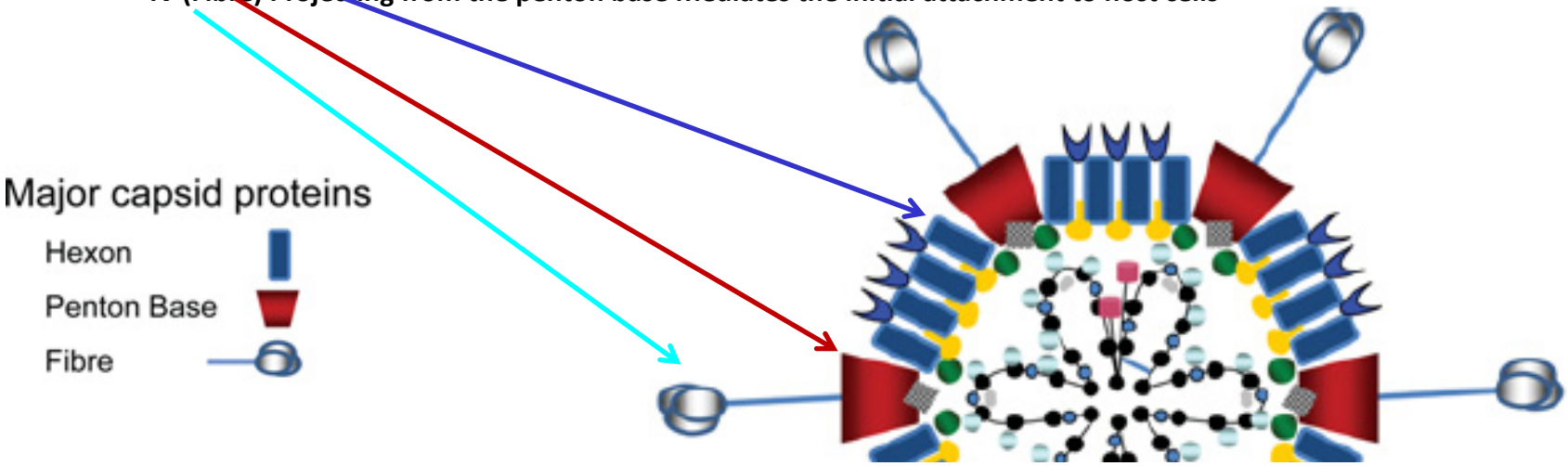


Major structural protein: the capsid

II (hexon) Facets of icosahedron **Major structural component, forms facets of the capsid**

III (penton base) Capsid vertices **Contains an RGD motif which facilitates interaction with cellular integrins***

IV (Fibre) Projecting from the penton base Mediates the initial attachment to host cells



Minor capsid proteins

IIIa

VI

VIII

IX

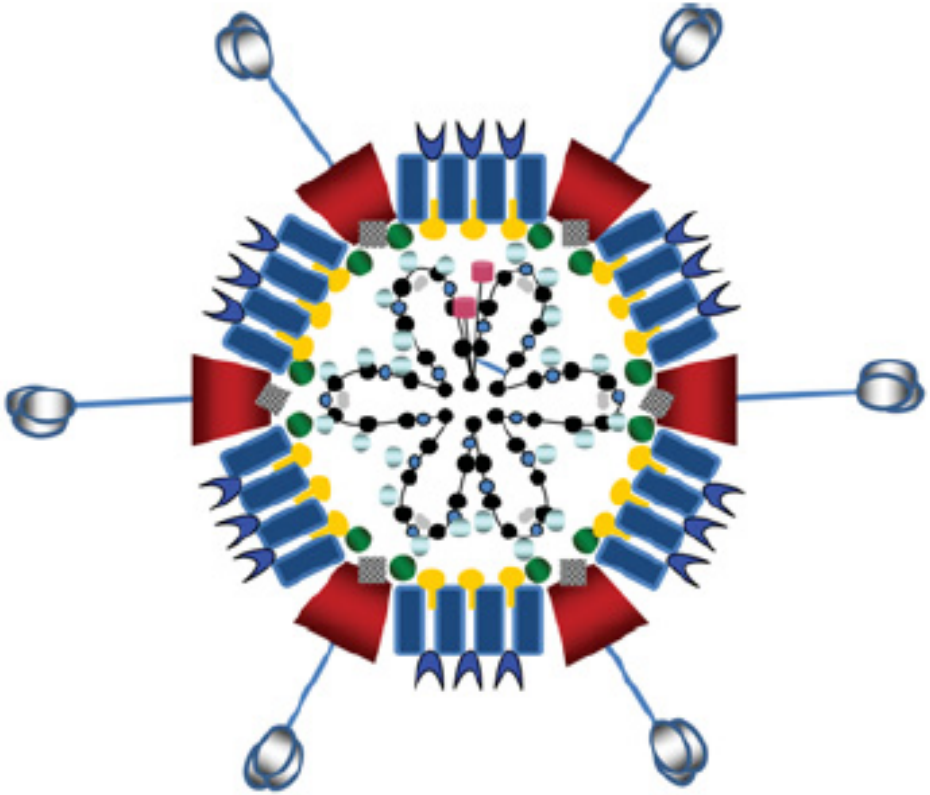
IIIa Underside of penton base Stabilizes the vertices

Inner hexon cavity Protease cofactor, assembly, endosome disruption and nuclear import of hexon

Between hexons Stabilization of peripentonal hexon–hexon interactions

IX External faces of the capsid Stabilization of virion. Transcriptional activator

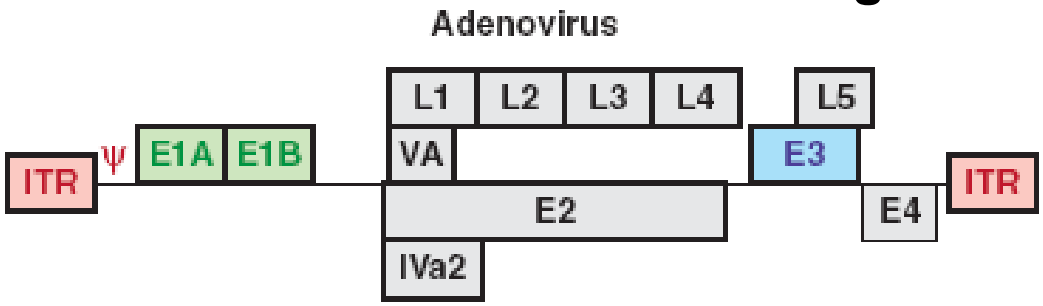
Major structural protein: the core proteins



Core proteins

V	**V Core Links core to capsid, possibly aids nuclear localization**
VII	**VII Core Targets viral genome to the nucleus and condenses DNA**
Mu	**Mu Core DNA condensation**
TP	**TP 5-End of the genome Primes DNA replication**
IVa2	**IVa2 Core DNA packaging**
Protease	**Ad protease Core Cleaves precursor proteins**

The Ad genome



Genome size

36 kb, dsDNS producing more than 50 proteins, origins of replication (*ori*) are located in the ITR (inverted terminal repeats), TP binds to 5'-end DNA and starts DNA replication

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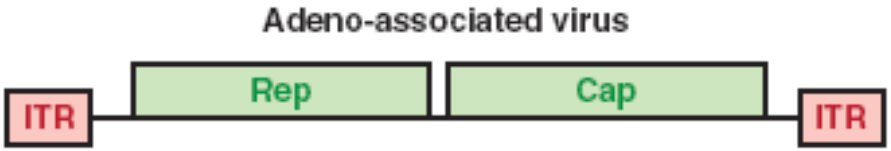


4,7 kb, ssDNA

X Essential elements retained in vectors

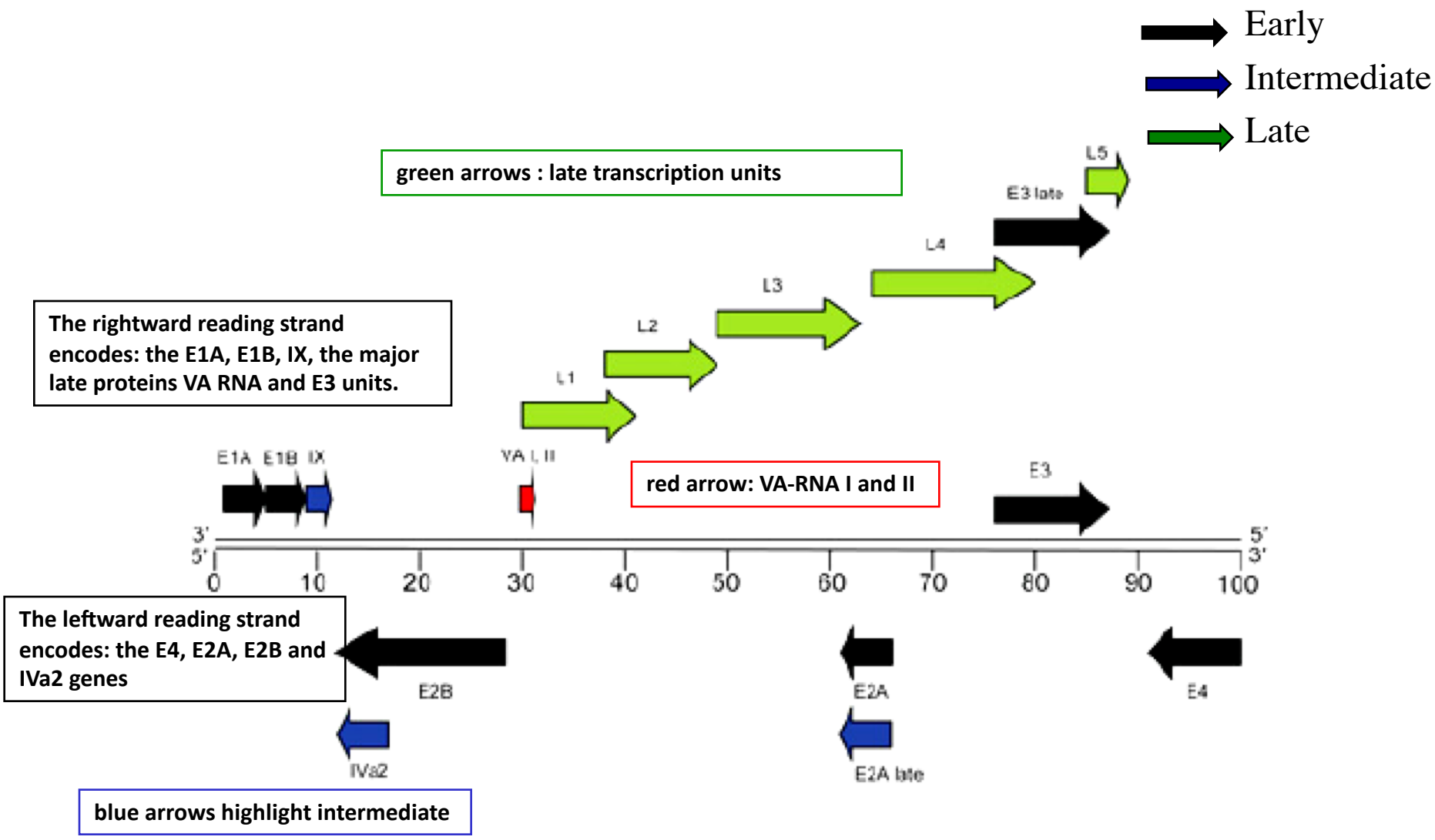
X Genes supplied by packaging construct / cell line

X Nonessential genes often deleted

adeno infection

- After infection, the viral DNA escapes from the lysosome and is transported to the nucleus of the cell, where it persists as an episome; multiple adeno-genome copies can coexist within the nucleus of an infected cell.
- The adenoviral genome has eight transcriptional units, expressed in temporal sequence as early (E), intermediate (I), and late (L) genes.
- There are four early genes (**E1–E4**), encoding proteins necessary for the replication of the viral genome.
- **E1A** is the first viral gene expressed, and its product **trans-activates the other promoters of early genes.**

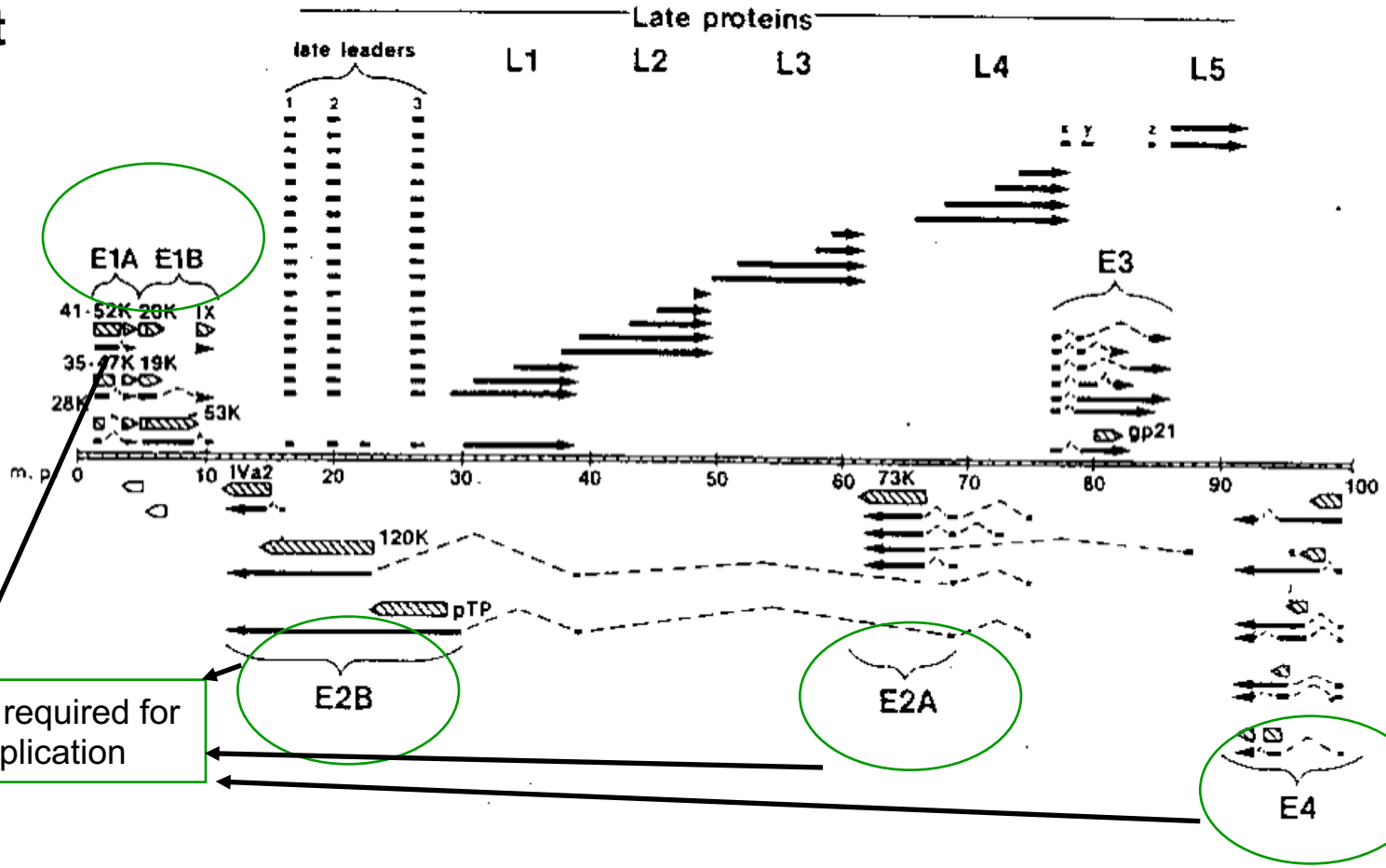
Ad genome organization



Ad transcripts

Pol II transcript

E:E1A, E1B, E2, E3, E4
Ritardati: IX, IVa2
L: processati in 5
RNA **L1-L5**



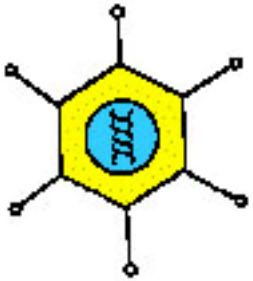
E1A and E1B are required for Ad genome replication

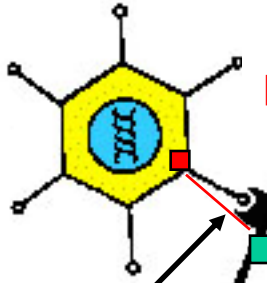
The Ad life-cycle is finely tunes
Early phase: expression of the early genes (E) ➔ genome duplication
Late phase: expression of the late genes (L) ➔ capside components and virion assembly

Infection

virus-host cell interaction (two steps)
1. Interaction of the fiber-knob with the CAR receptor
2. Interaction between the RGD (arginin, glycin, aspartic acid) motif of the penton-base with the integrin

Mature virion



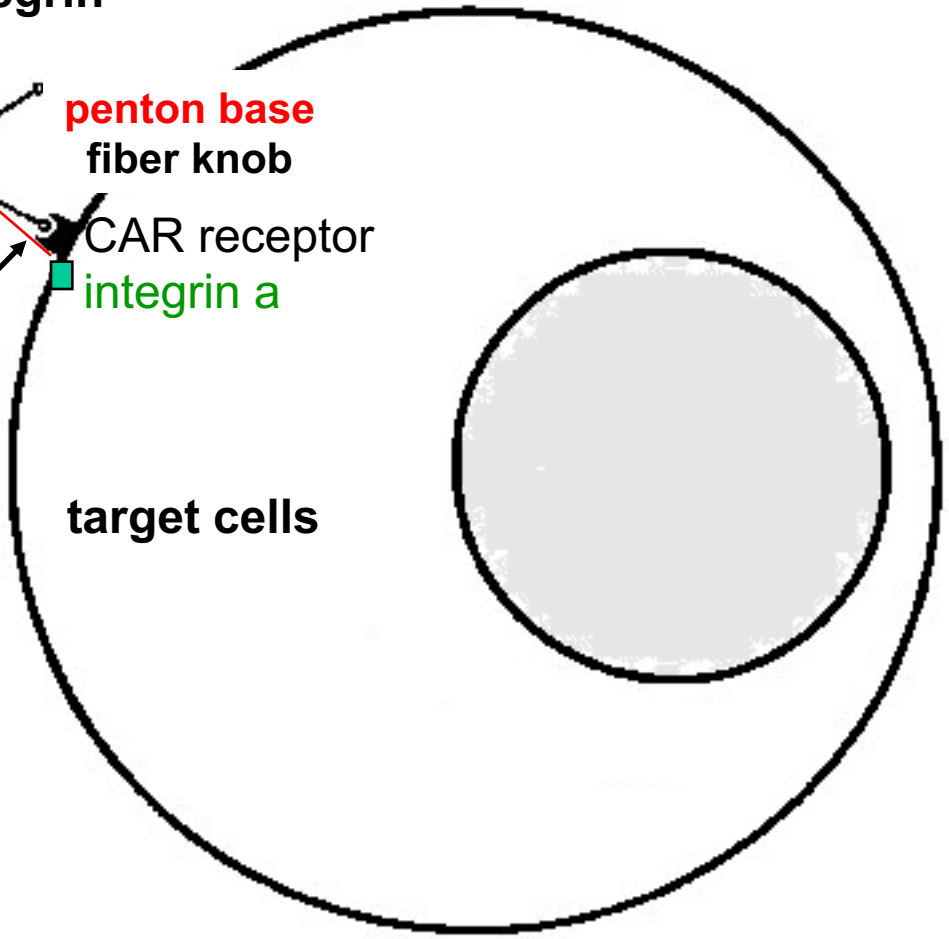


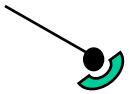
penton base
fiber knob
CAR receptor
integrin a

Attachment

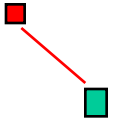
C-term
Fiber knob

target cells





Fiber knob-CAR



Interaction penton-base integrin a (integrins are membrane proteins)

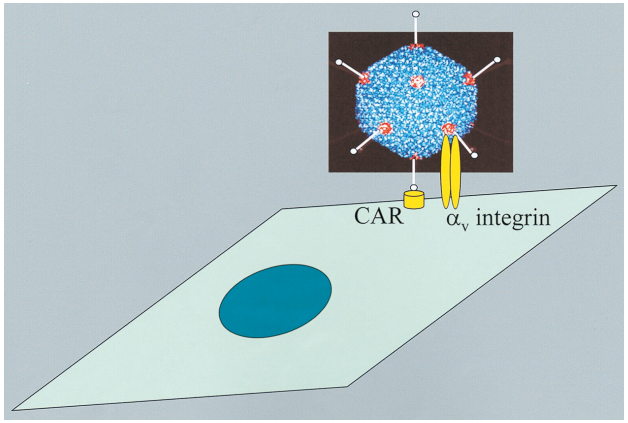
integrins

Integrins are heterodimeric transmembrane receptors that mediate cell-adhesion. With their extracellular head region, most integrins bind extracellular matrix (ECM) glycoproteins such as laminins and collagens in basement membranes or connective tissue components like fibronectin. **Others bind counterreceptors on neighboring cells, bacterial polysaccharides, or viral coat proteins**. Through all these interactions integrins mediate stable adhesion to basement membranes, the formation of extracellular matrices and migration on such matrices, the formation of platelet aggregates, the establishment of intercellular junctions in the immune system, and bacterial and viral entry during infectious diseases. Furthermore, integrin-mediated adhesion modulates signaling cascades in control of cell motility, survival, proliferation, and differentiation.

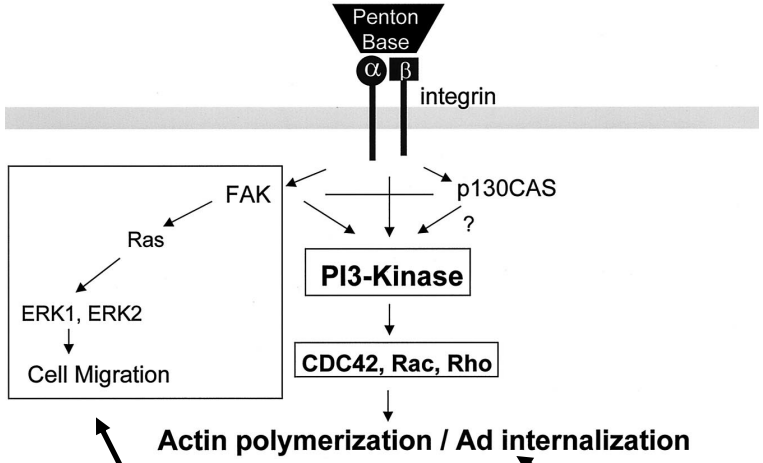
There are over 20 different members of the integrin family, many of which recognize an **arginine, glycine, aspartic acid** (RGD) se- quence in host extracellular matrix proteins

Ad entry

Ad internalization was shown to be regulated by a lipid kinase, phosphatidylinositol-3-OH kinase (PI3K)



Schematic illustration of the interaction of Ad2 with different cellular receptors involved in infection. High-affinity virus attachment is mediated by the interaction of the fiber capsid protein (white) with a 46-kDa receptor known as CAR. A second interaction of the penton base capsid protein (red) with v integrins promotes virus internalization.



Schematic diagram of the signaling events involved in Ad internalization and cell migration.

CAR (coxsackie and Ad receptor): a major Ad attachment molecule

Although CAR has been shown to support Ad entry to cultured cell lines, in the airway epithelium of the host, CAR expression is restricted to tight junctions and the basolateral membrane. Therefore there has been some debate as to whether CAR is accessible to the virus.

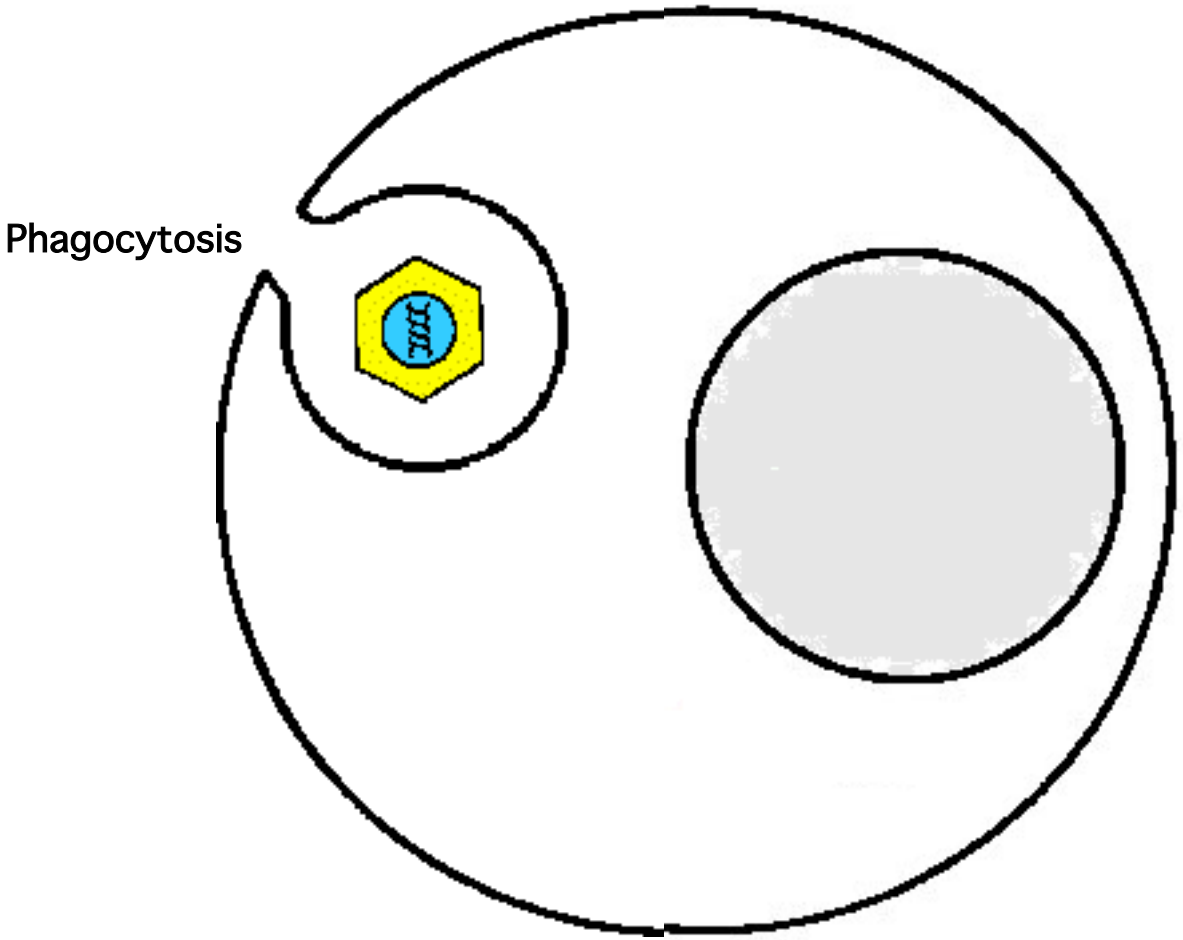
CAR-independent virus entry

cell-surface molecules have also been proposed to function as attachment sites for certain Ads (species C) including **VCAM-1** (vascular cellular adhesion molecule-1) and **HS-GAGs** (heparan sulfate glycosaminoglycans)

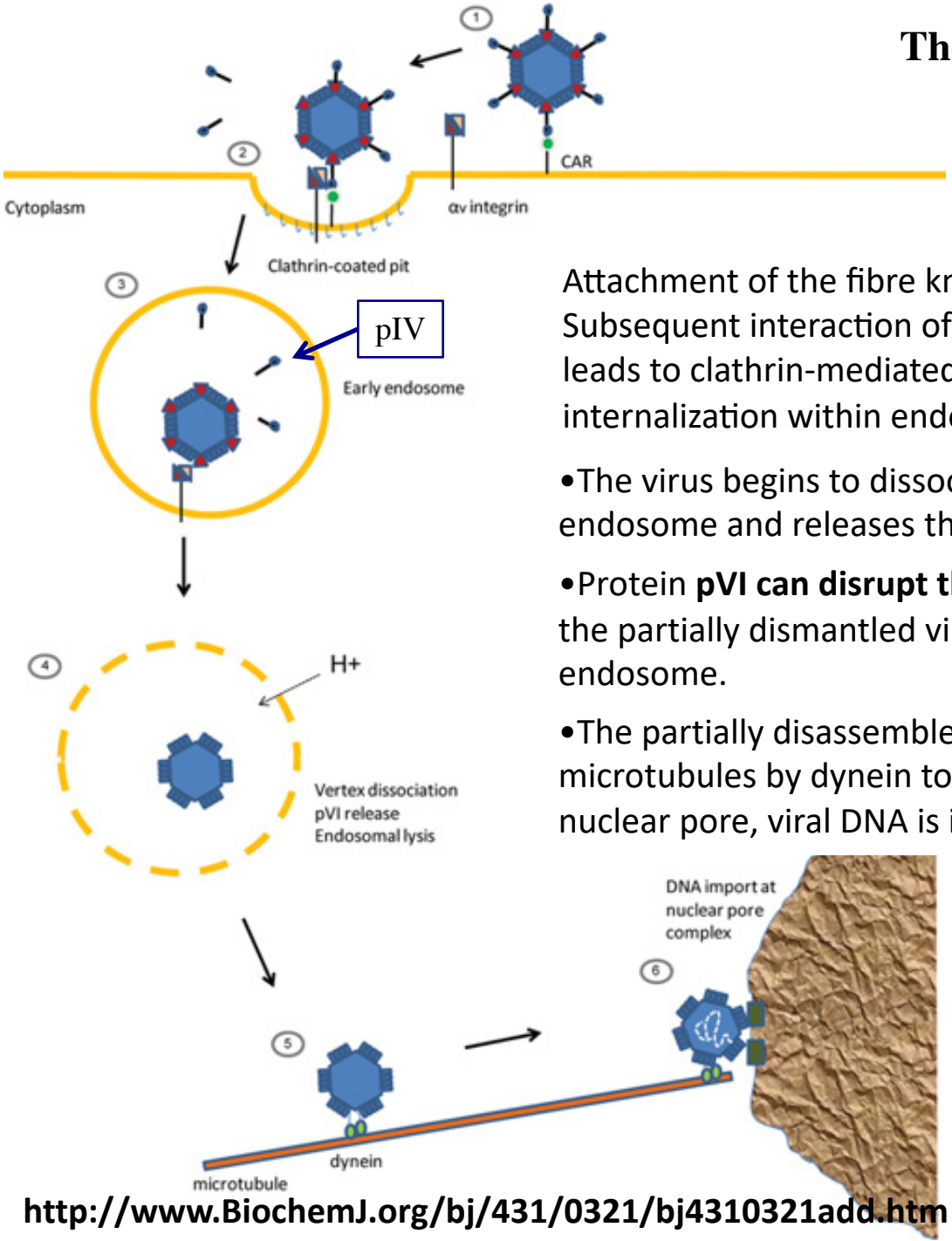
Internalizzazione

Adenovirus di tipo C → **fagocitosi mediata da "clathrin-coated vescicles"**

Adenovirus di tipo B → **micropinocitosi**



The entry pathway of species C Ads

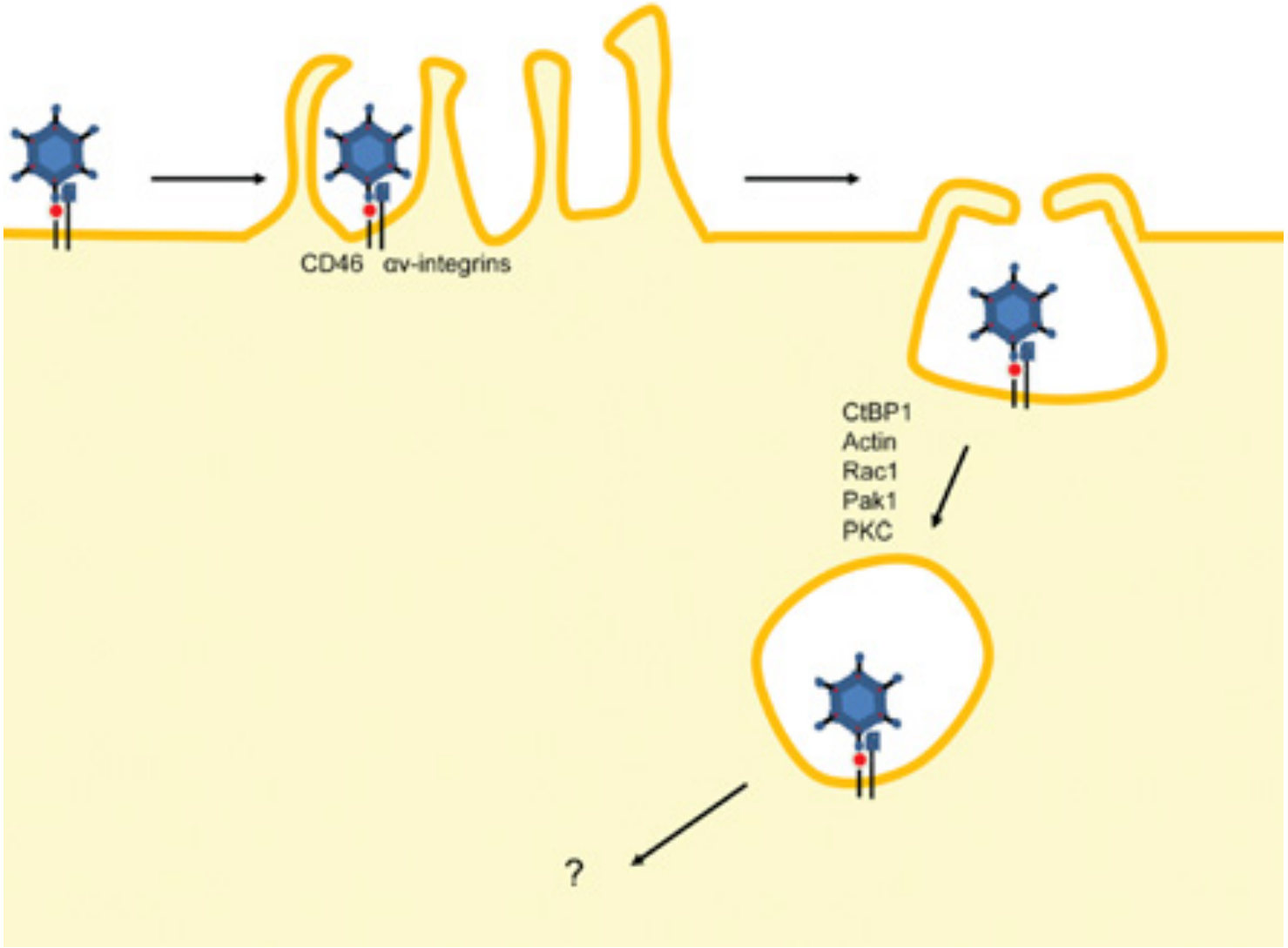


Attachment of the fibre knob to the primary receptor CAR. Subsequent interaction of the penton base with *αv* integrins. This leads to clathrin-mediated endocytosis resulting in virus internalization within endosomes

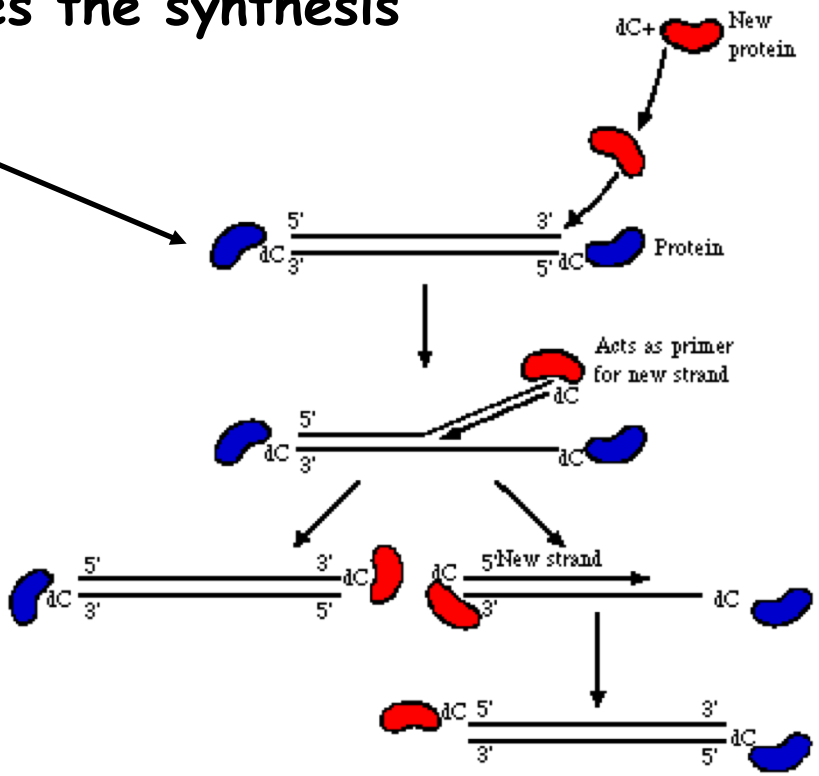
- The virus begins to dissociate in the low pH environment of the endosome and releases the vertex proteins including pVI.
- Protein **pVI can disrupt the endosomal membrane**, allowing the partially dismantled virus particle to escape from the endosome.
- The partially disassembled virus is then transported along microtubules by dynein to the nuclear pore complex. At the nuclear pore, viral DNA is imported into the nucleus.

http://www.BiochemJ.org/bj/431/0321/bj4310321add.htm

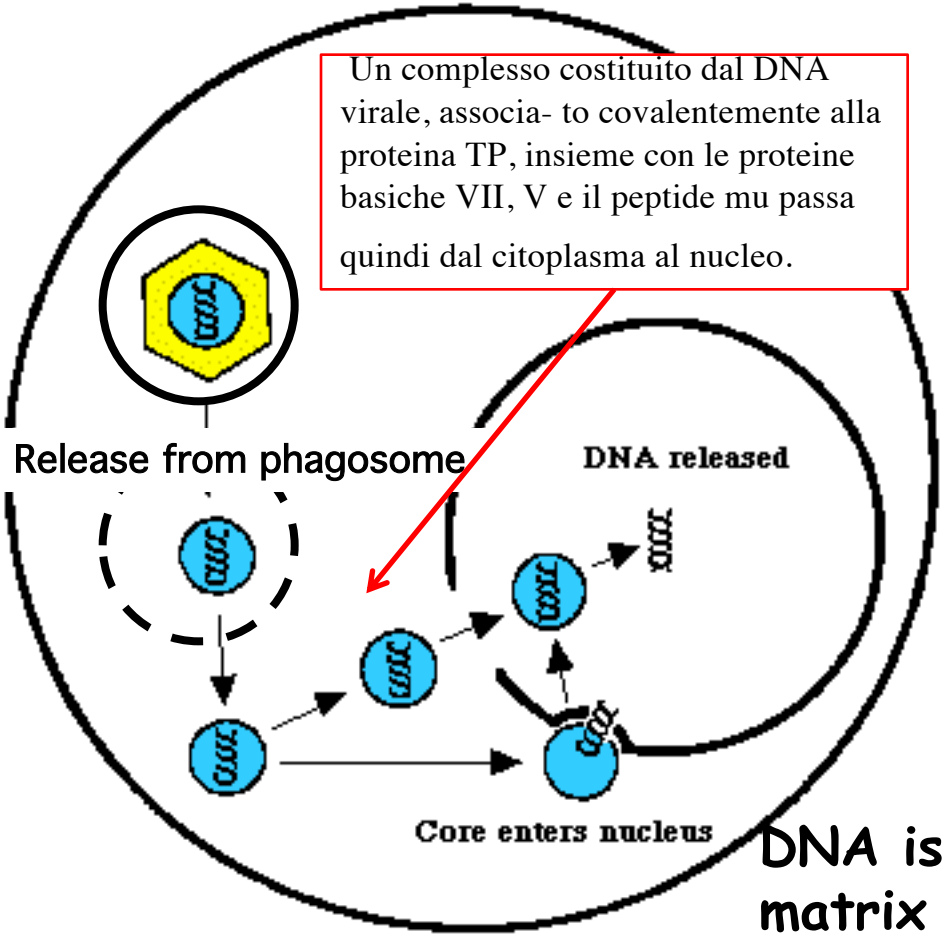
Alternative virus entry: the uptake of species B Ads into epithelial cells by macropinocytosis



Ad viral replication require viral proteins that primer DNA synthesis – Tp primes the synthesis



Un complesso costituito dal DNA virale, associa- to covalentemente alla proteina TP, insieme con le proteine basiche VII, V e il peptide mu passa quindi dal citoplasma al nucleo.



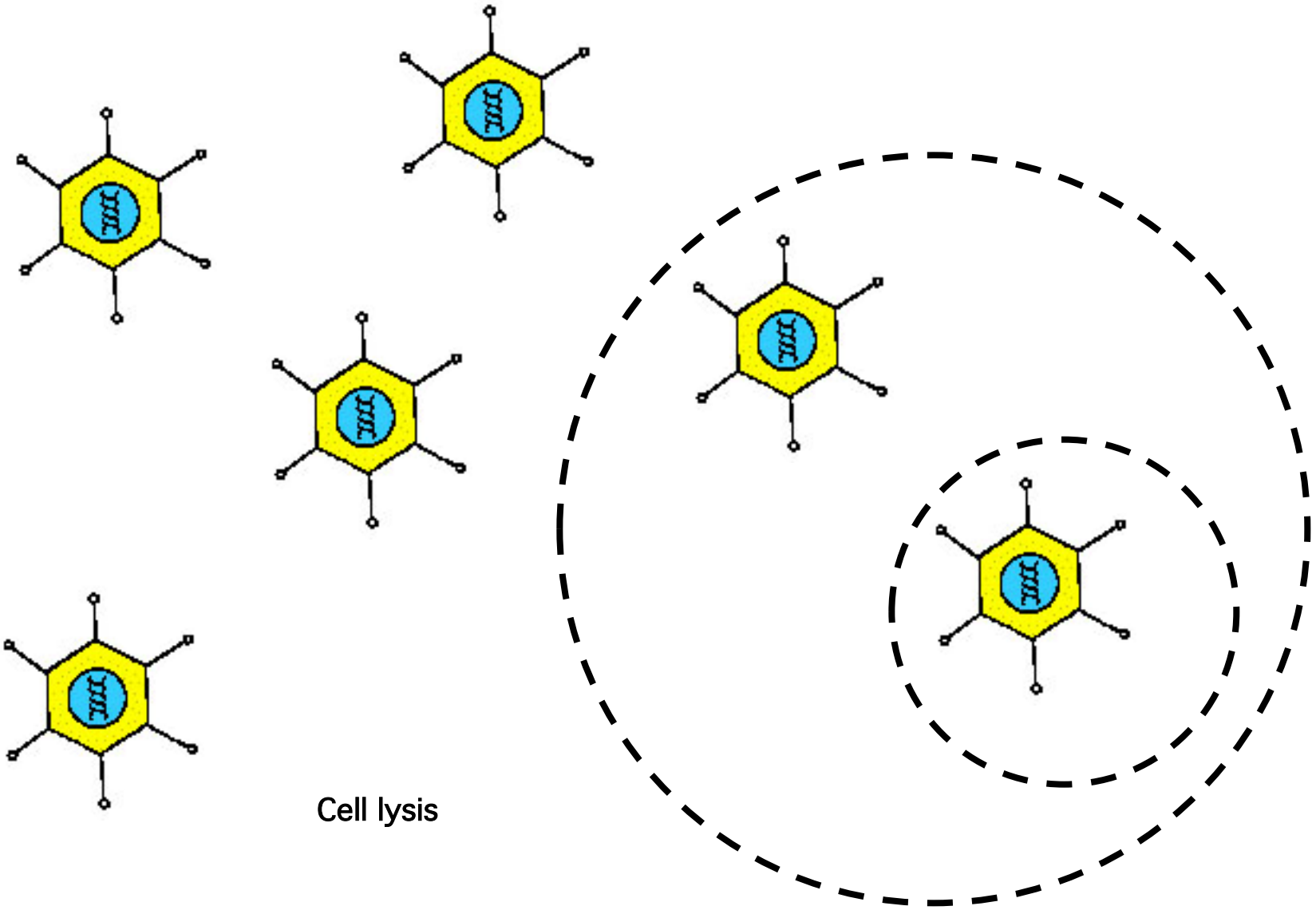
DNA is associated with nuclear matrix and this favour replication

Ad production

1) **indurre la cellula ospite ad entrare nella fase S** del ciclo cellulare e generare quindi un ambiente cellulare ottimale per la replicazione del virus – questa funzione è esercitata dai prodotti dei **geni E1A, E1B ed E4** -;
2) proteggere la cellula infettata dai vari sistemi di difesa antivirale dell'organismo – **geni E1A, E3 e VA RNA**;
3) sintetizzare proteine virali indispensabili per la replicazione del DNA virale **– gene E2**.
Tutti e tre questi obiettivi dipendono dall'attivazione trascrizionale del genoma virale mediata dal prodotto del gene E1A

E1A interagisce con l'oncosoppressore pRb, stimolando l'ingresso nella fase S.
E1A si lega a diversi componenti del complesso basale di trascrizione, tra cui i coattivatori trascrizionali e istone-acetiltrasferasi p300/CBP e P/CAF.
La presenza delle proteine **E1A nella cellula ha anche la caratteristica di attivare la proteina p53**, tramite l'attivazione trascrizionale dell'oncosoppressore p19ARF, che si associa a p53 e ne modula l'attività; **una delle conseguenze di questa attivazione è l'induzione di apoptosi nella cellula infettata**. Tuttavia, almeno tre proteine di adenovirus svolgono **attività anti-apoptotica**: i due prodotti generati dal gene **E1B** (E1B-55K, che si lega e inattiva p53 e E1B-19K, un omologo del gene cellulare antipoptotico Bcl-2) e la proteina **E4orf6**, che anch'essa si lega e inatti- va p53.

Ad is a lytic virus: mature Ad virions are released following cell lysis



Host immune response

- cell mediated immune response with cytokines production
- T cytotoxic response directed against infected cells
- activation of linfocytes B and antibodies production

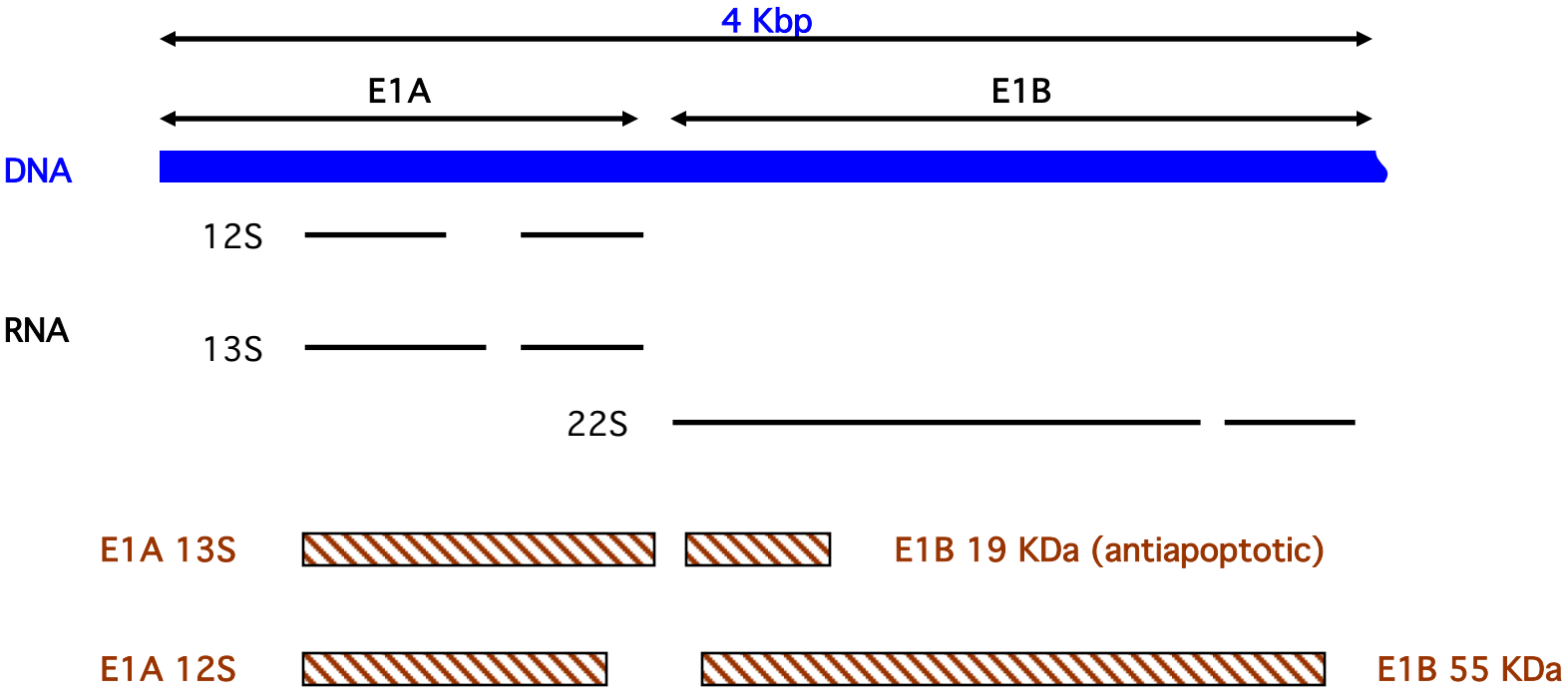
40-60% of infants show anti-Ad Abs against the serotypes 1, 2 and 5

Oncogenesis

the viral E1 has been associated with oncogenesis

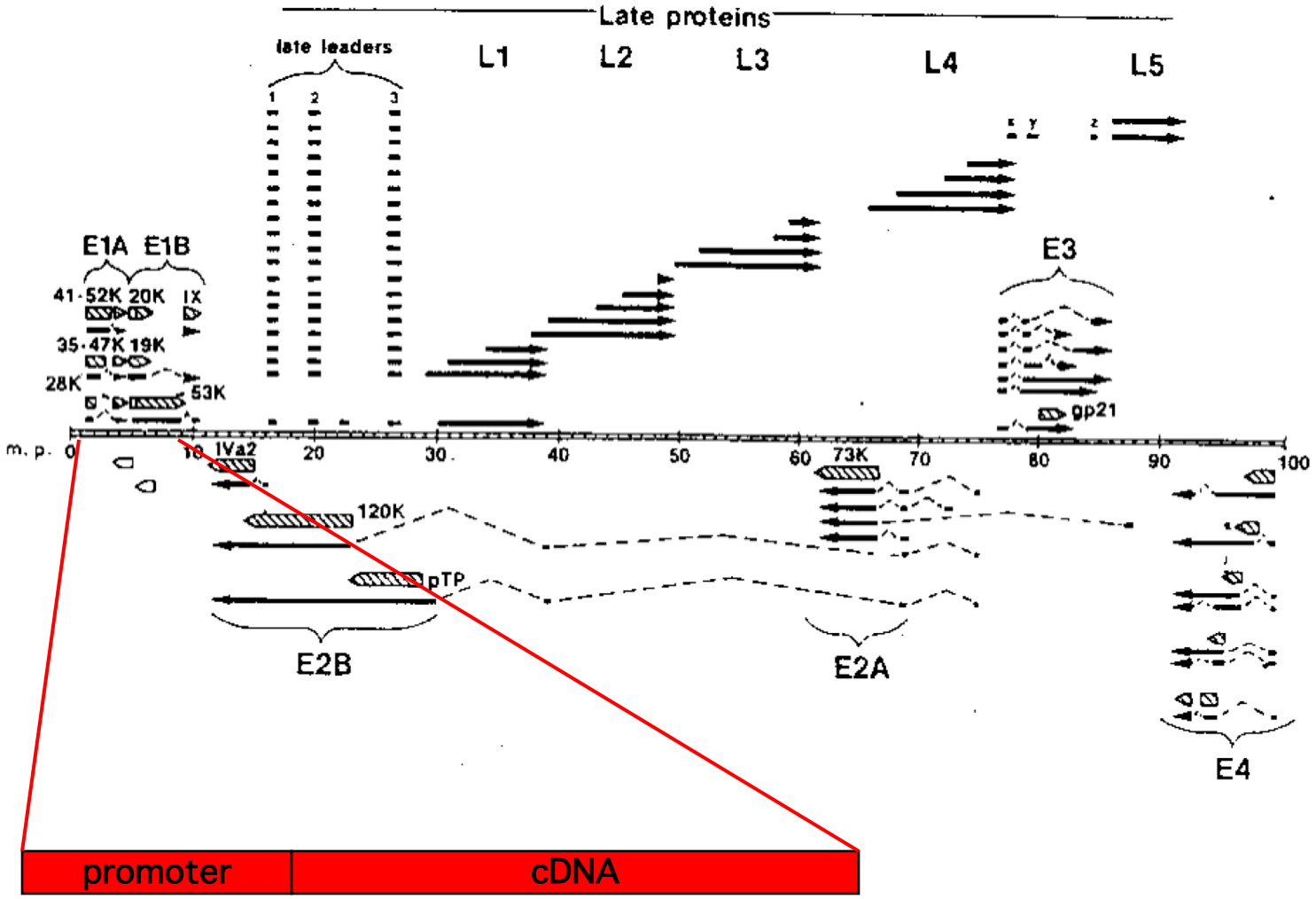
First genetion Adeno-vector:E1 deleted recombiant adenoviral vector

the E1: essential for transcription regulation and viral genome replication



Delezione totale di E1a e parziale di E1b; il transgene è clonato in sostituzione di E1

vettori deleti di E1 (E1D) possono essere prodotti in cellule packging che esprimono E1 in trans (293)



4,7-4,9 kb cDNA

prestazioni dei vettori adenovirali di prima generazione

Derivano prevalentemente dal serotipo 5
Sono difettivi nella replicazione (rimoziene del gene E1 che è richiesto per la replicazione)

Analisi delle barriere immunologiche al trattamento con vettori adenovirali somministrazione di un vettore adeno/lacZ per via biliare in:
•Topi immunocompetenti ➔ gli animali trattati mostravano 80% degli epatociti positivi a 2gg dal trattamento, l'espressione del transgene non era più rilevabile a 20gg dal trattamento
•Topi atimici (non-immunocompetenti) ➔ non mostravano riduzione di espressione del transgene a 60gg dal trattamento

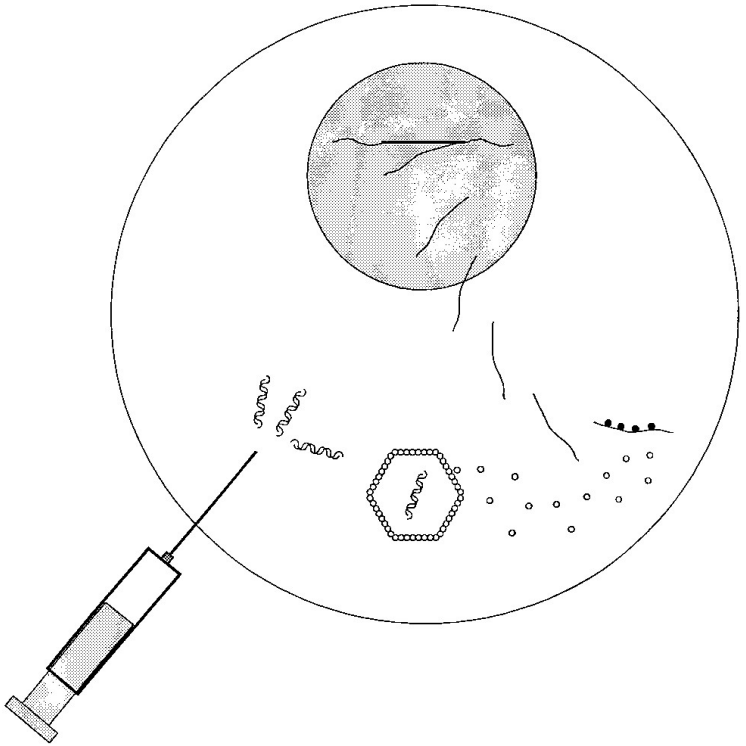
Questi risulatati suggeriscono che **le cellule infettate dal vettore erano eliminate dalla risposta immune cellulo-mediate.**

limitation of the first generation Ad vectors

The utility of these vectors, however, is limited by the continued synthesis of viral proteins by infected cells, despite the genetic deletions.
These proteins make infected cells antigenic and thus reliable to elimination by the immune system.

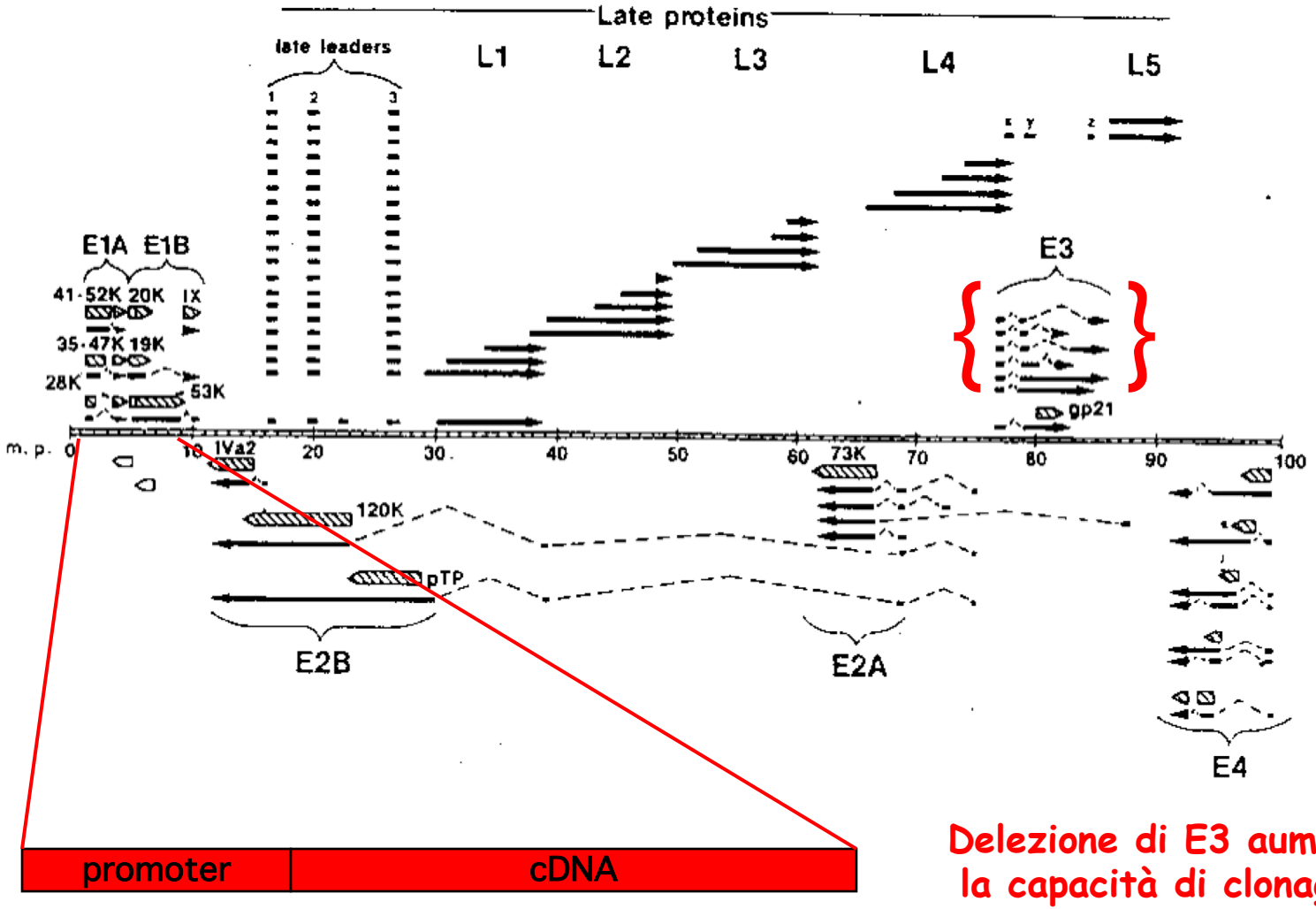
Inoltre

La somministrazione ripetuta dei vettori adenovirali era inefficace per l'insorgenza di una risposta umorale a seguito della prima somministrazione con produzione di anticorpi che neutralizzano il virus impedendo di fatto l'infezione delle cellule bersaglio



per migliorare il potenziale terapeutico è stata proposta la eliminazione del gene E3 che codifica per proteine che modulano la risposta immunitaria

vettori deleti di E1 ed E3 possono essere prodotti in cellule packging che esprimono E1 in trans (293)



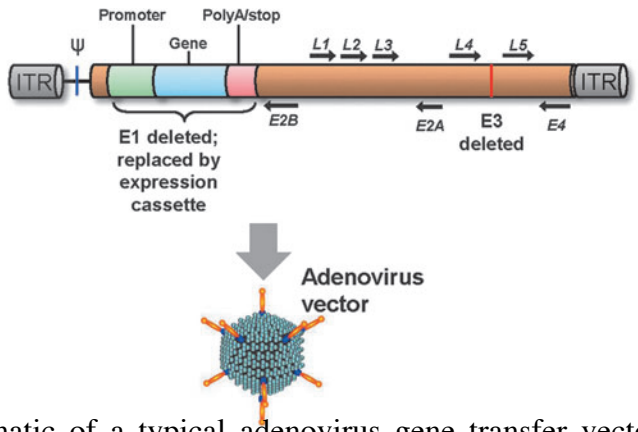
Delezione di E3 aumenta la capacità di clonaggio

4,7-4,9 kb cDNA → 8,3 kb

Early In Vivo Studies

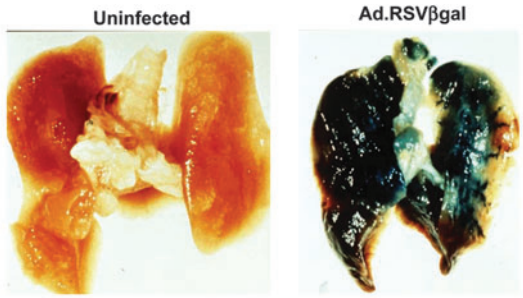
By deleting the E1 genes to prevent replication, and the E3 genes to make more room for the transgene, the common human serotype 5 adenovirus could be converted to a vector that had sufficient room for a promoter and transgene and was replication deficient (A).

We quickly established the system in our laboratory, and in one of those rare eureka moments in any scientist's career when you recognize that an observation in your laboratory may have significant implications, we observed that an E1 - E3 - adenovirus coding for b-galactosidase was strikingly effective in transferring genes in vivo.



Schematic of a typical adenovirus gene transfer vector genome. In an adenovirus vector, the early (E) genes in the E1 region are deleted (to prevent replication) as is the E3 region (to make more room for the expression cassette). The inverted terminal repeats (ITR), packaging signal (w), and the late (L) genes remain in the vector. The deletions allow for an **expression cassette of up to 7–8 kb**. A typical expression cassette, including a **promoter, the transgene, and stop/polyA sequences**, is inserted into the deleted El region. The construct is typically packaged in 293 cells, a cell line that expresses the human adenovirus E1 region, thus providing the components necessary for replication. The vector enters cells through the fiber interacting with the coxsackie- adenovirus (CAR) receptor and secondary integrin receptors

cotton rat model



The first example of effective *in vivo* gene transfer using an adenovirus vector. Examples from a notebook in 1991 from the Crystal laboratory (Pulmonary Branch, the National Heart, Lung, and Blood Institute) of a lung of a cotton rat that had received intratracheal E1Δ- E3Δ– adenovirus coding for β-galactosidase under control of an RSV promoter 7 days earlier. Shown is a control and with AdRSVbgal vector. There is extensive β–galactosidase expression throughout the lung.

Adenovirus-Mediated Transfer of a Recombinant α1-Antitrypsin Gene to the Lung Epithelium in Vivo

MELISSA A. ROSENFELD, WOLFGANG SIEGFRIED, KUNIHIKO YOSHIMURA, KOICHI YONEYAMA, MASASHI FUKAYAMA, LARUE E. STIER, PAAVO K. PÄÄKKÖ, PASCALE GILARDI, LESLIE D. STRATFORD-PERRICAUDET, MICHEL PERRICAUDET, SOPHIE JALLAT, ANDREA PAVIRANI, JEAN-PIERRE LECOCQ, RONALD G. CRYSTAL*

19 APRIL 1991 SCIENCE, VOL. 252

Soon afterward, we demonstrated that an adenovirus vector could be used to effectively transfer and express the normal human AAT cDNA to the liver, the natural site of AAT (α1- antitripsin) expression (Jaffe et al., 1992).

L'Alfa 1-antitripsina o α1-antitripsina (A1AT) è una glicoproteina. È un Inibitore della serin proteasi. Il deficit di alfa 1-antitripsina è un disordine genetico a trasmissione autosomica recessiva, nel quale si ravvisa una diminuzione della proteina sia nel sangue che nel fegato. A livello epatico si registra la deposizione in eccesso di una variante anormale della alfa 1-antitripsina che si accumula negli epatociti causando ingombro meccanico e, a lungo termine, insufficienza epatica. Deficit acuti della proteina causano enfisema e broncopneumopatia cronica ostruttiva nel soggetto adulto così come disordini epatici nei soggetti giovani.

the back side of the moon

In our initial enthusiasm, however, we and others did not recognize an important biologic fact that the human adenovirus is highly immunogenic, and that this immunogenicity would limit the time of expression, despite how highly efficient gene transfer is by adenovirus.

second generation Ad vectors 1996

Two approaches have been followed to construct second generation Ad-vectors: 1) functional deletions in the E2 gene; 2) deletion of E4.
Both showed a clear improvements with respect to immunogenicity and toxicity. However, inactivation of proteins encoded by the E4 gene has been shown to impair seriously expression from heterologous promoters

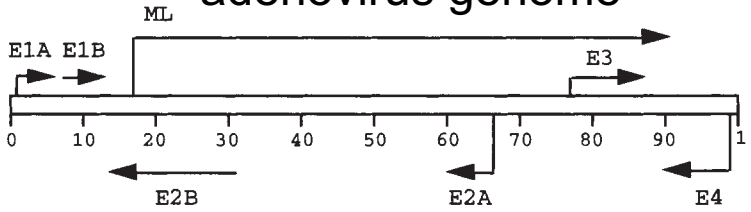
Alternatively, it has been considered the possibility to add immune-suppressing genes.

Second generation Ad vectors - *ts* E2A

E2A encods for a ssDNA binding protein essential for DNA initiation

the first approach to improve 1st Ad vector was to introduce a mutation into the E2A gene making the gene product ts (inactive at 39° C).

adenovirus genome



- starting from a ΔE1 vector
- tsE2, temperature sensitive phenotype, lethal at 39 ° C; expression of late proteins is reduced at the non permissive temp.

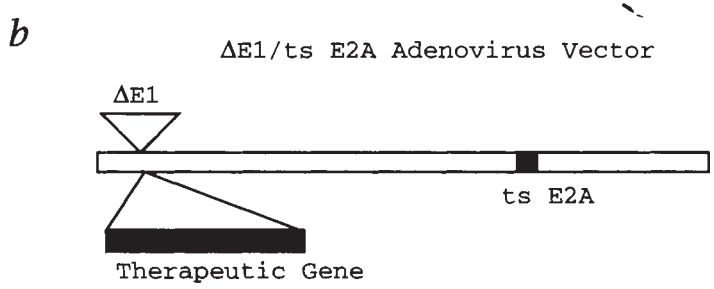


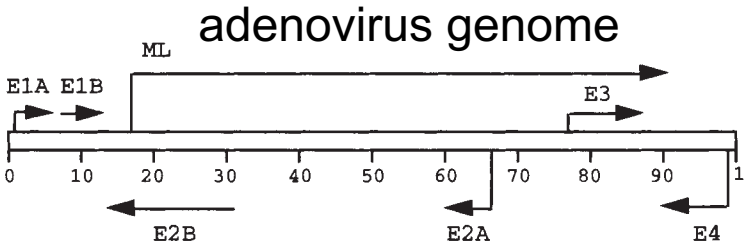
Fig. 1 Second-generation adenovirus vectors. *a*, The adenovirus genome (36 kb) is divided into 100 map units. The E1A, E1B, major late (ML) and E3 gene regions are transcribed from the negative strand. *b*, The ΔE1/*ts* E2A adenovirus vectors contain a G→A transition at nucleotide 1064 of the DBP cDNA resulting in a temperature-sensitive phenotype (lethal at 39 °C),

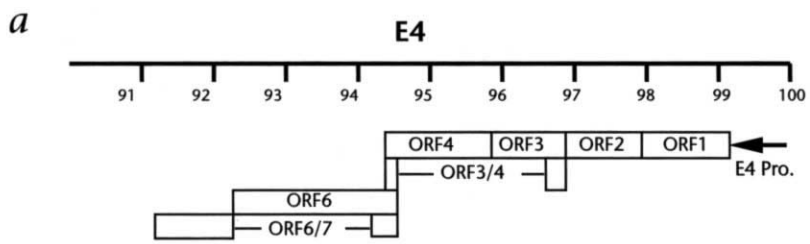
Second Generation Ad vector in animal model: lack of persistence of Factor IX gene expression by this vector in animal model was observed

Second generation Ad vectors - ΔE4

the second approach to improve 1st Ad vector was to introduce a deletion of E4

E4 encodes for 7 ORFs that impact many events of the viral life cycle.

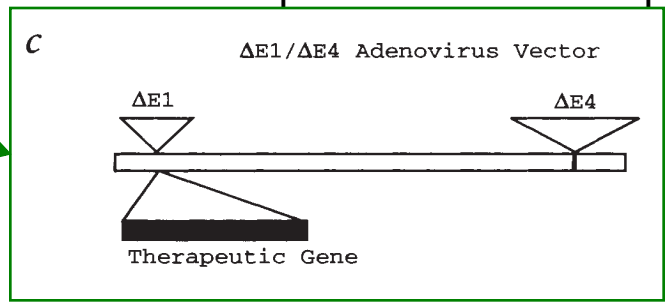




To allow vector production by ΔE1 ΔE4 vectors, the deleted functions need to be provided in trans.

E4 was successfully expressed from an integrated copy within 293 cells **(293-E4)**, the transgene was expressed by an heterologous promoter, so that to makes the expression of E4 independent from E1

Ad - ΔE1 ΔE4 vector , in addition to the E1 region deletion[12]. *c*, The DE1/DE4 adenovirus vectors contain a lethal deletion in the E4 region, in addition to the E1 region deletion[25].



by this vector high level expression and persistence of the transgene *in vivo* in animal models for at least 6 months

These attempts to limit antivector immunity proved fruitless: the adenovirus is too immunogenic in humans, and the immune system too subtle in its ability to recognize adenovirus epitopes.

possible solution

Gutless-vector. The development of helper-dependent, so-called ‘‘gutless’’ adenovirus vectors, where all of the adenovirus genes are removed, with the necessary genes to create the vector provided by the producer cells.

Seroswitch. to circumvent the antiadenovirus immunity elicited by the initial administration of an adenovirus gene transfer vector by administering an adenovirus vector comprised of a different serotype carrying the same gene, a strategy that is effective in experimental animals.

gutted adenovector 2000

Is it possible to eliminate all the trans-acting viral sequences?

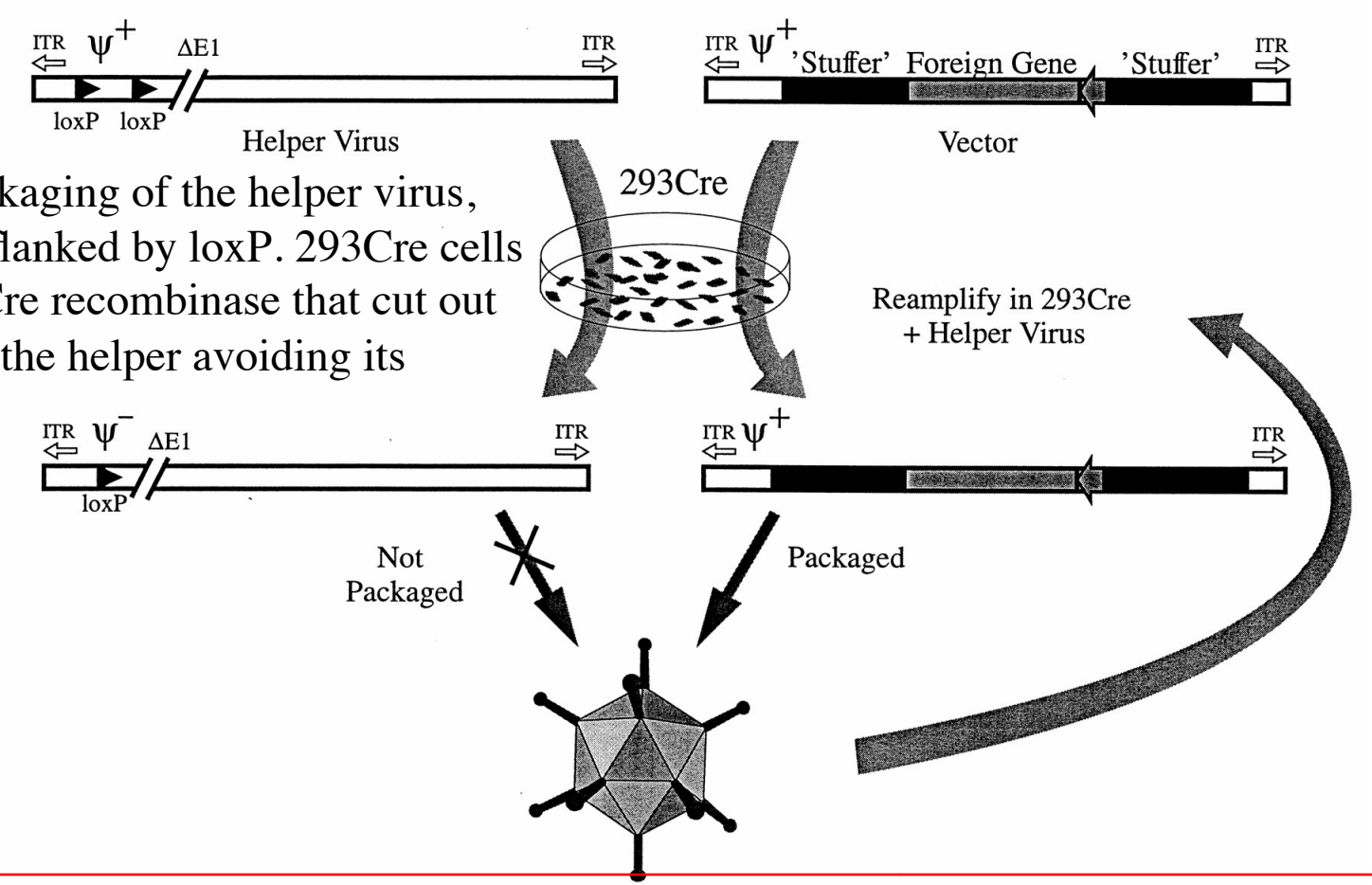
Attempt to do so gave rise to genome rearrangments when the size of the deleted viral genome was less than 75% (27 Kb) of the wt DNA (36 Kb).

Is it possible to circumvent this problem? and if yes, how?

In the latest versions of adenoviral vectors, all viral coding sequences have been eliminated.

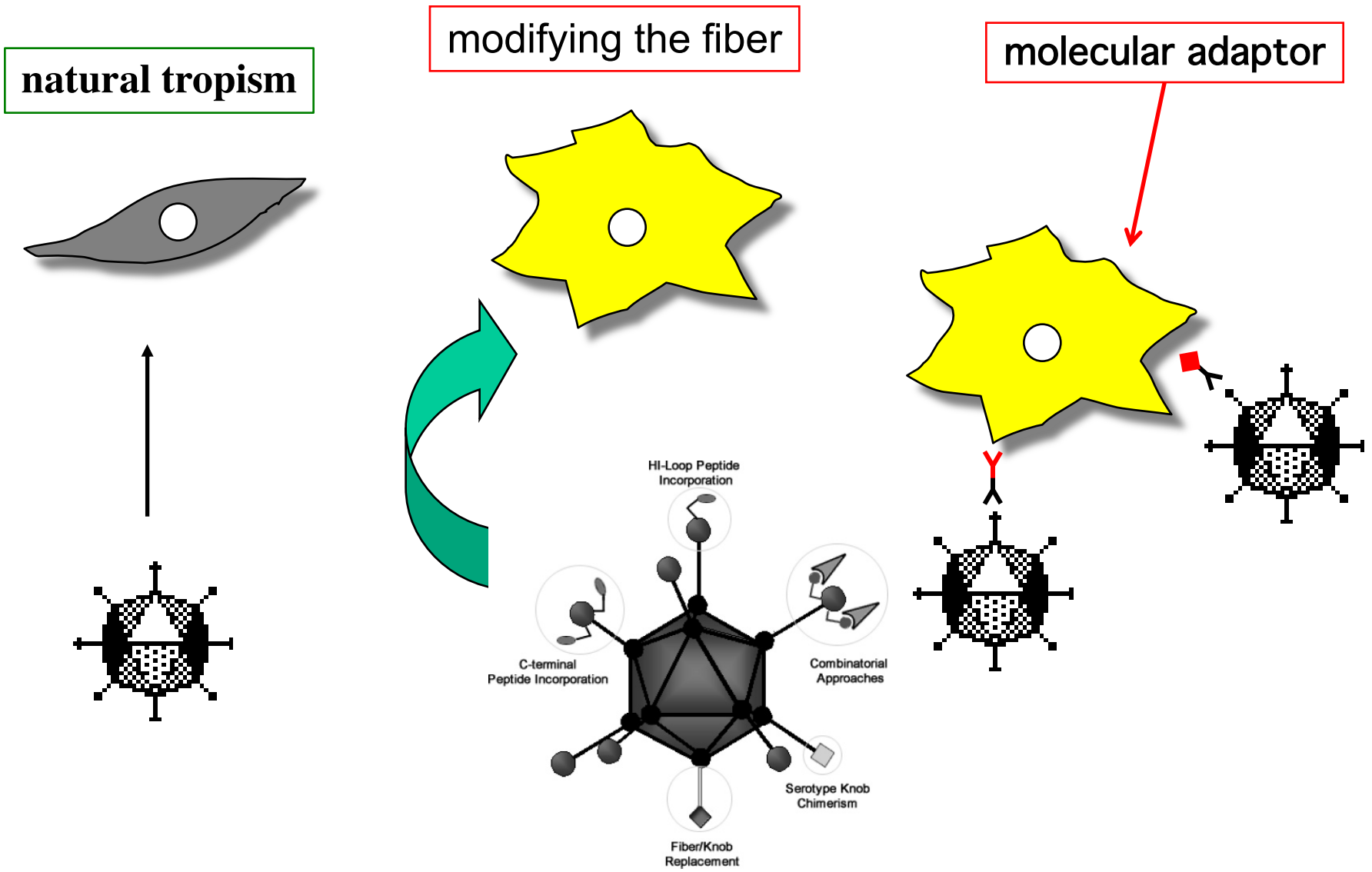
In the "Gutted" adenoviral vector the entire Ad genome, except the ITR and ψ site, has been eliminated. The vector was provided with stuffer DNA that aids to rich the size required to avoid vector rearrangments.

To avoid packaging of the helper virus, the ψ site is flanked by loxP. 293Cre cells express the Cre recombinase that cut out the site from the helper avoiding its packaging.

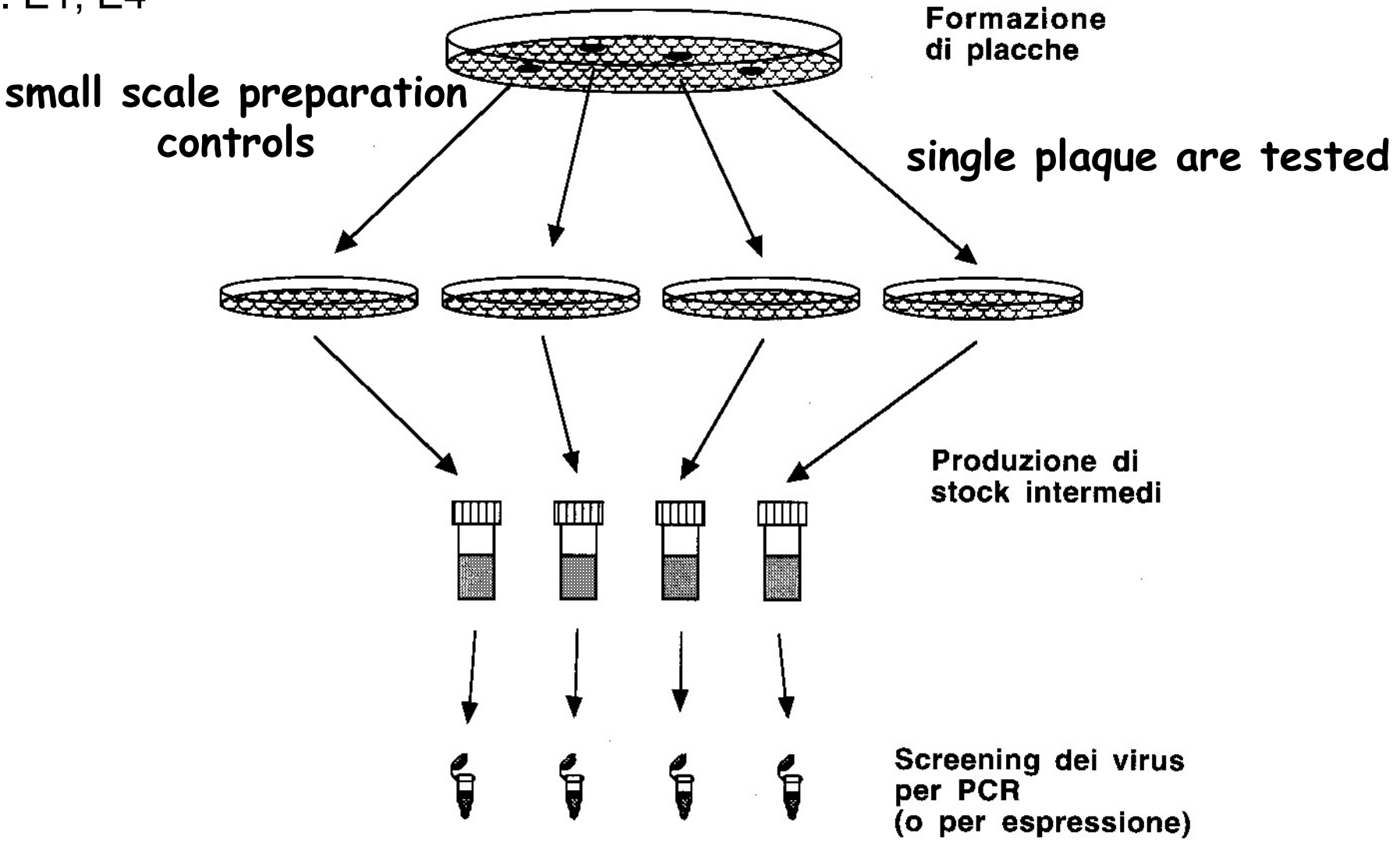


A challenge for the Ad vector system is that the vector preparations are contaminated with low levels of helper Ad virus

The Ad virus is non-envelopped, how to change the target specificity?
this may be important to enlarge application of this vectors to cell types other than CAR-positive cells.
Two strategies may be used: 1) modify the fiber; 2) use adaptor

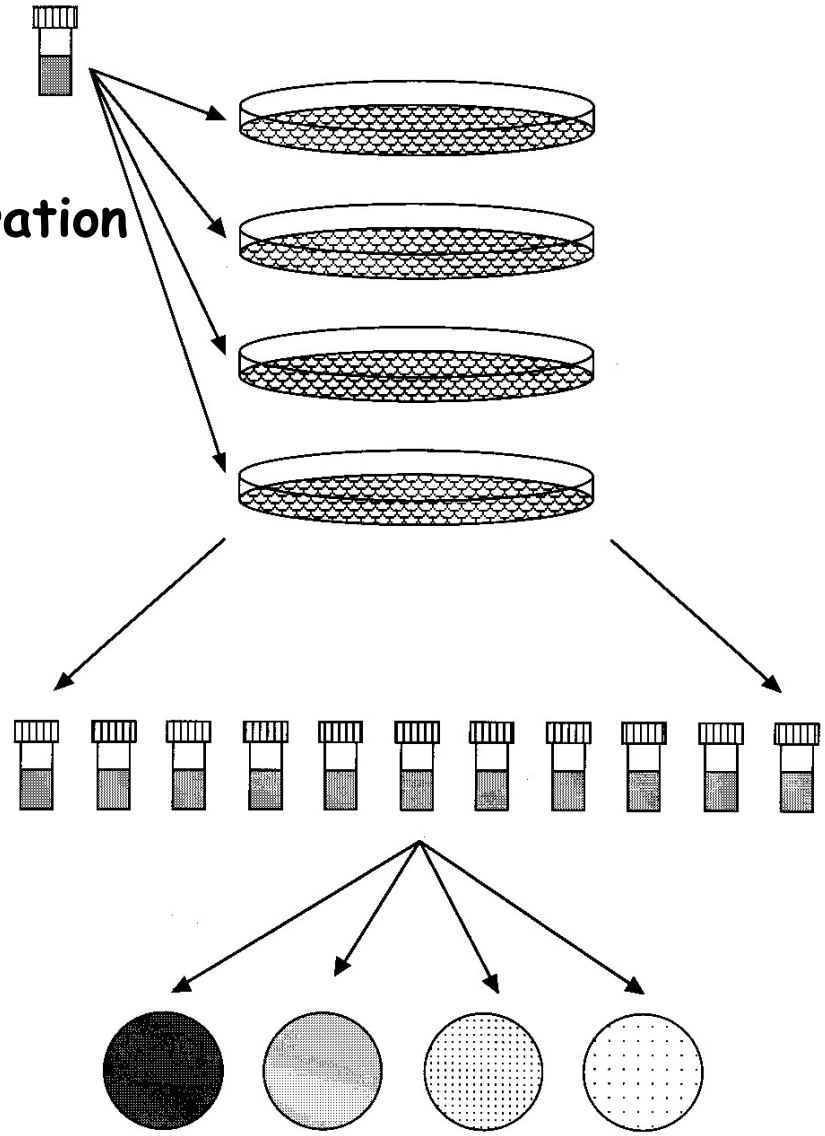


Ad-vector production: the packging cells are transfected with the Ad-vector containing the transgene or the Ad-vector + the helper.
Depending on the vector used, packaging cells require trans actiing viral genes i.e. E1, E4



Produzione stock virale su larga scala

large scale preparation
and titration



Titolazione per plaque test

Biblio

Crystal 2014 Adenovirus:The First Effective In Vivo Gene Delivery Vector. Hm Gene Therapy 23, 3-11

Cots D. et al “Helper Dependent Adenovirus Vectors: Progress and Future Prospects” Current Gene Therapy, 2013, 13, 370-381

Russel 2012 “Oncolytic virotherapy” Nature Biotechnology

Daya et al 2008. Gene Therapy Using Adeno-Associated Virus Vectors. Clin Microbiol Rev.