Thema Gentechnologie

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2. VL

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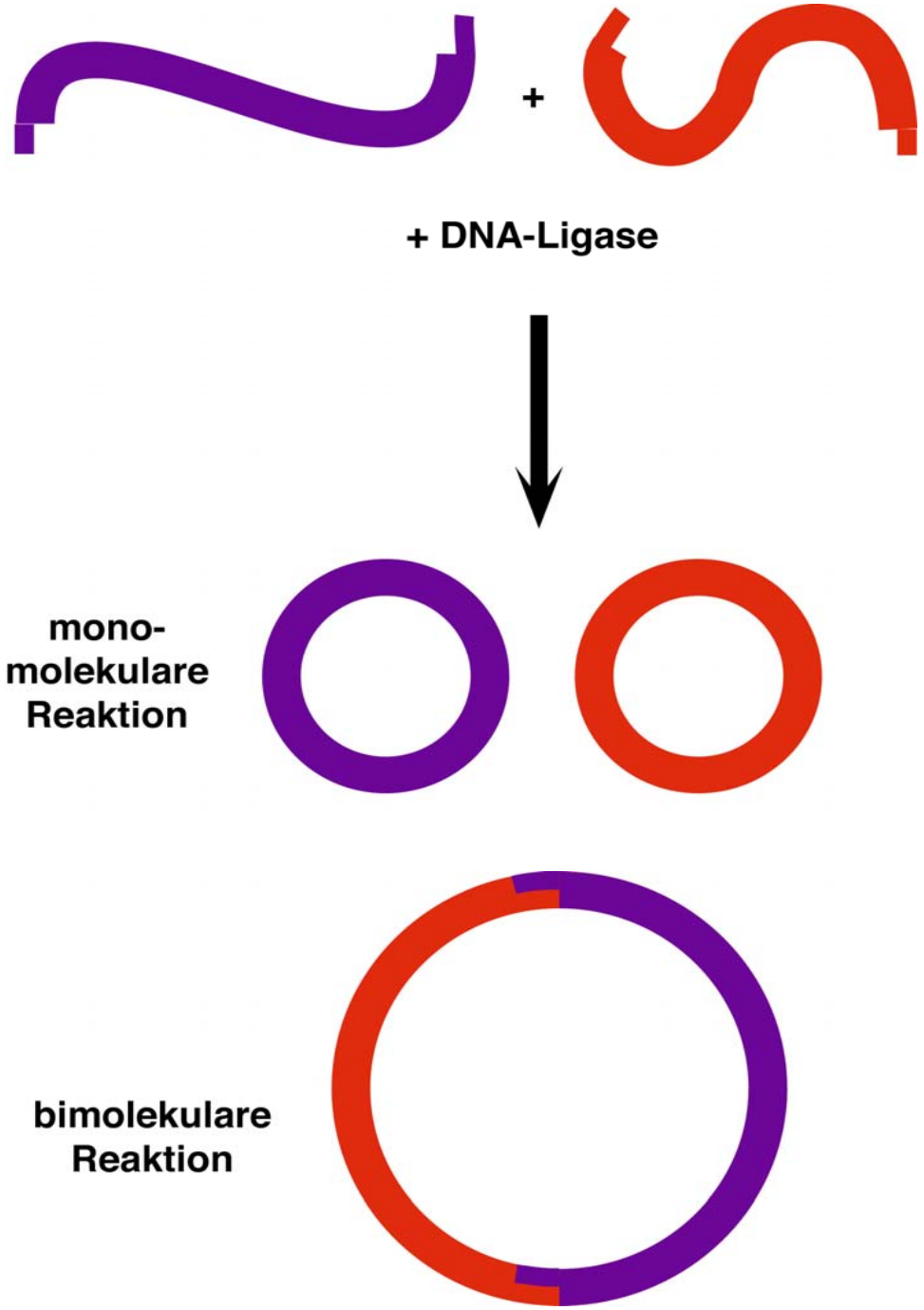
Die Voraussetzungen für Gentechnologie

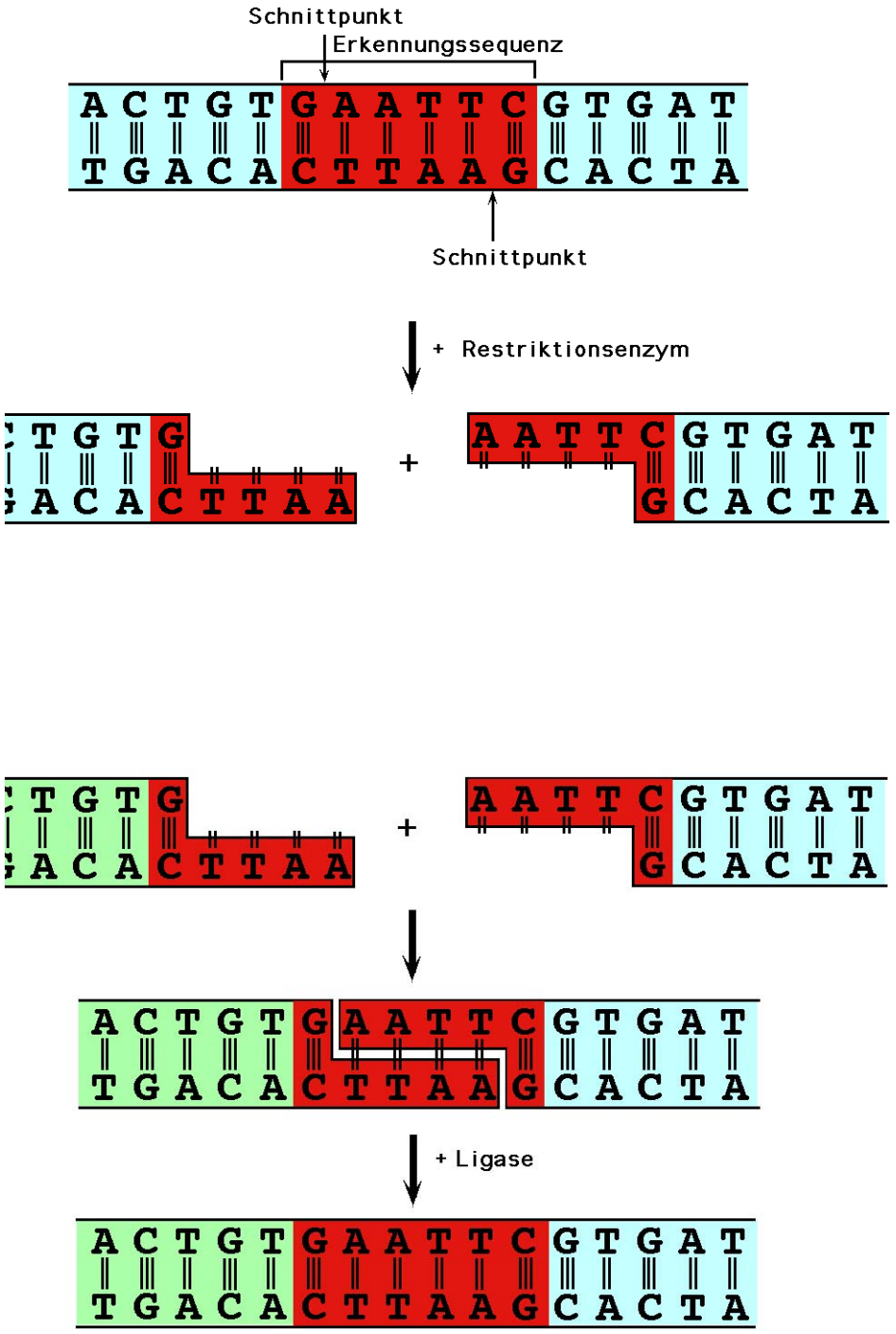
- Spender-DNA (z. B. ein Gen)
- Vektor-DNA
- Restriktionsenzyme
- DNA-Ligase
- DNA-Transfermethoden
- Wirtsorganismen

In der Gentechnologie
ist die Neukombination unterschiedlicher DNA-Moleküle wichtig.

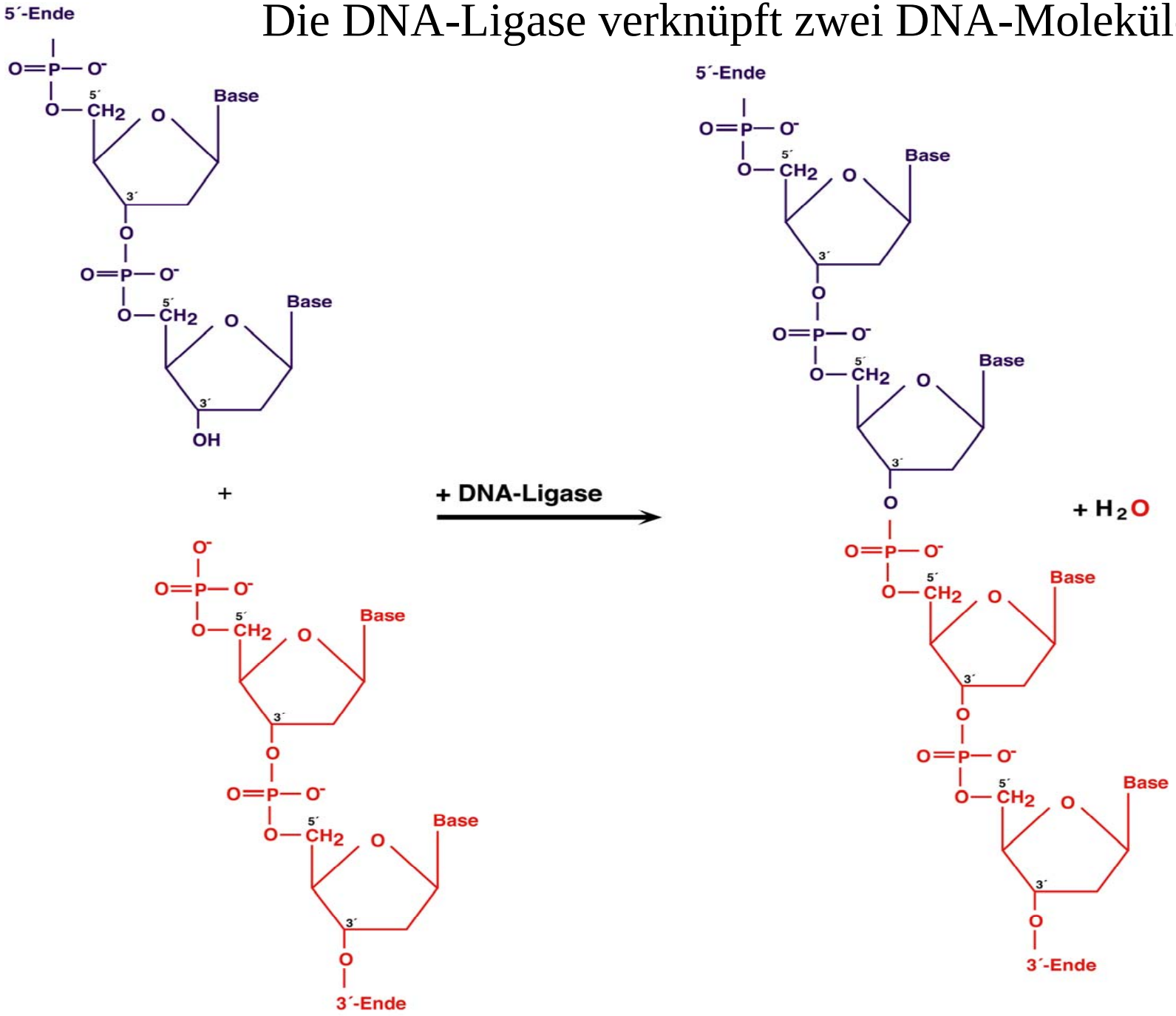
Das Verknüpfen von DNA-Molekülen erledigt das Enzym „DNA-Ligase“

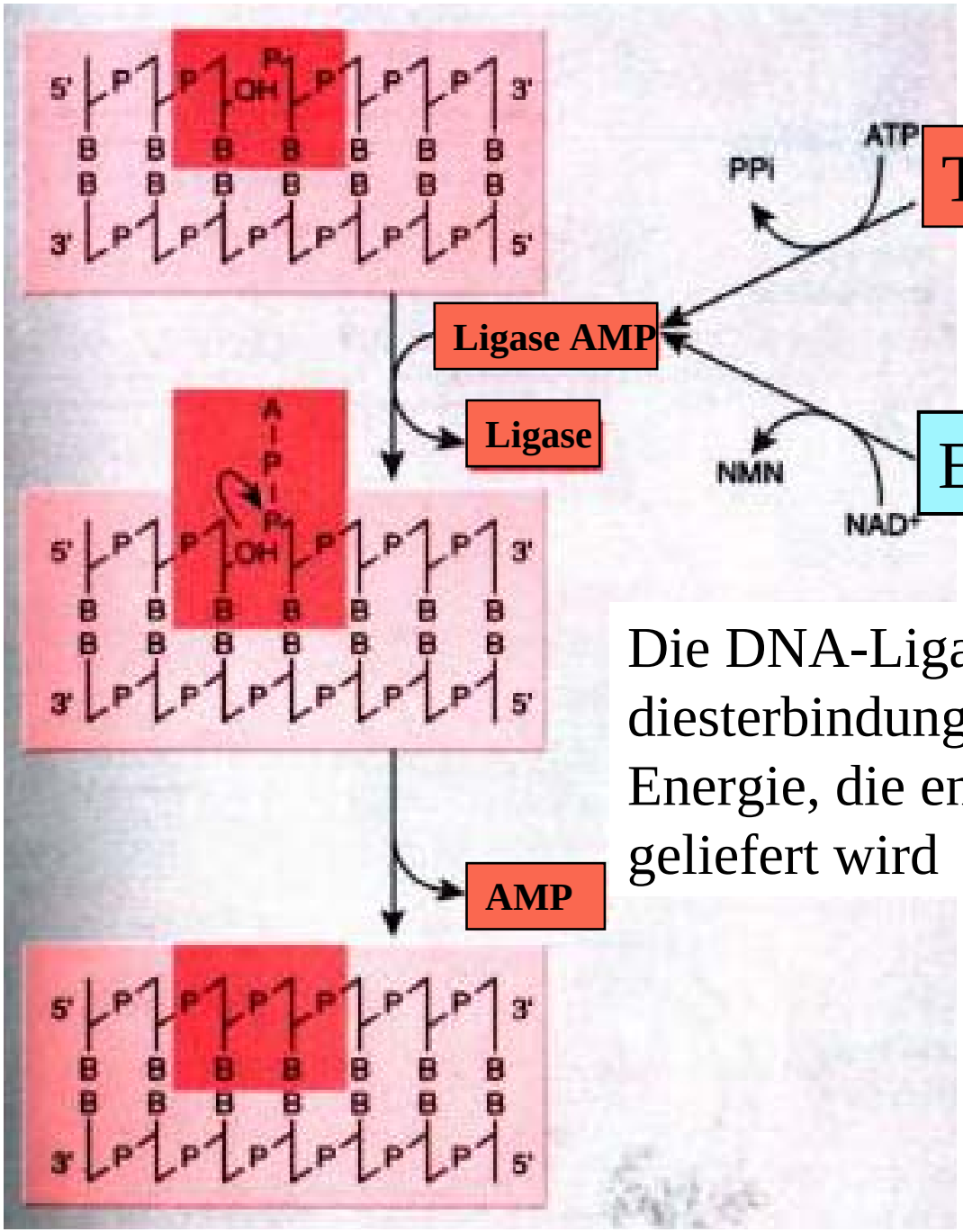


Komplementäre überhängende Enden („Sticky ends“) erleichtern das Wiederverknüpfen von DNA-Molekülen



Die DNA-Ligase verknüpft zwei DNA-Moleküle



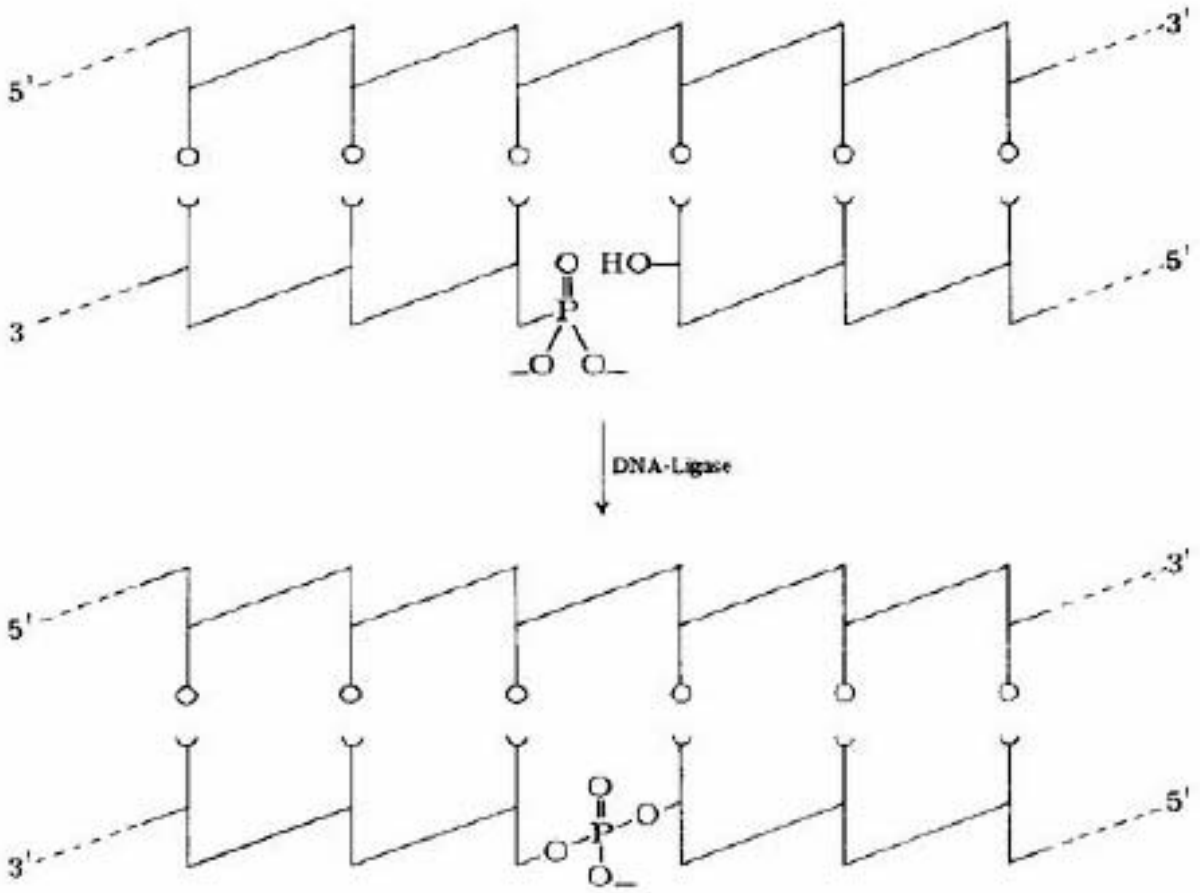


T4-DNA-Ligase

E. coli DNA-Ligase

Die DNA-Ligasen sind in der Lage, Phospho-diesterbindungen zu knüpfen. Sie brauchen dafü Energie, die entweder durch ATP oder NAD+ geliefert wird

Die DNA-Ligase schließt nur „nicks“



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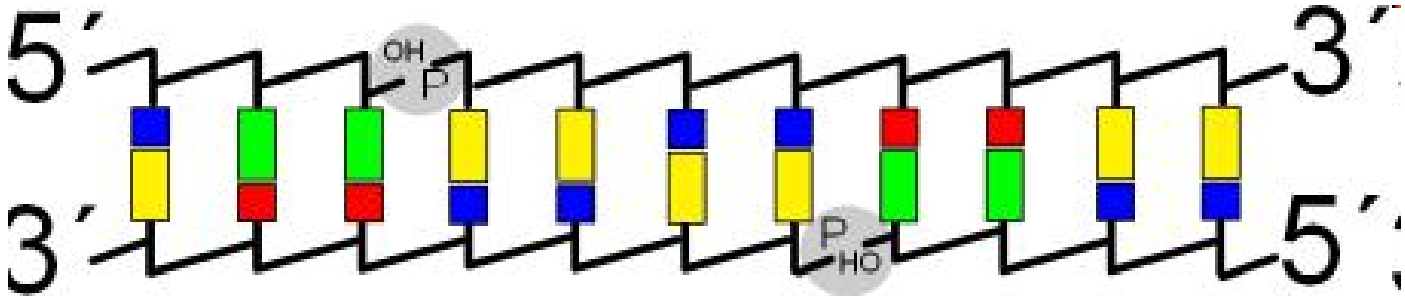
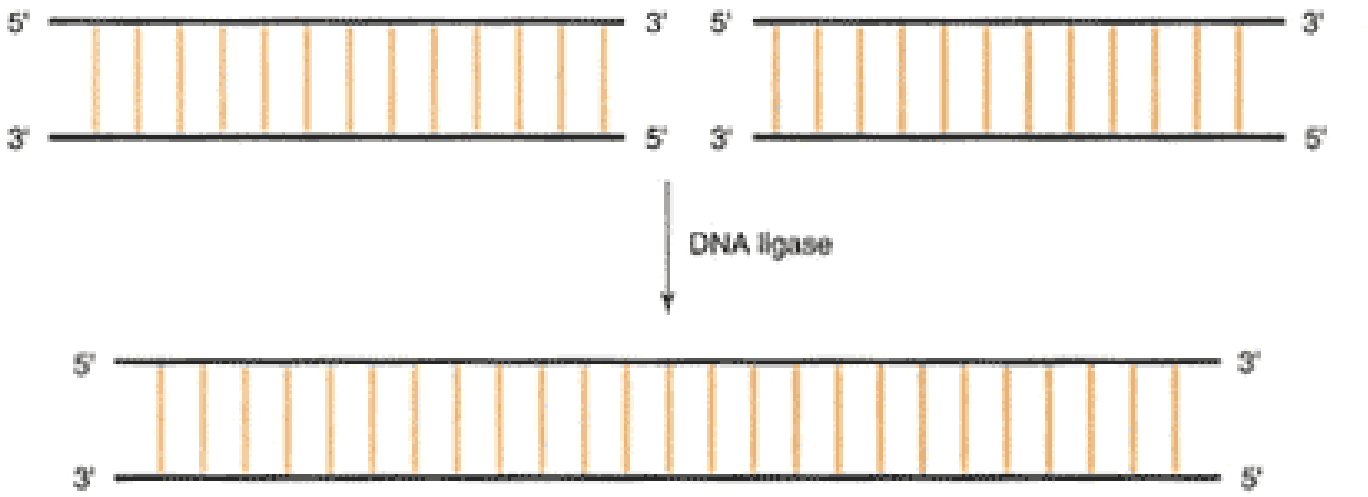
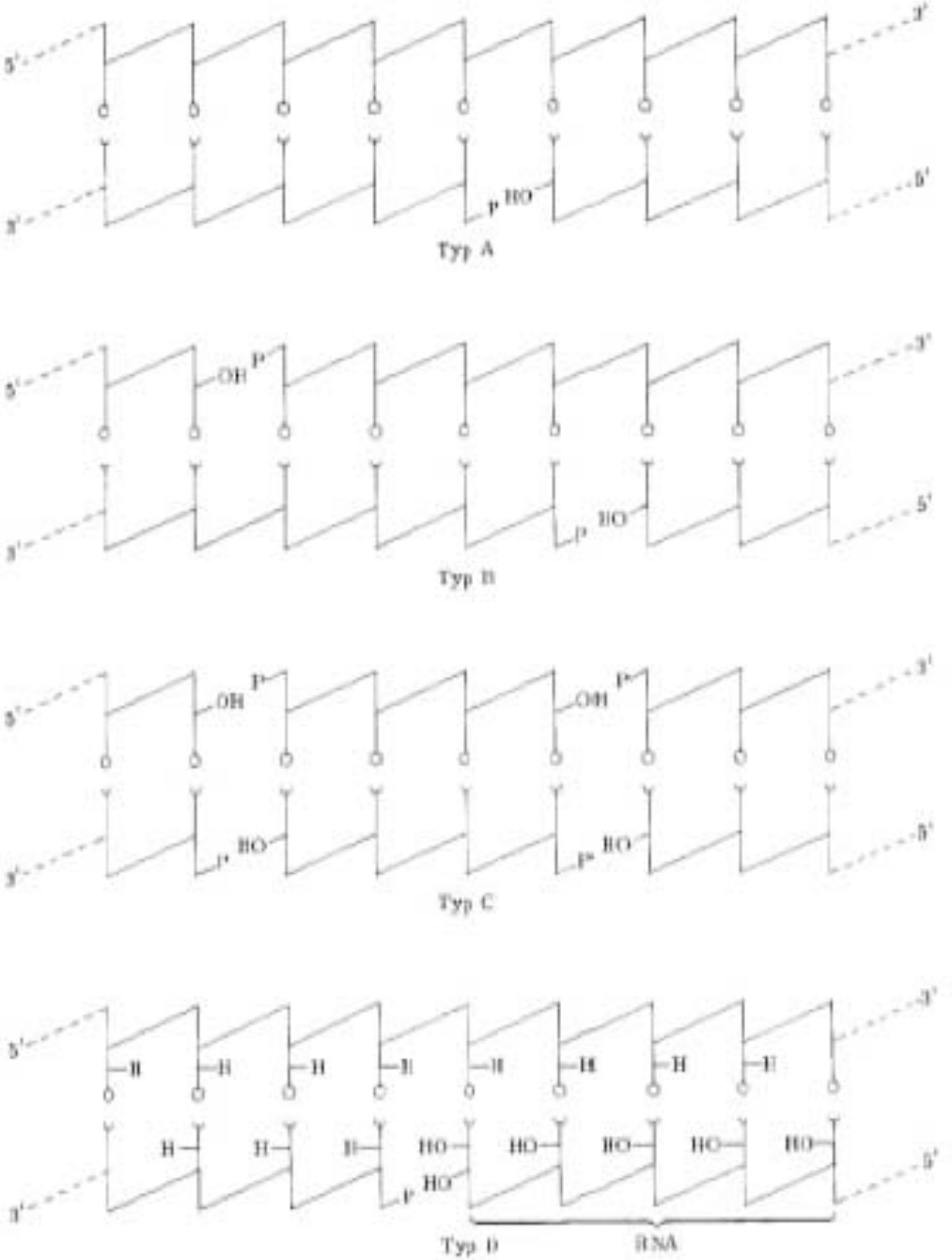


Oder verbindet „glatte“ Enden miteinander



Substrate der verschiedenen Ligasen



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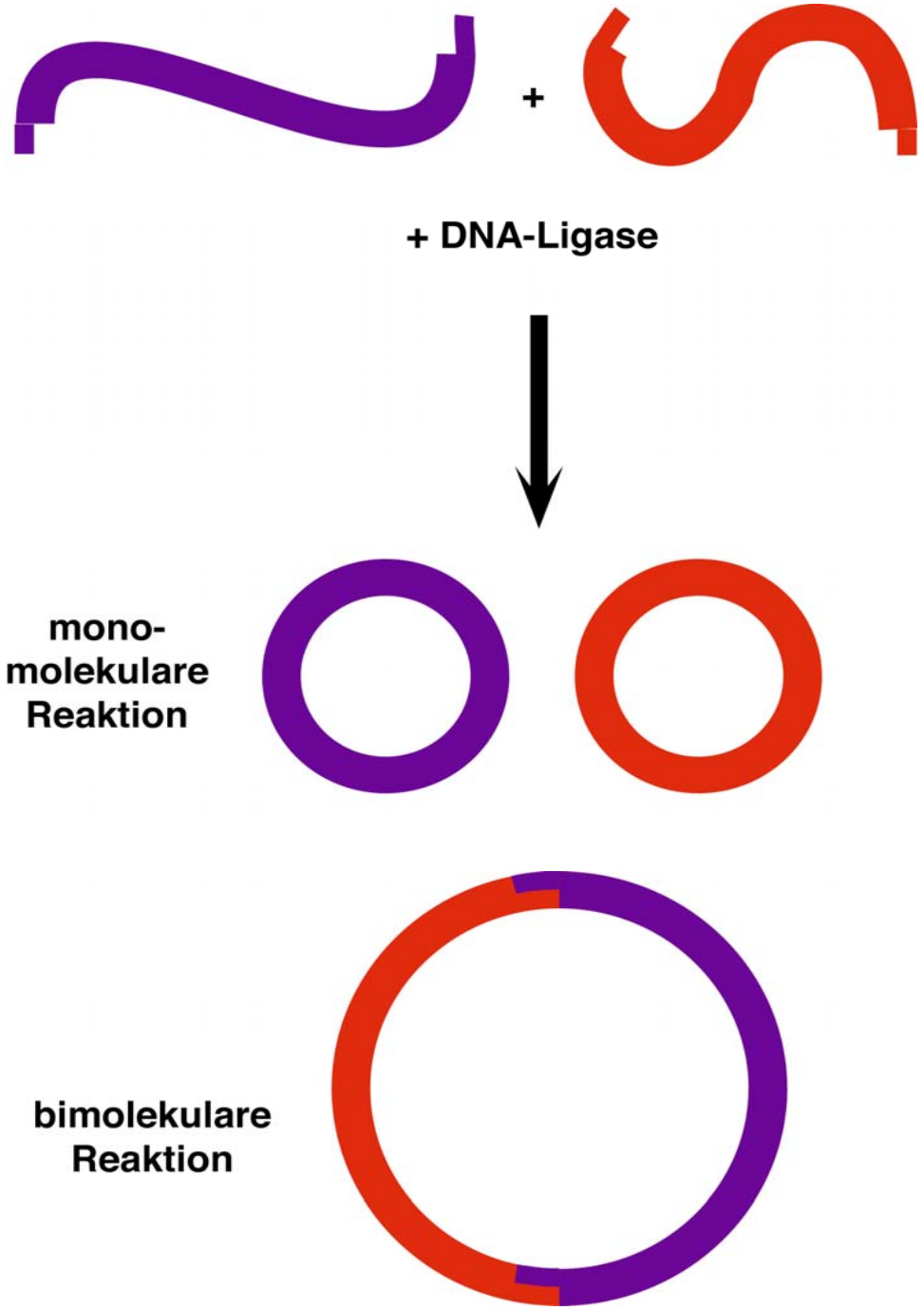
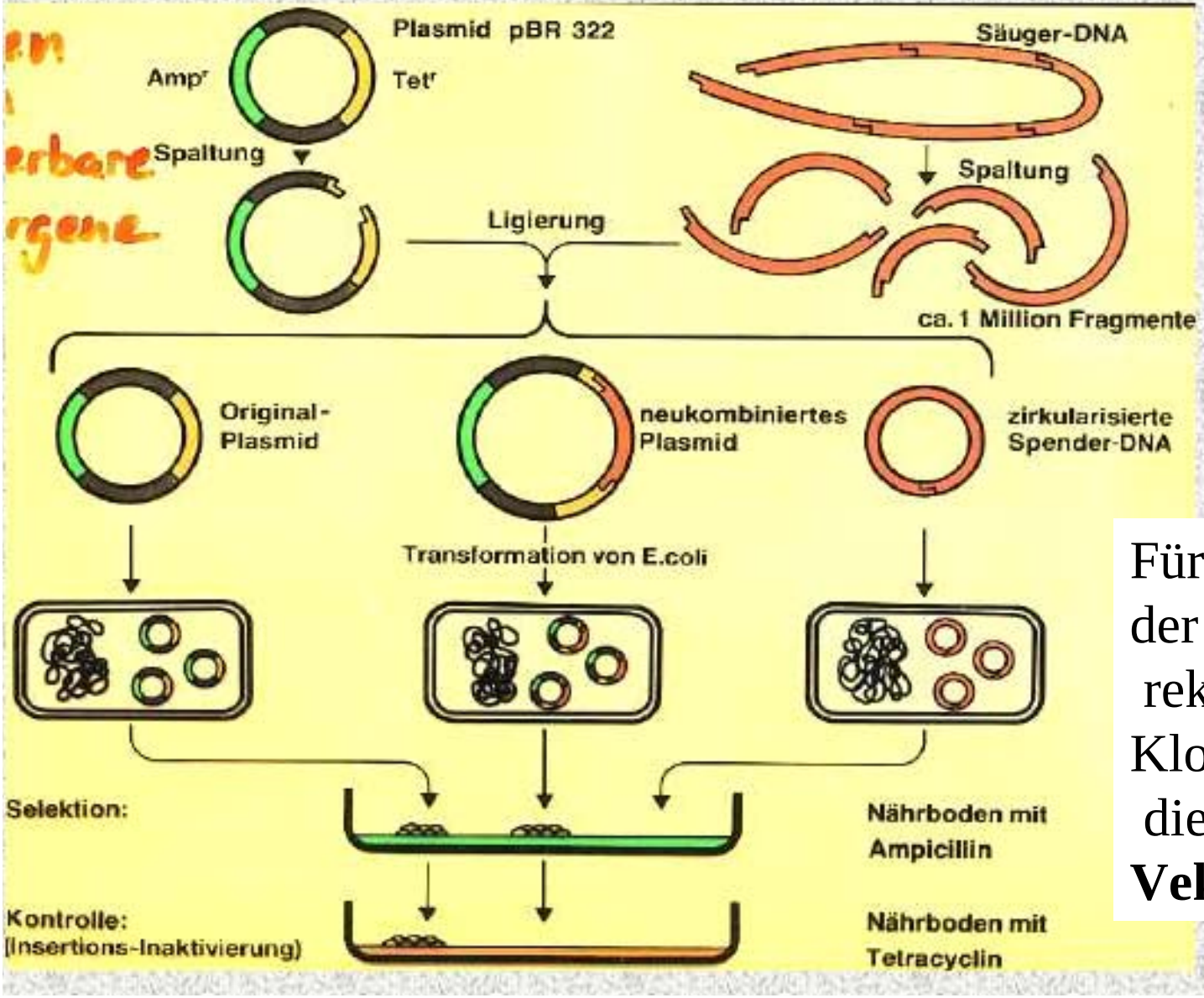


Bei der Ligation entstehen nicht nur die gewünschten rekombinanten Moleküle



Für die Selektion der gewünschten rekombinanten Klone braucht man die **Vektor-DNA**

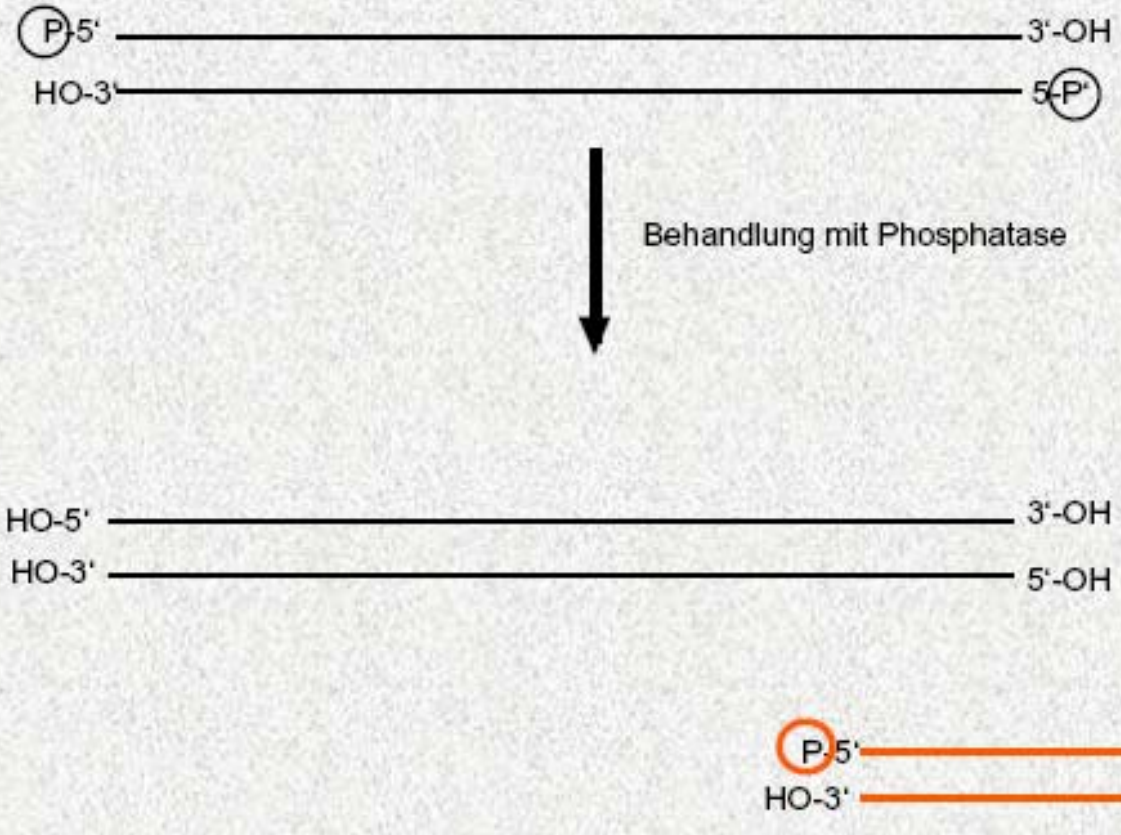
Wie kann man die Ligationsreaktion in die gewünsche Richtung treiben?

Vektor und Integrat-DNA werden mit je zwei R-Enzymen geschnitten, so dass jeweils ein Molekül inkompatible Enden hat

Die Vektor - DNA wird mit Hilfe von Phosphatase dephosphoryliert, so dass der Vektor nicht rezirkularisieren kann

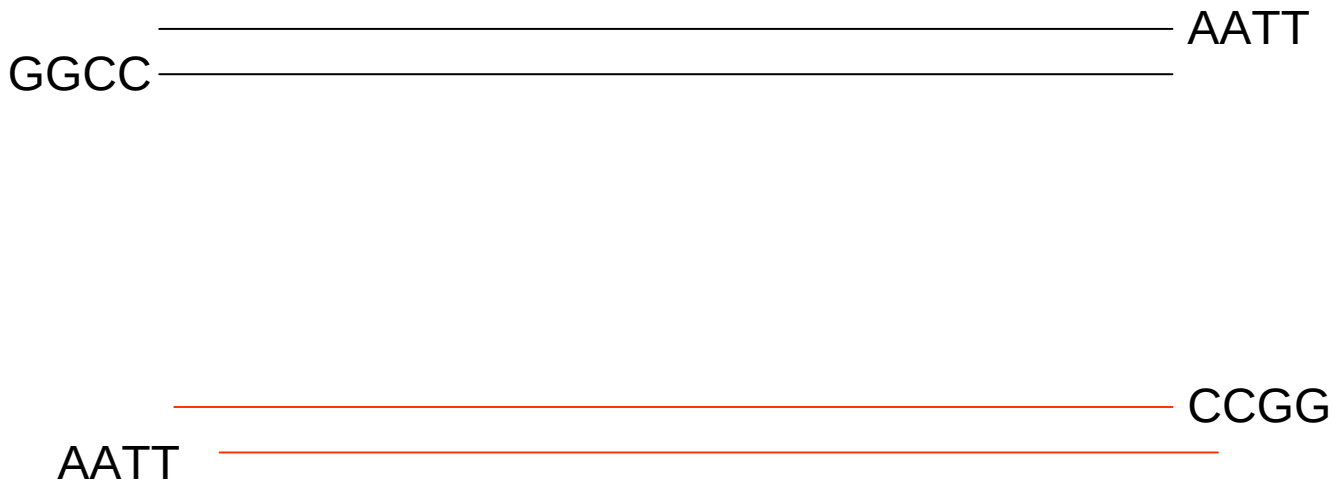
Die Konzentrationen von Vektor- und Integrat-DNA werden so, gewählt, dass überwiegend bimolekulare Reaktion erfolgt

Phosphatase-Behandlung verhindert die Religation des Vektors



nur mit Integrat-DNA kann eine Ligation erfolgen!

Asymmetrische Restriktion von Vektor und Integrat-DNA



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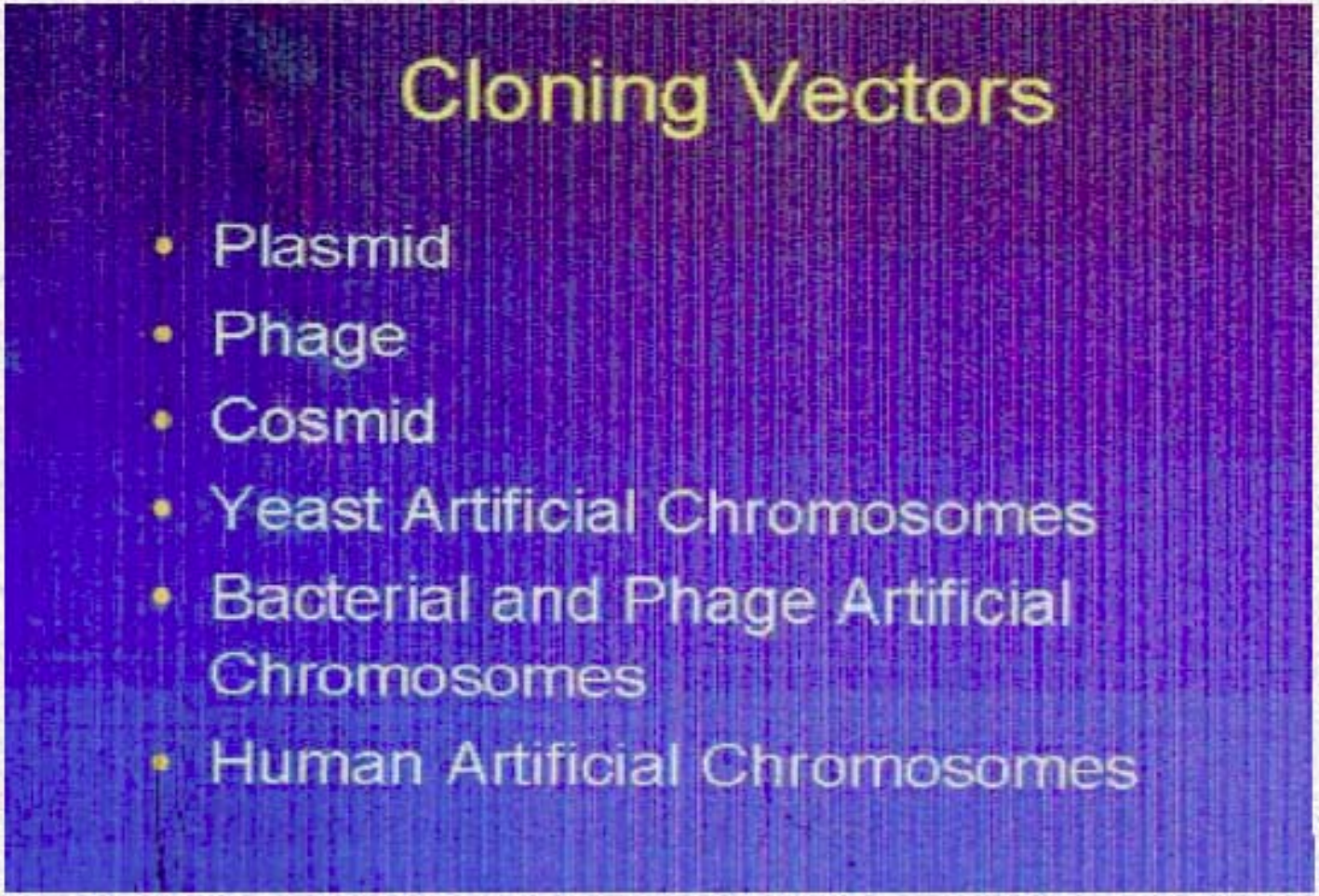
- Wirtsorganismen

Funktionen der Vektor DNA

- Sorgt für Replikation in der Wirtszelle
- Stellt selektierbare Markergene bereit
- Hat Vielzweck-Klonierungsstelle
- Trägt Signalsequenzen für Genexpression
- Verschiedenste Modifikationen für spezielle Anwendungen (z. B. „Shuttle“ zwischen Pro- und Eukaryoten; Elemente für künstl. Chromosomen)

Entscheidend für den Erfolg in der Gentechnologie ist die Wahl des geeigneten Vektors



Typische **Vektoren** besitzen einen **Replikationsorigin**,
selektierbare **Marker-Gene** und eine **multiple Klonierungsstelle**

Typische **Vektoren** besitzen einen **Replikationsorigin**, selektierbare **Marker-Gene** und eine **multiple Klonierungsstelle**

Marker-Gene sind wichtig für die Selektion der gentechnisch veränderten Zellen

- Antibiotika-Resistenz
- ß-Galactosidase
- LUX-gene/Luciferase
- fluoreszierende Proteine
- Auxotrophie
- Suizid-Gene
- Augenfarbe
- (bei Drosophila)

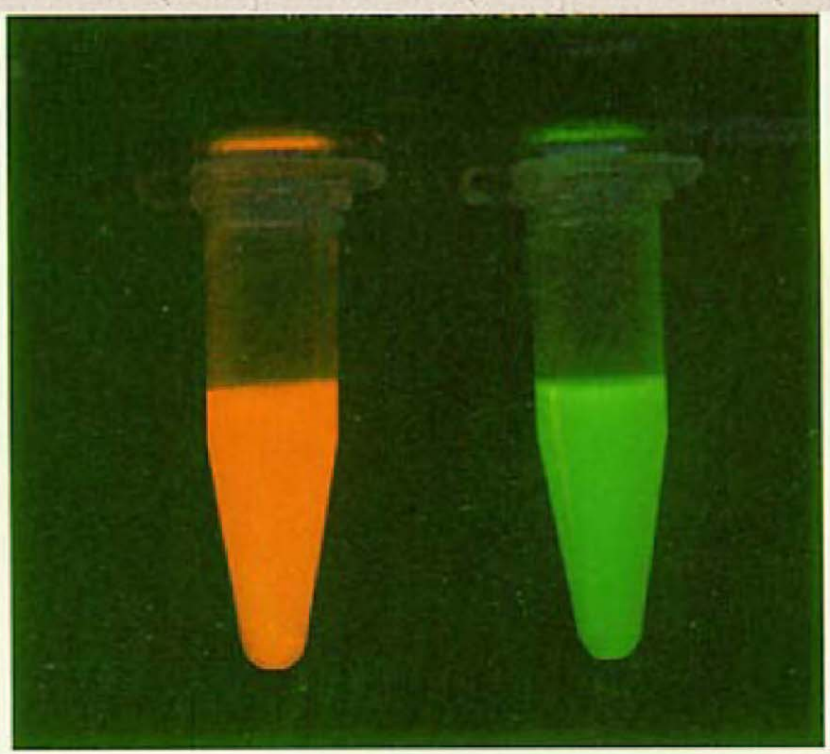


Figure 1. Bright fluorescence of DsRed and GFP.

Inzwischen gibt es die verschiedensten Farbvariationen als Marker oder Reportergene

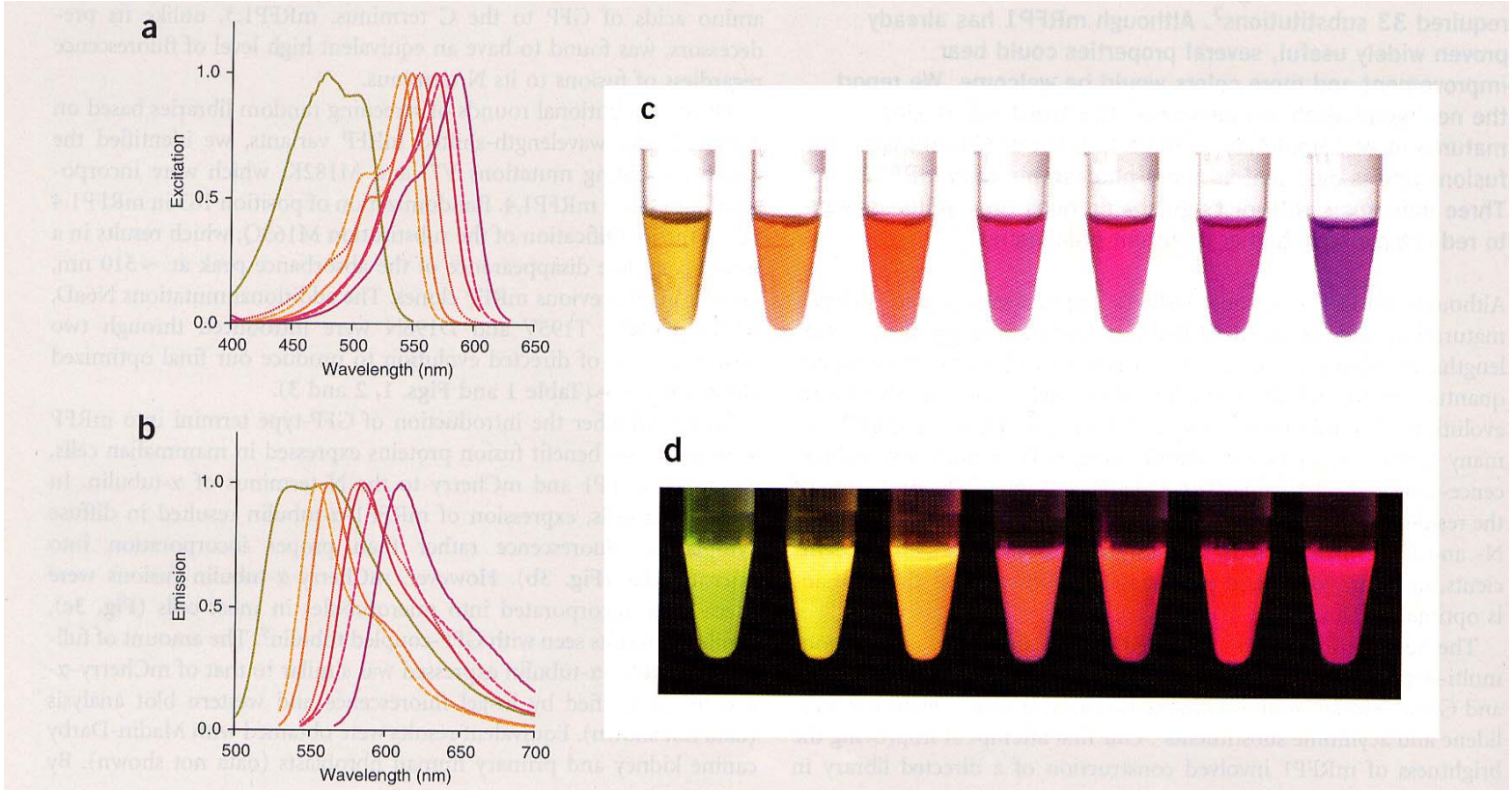
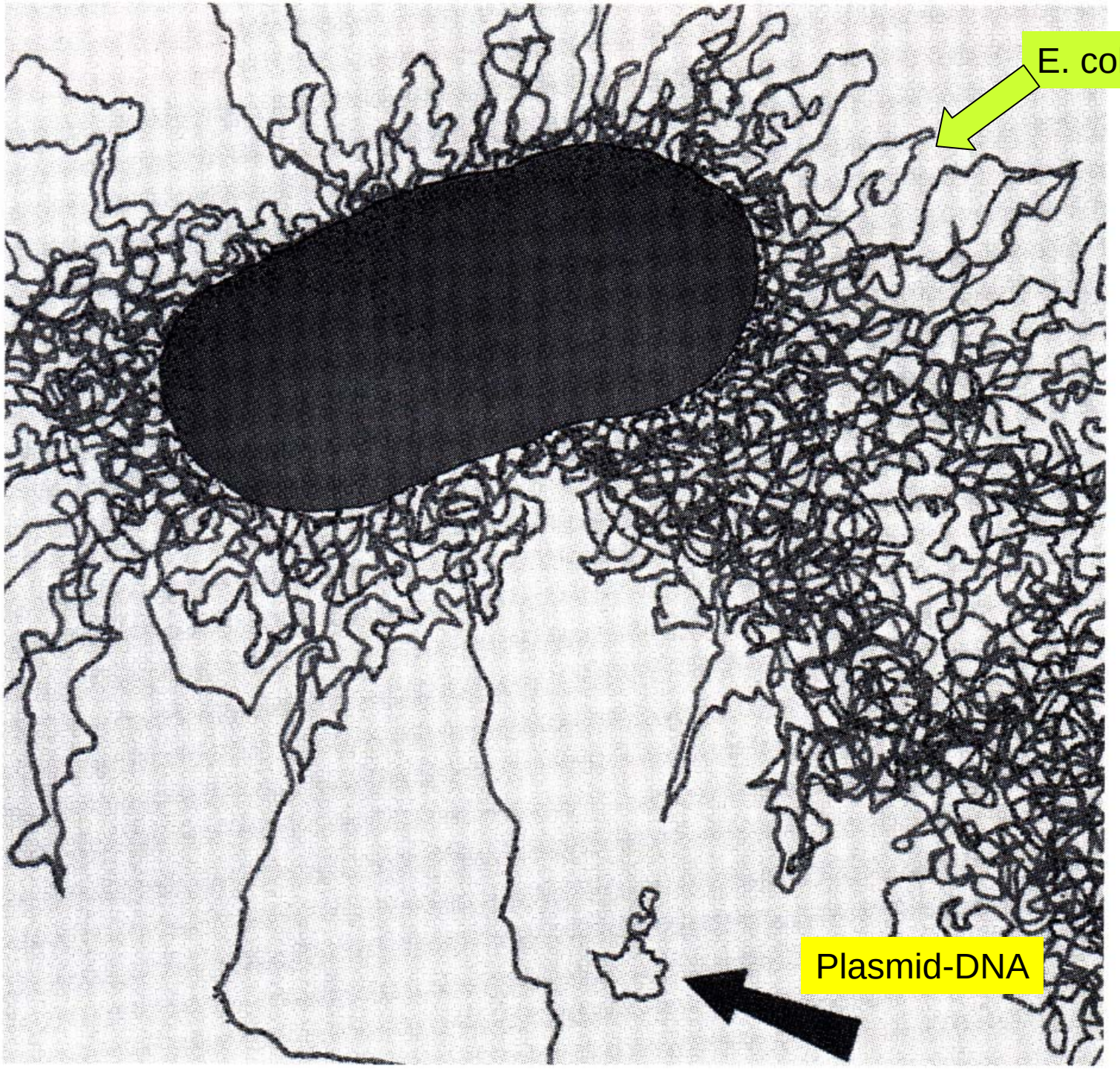


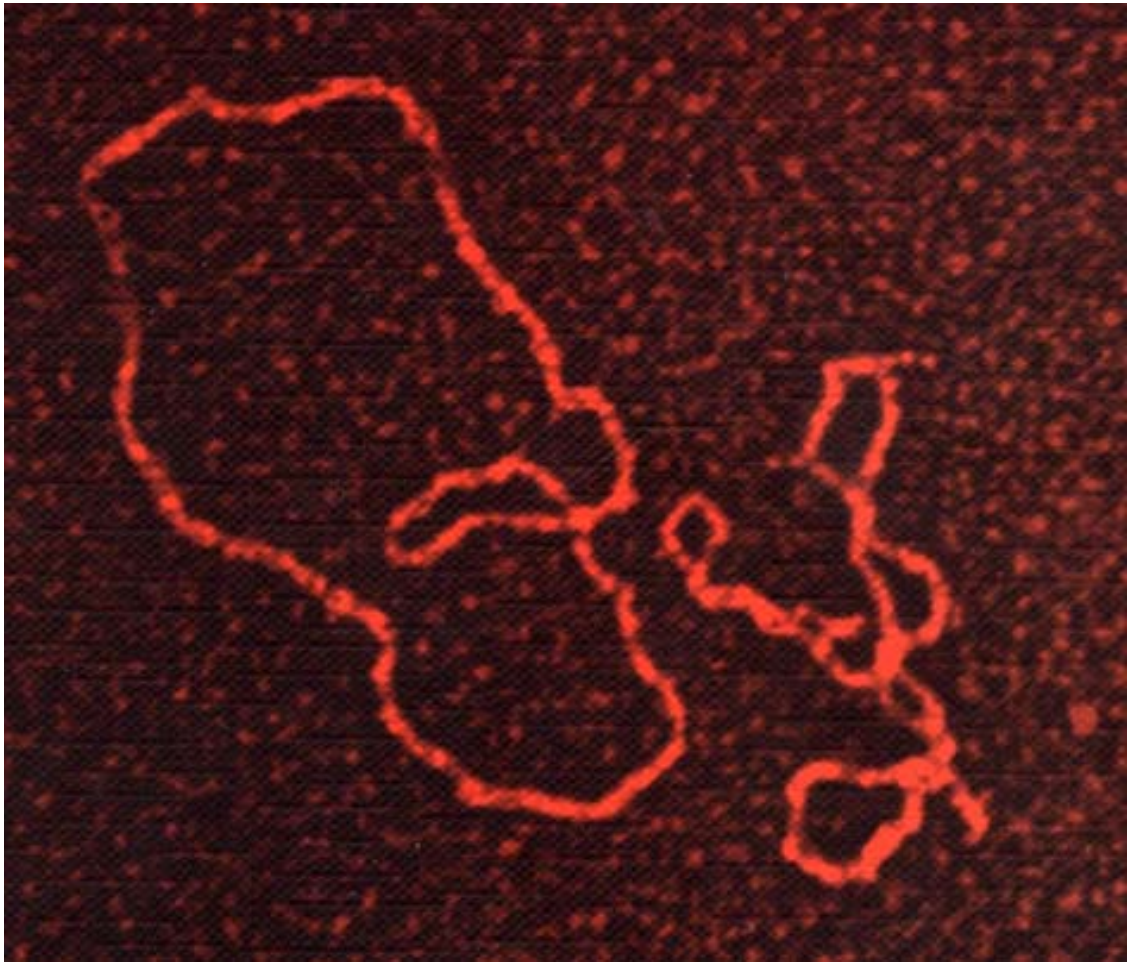
Figure 1 Excitation and emission spectra for new RFP variants. Spectra are normalized to the excitation and emission peak for each protein. (**a**,**b**) Excitation (**a**) and emission (**b**) curves are shown as solid or dashed lines for monomeric variants and as a dotted line for dTomato and tdTomato, with colors corresponding to the color of each variant. (**c**,**d**) Purified proteins (from left to right, mHoneydew, mBanana, mOrange, tdTomato, mTangerine, mStrawberry, and mCherry) are shown in visible light (**c**) and fluorescence (**d**). The fluorescence image is a composite of several images with excitation ranging from 480 nm to 560 nm.

Plasmide als Vektoren

Plasmide sind extrachromosomale,
autonom replizierende,
meist zirkuläre DNA-Moleküle,
die natürlicherweise in vielen
Bakterien vorkommen

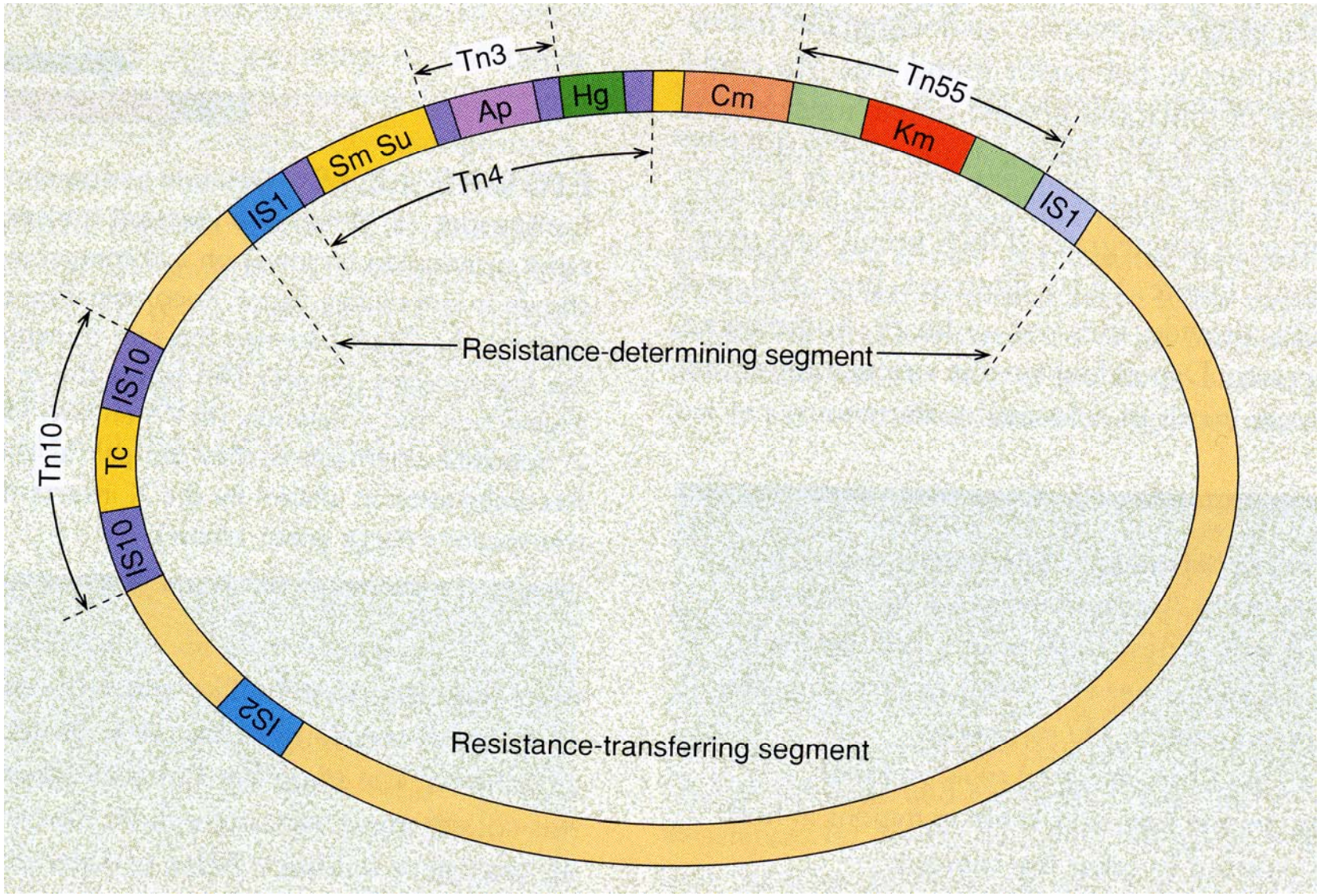

E. coli DNA
Plasmid-DNA

Elektronenmikroskopische Aufnahme eines Plasmids in der Zustandsform „supercoiled“(rechts) bzw. „relaxed circle“ (links)



http://www.gen.cam.ac.uk/Images/summers/plasmids.jpg

z.B. Resistenzplasmide



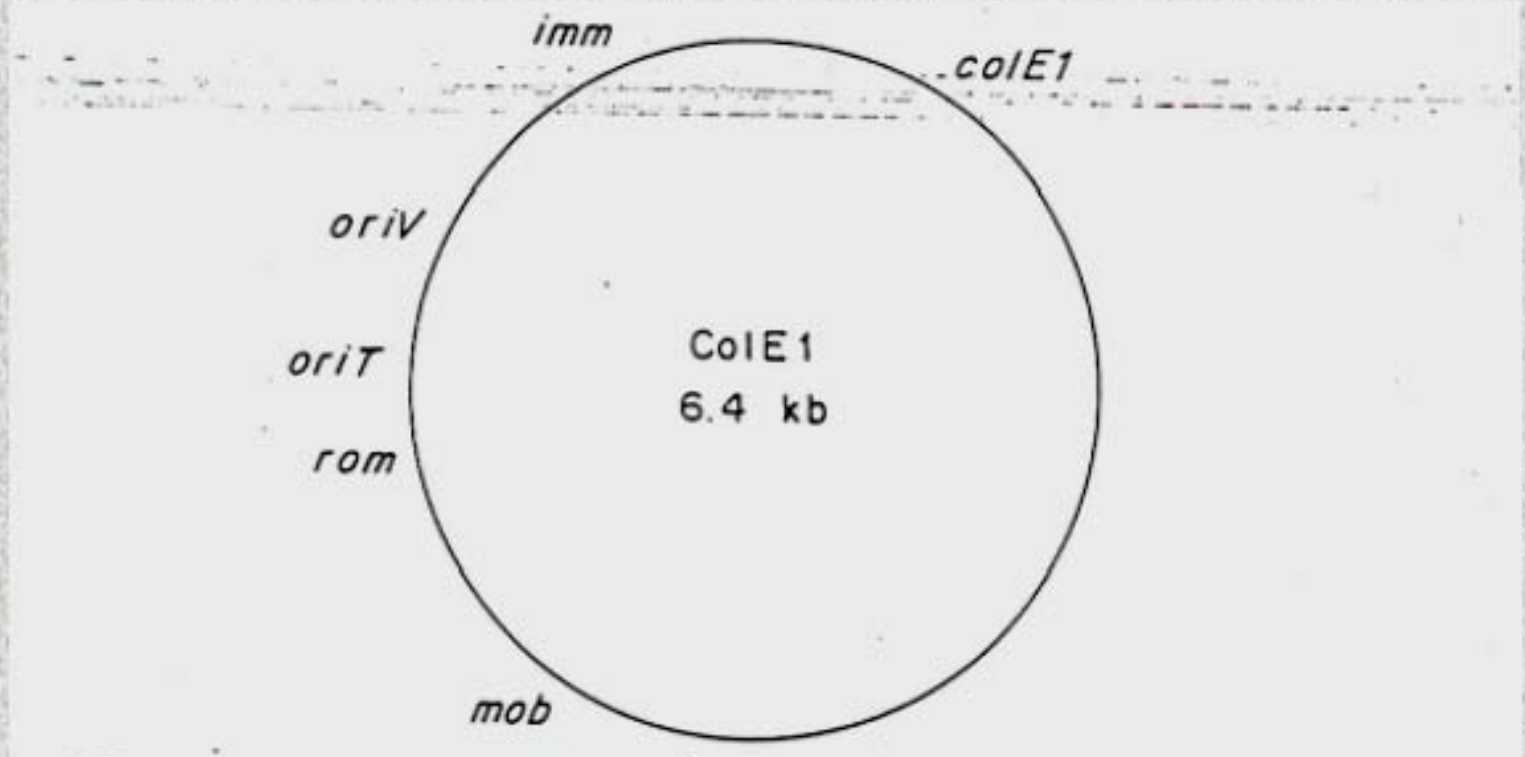
aus Weaver/Hedrick

Eigenschaften einiger natürlicher Plasmide

Table 4.2 Properties of some conjugative and non-conjugative plasmids of Gram-negative organisms

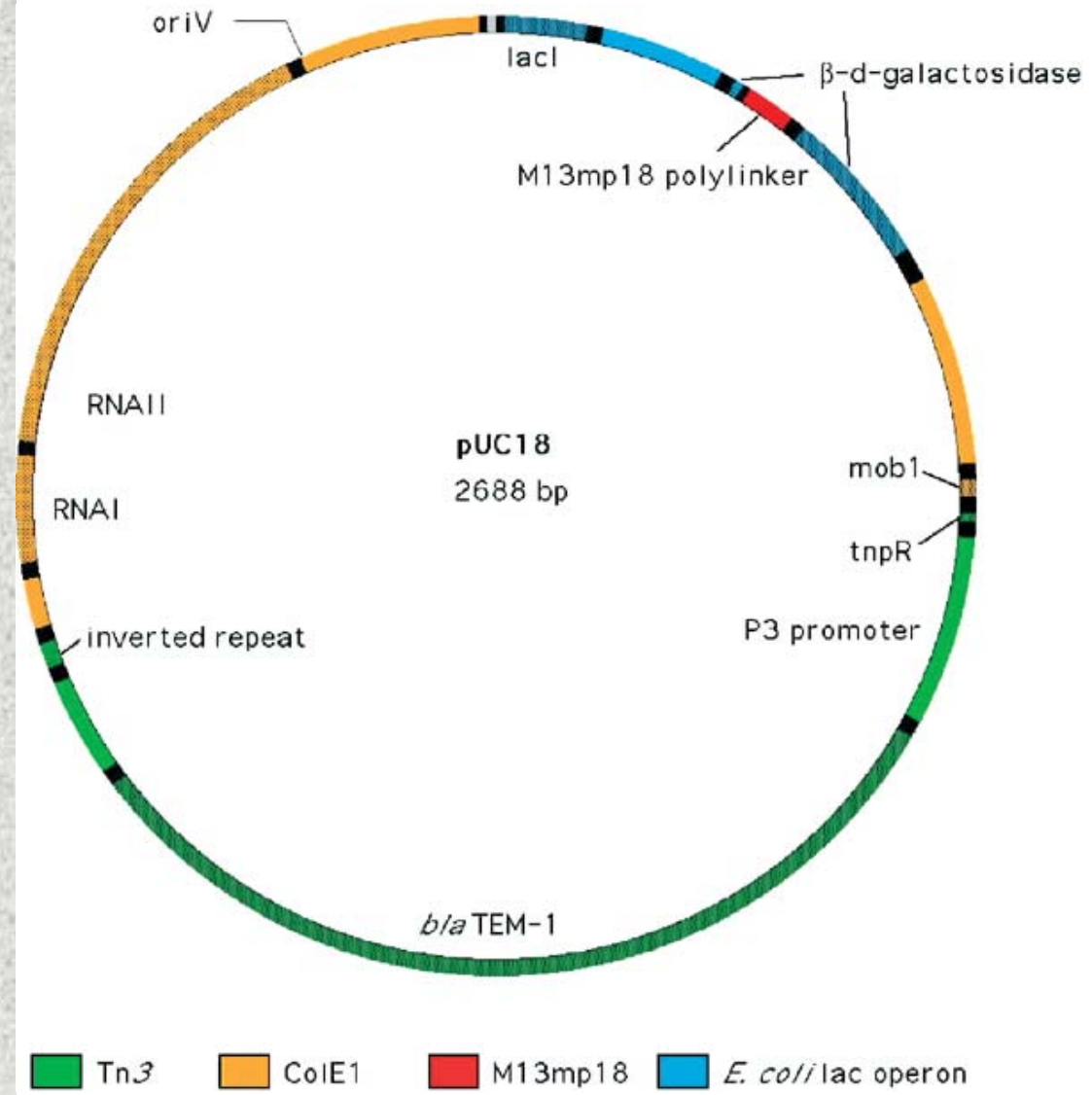
Plasmid	Size (MDa)	Conjugative	No. of plasmid copies/chromosome equivalent	Phenotype
Col E1	4.2	No	10–15	Col E1 production
RSF 1030	5.6	No	20–40	Ampicillin resistance
clo DF13	6	No	10	Cloacin production
R6K	25	Yes	13–38	Ampicillin and streptomycin resistance
F	62	Yes	1–2	–
RI	62.5	Yes	3–6	Multiple drug resistance
Ent P 307	65	Yes	1–3	Enterotoxin production

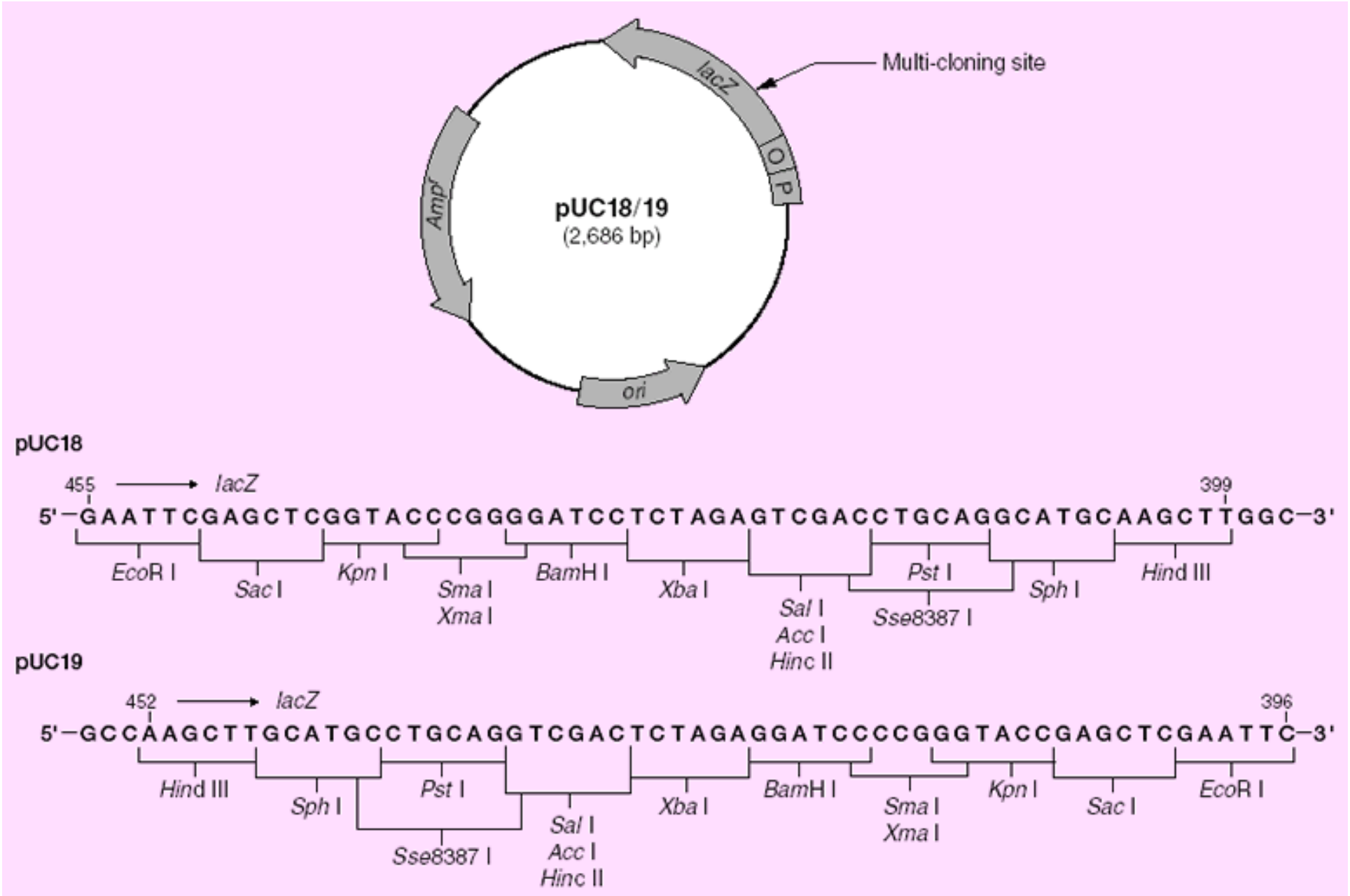
ColE1-Ursprung unzähliger Plasmid-Vektoren



colE1, *imm*: genes for production of, and immunity to, colicin E1
mob :codes for nuclease required for mobilisation
rom :codes for protein required for effective control of copy number
oriT :origin of conjugal transfer
oriV :origin of replication

Vektoren der pUC-Serie, die erfolgreichsten Vektoren aller Zeiten




Multi-cloning site
lacZ
O
P
Amp
pUC18/19
(2,686 bp)
ori
pUC18
455
lacZ
399
5'—GAATTCGAGCTCGGTACCCGGGGATCCTCTAGAGTCGACCTGCAGGCATGCAAGCTTGGC—3'
EcoR I
Sac I
Kpn I
Sma I
Xma I
BamH I
Xba I
Sal I
Acc I
Hinc II
Pst I
Sse8387 I
Sph I
Hind III
pUC19
452
lacZ
396
5'—GCCAAGCTTGCATGCCTGCAGGTCGACTCTAGAGGATCCCCGGGTACCGAGCTCGAATTC—3'
Hind III
Sph I
Pst I
Sse8387 I
Sal I
Acc I
Hinc II
Xba I
BamH I
Sma I
Xma I
Kpn I
Sac I
EcoR I

Plasmide als Vektoren

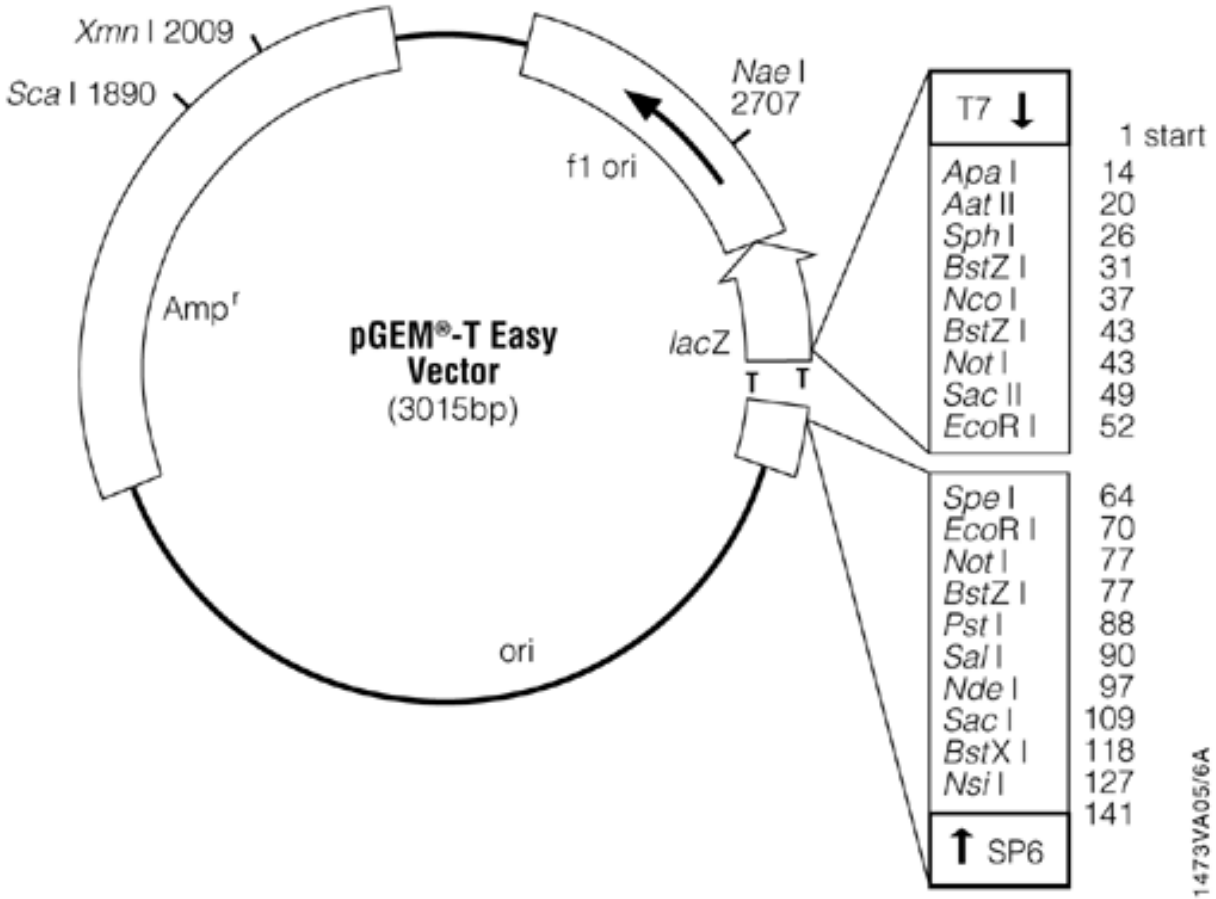
Der Replikationsursprung bestimmt die Art der Replikation

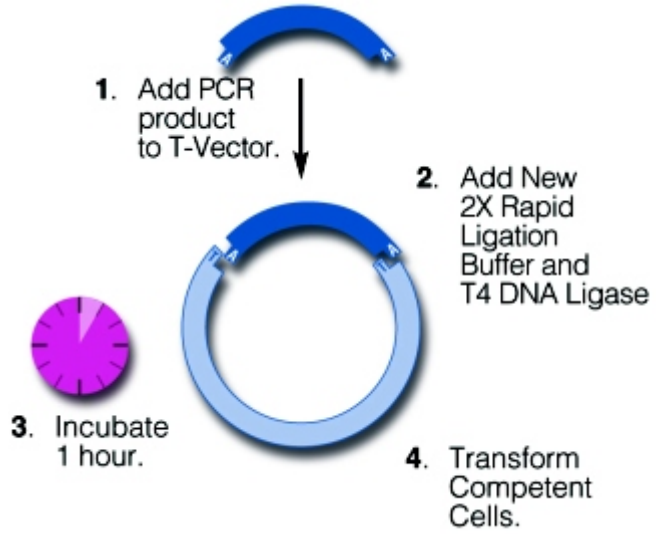
ColE1- ori:
relaxierte Replikation → **multicopy Plasmide**

F-Plasmid - ori:
Stringente Replikation → **„single copy“ Plasmide**

f1-Phagen - ori:
Einzelstrang Replikation → **„single strand“ Phagen**

Vektor zur Klonierung von PCR-Produkten





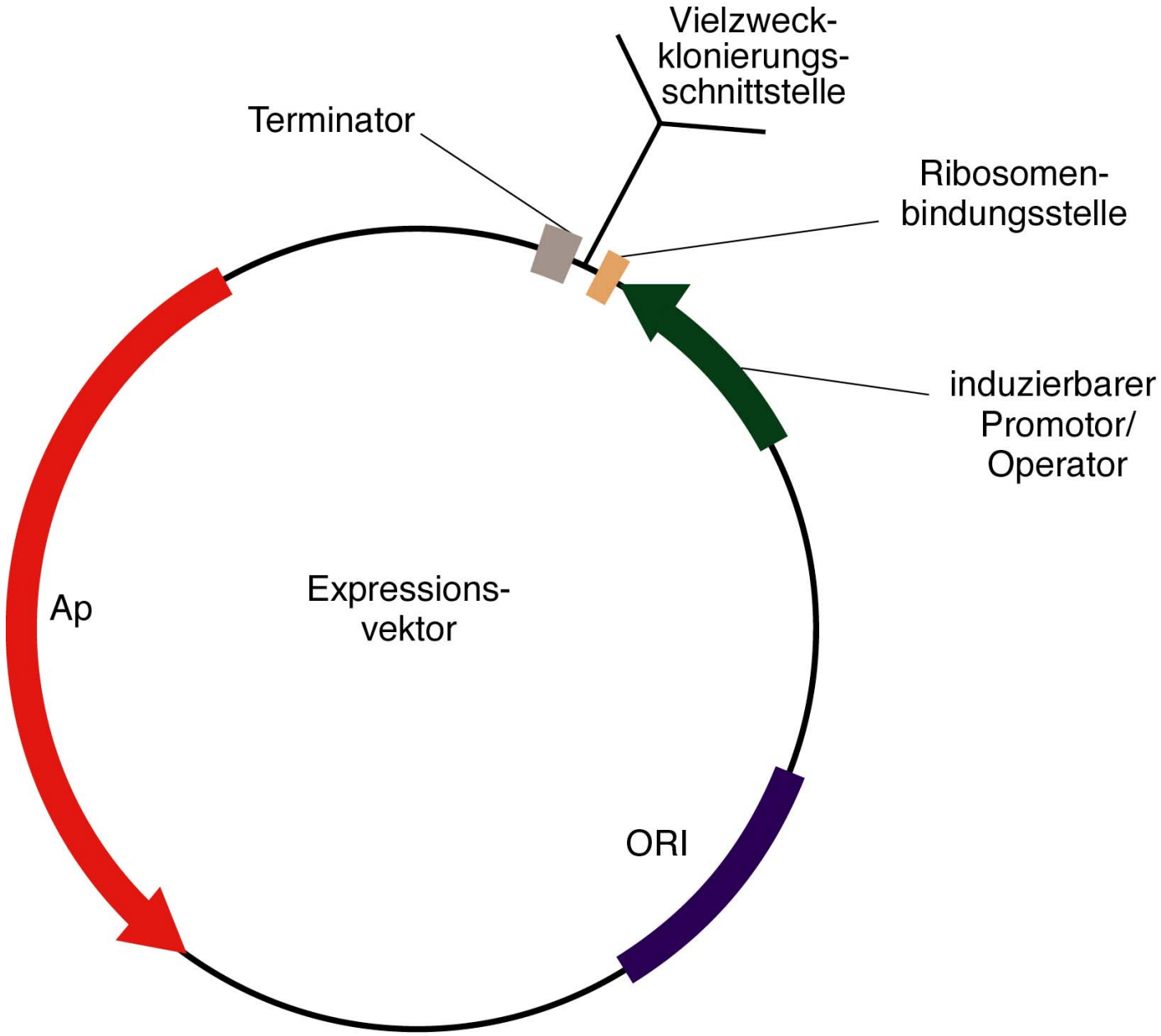
Eigenschaften einiger PCR-Polymerasen

Table 1. Comparison of PCR Product Properties for Some Thermostable DNA Polymerases.

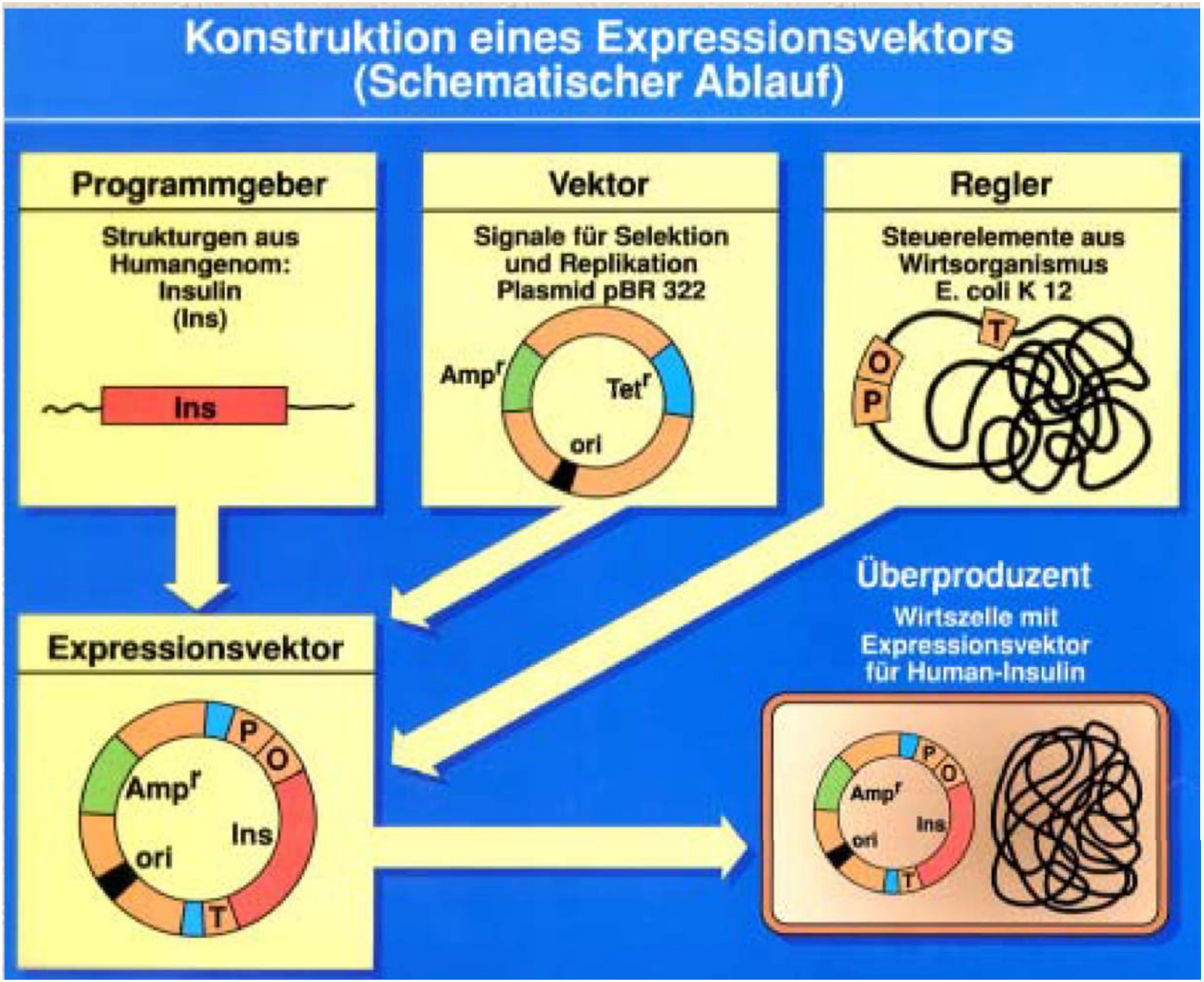
Characteristic	Taq/ AmpliTaq®	VentR®/ Tfl	Tth	Deep (Tli)	VentR®	Pfu	Pwo
Resulting DNA ends	3´ A	>95% 3´ A	>95% 3´ A	Blunt	Blunt	Blunt	
5´®3´ exonuclease Activity	Yes	Yes	Yes	No	No	No	No
3´®5´ exonuclease activity	No	No	No	Yes	Yes	Yes	Yes

N.A.: not available

Für die Expression fremder Gene gibt es spezielle Expressionsvektoren mit Signalsequenzen für die korrekte Transkription und Translation in Bakterien



Plasmidvektoren, zur Expression von Fremdgenen



Komponenten eines bakteriellen Expressionsvektors

Künstliche Promotoren verstärken die Expression

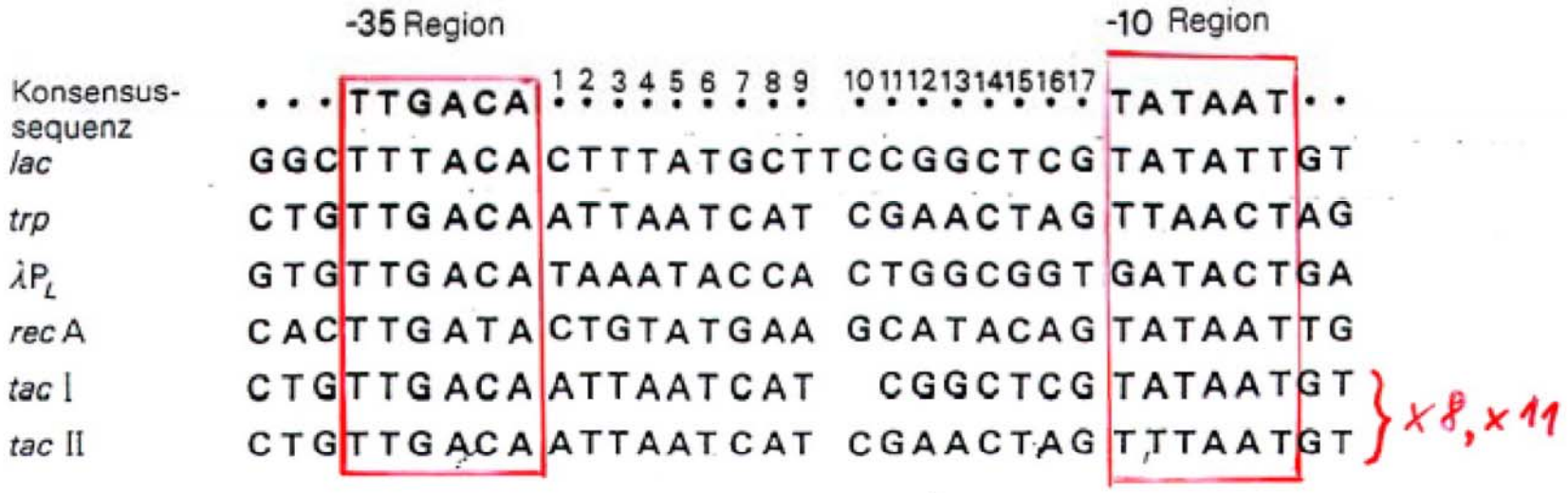


Abb. 2.70 Nucleotid-Sequenz der -10- und -35-Regionen von vier natürlichen Promotoren und zwei Hybrid-Promotoren

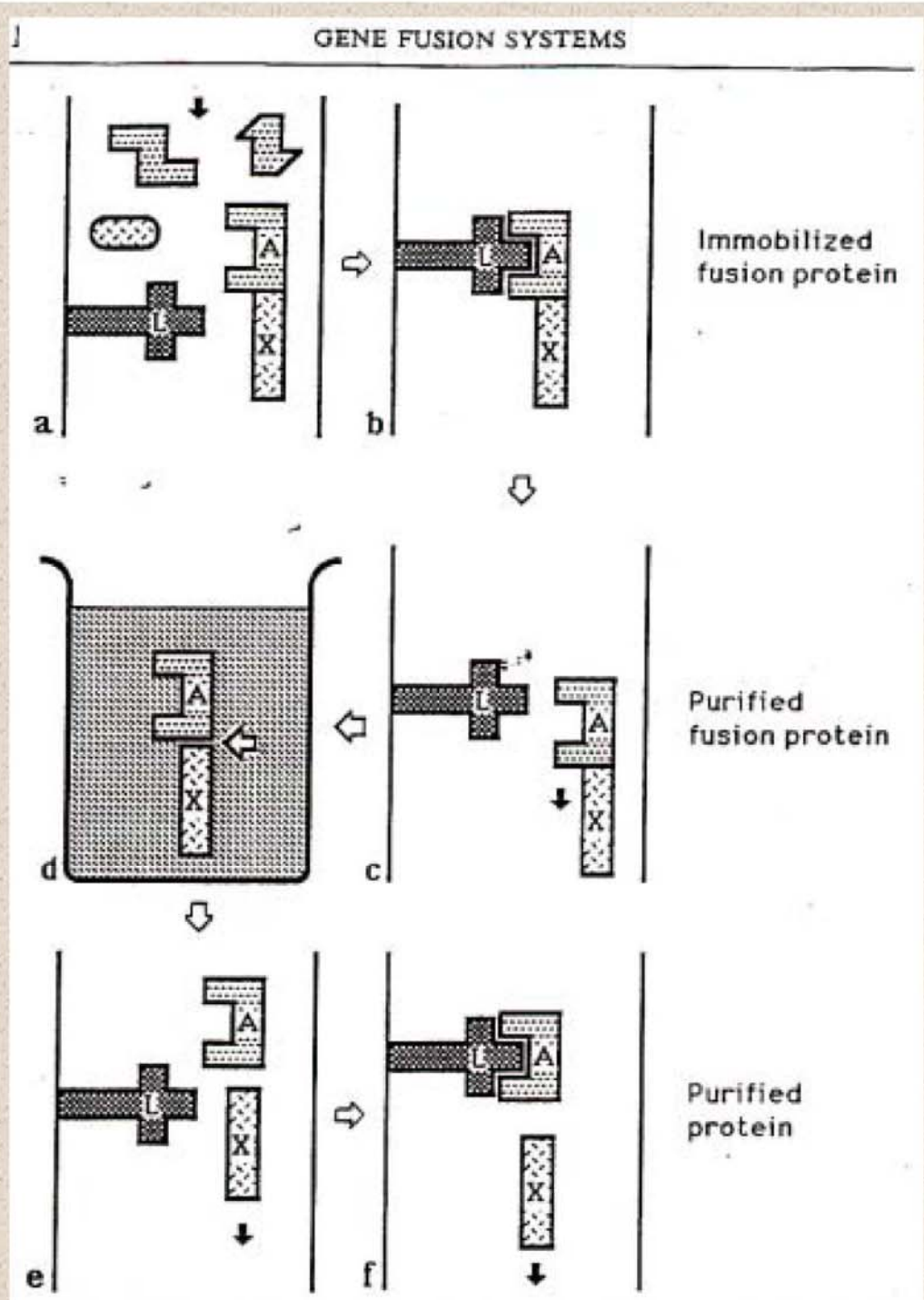
Komponenten eines bakteriellen Expressionsvektors

UAAGGAGGU

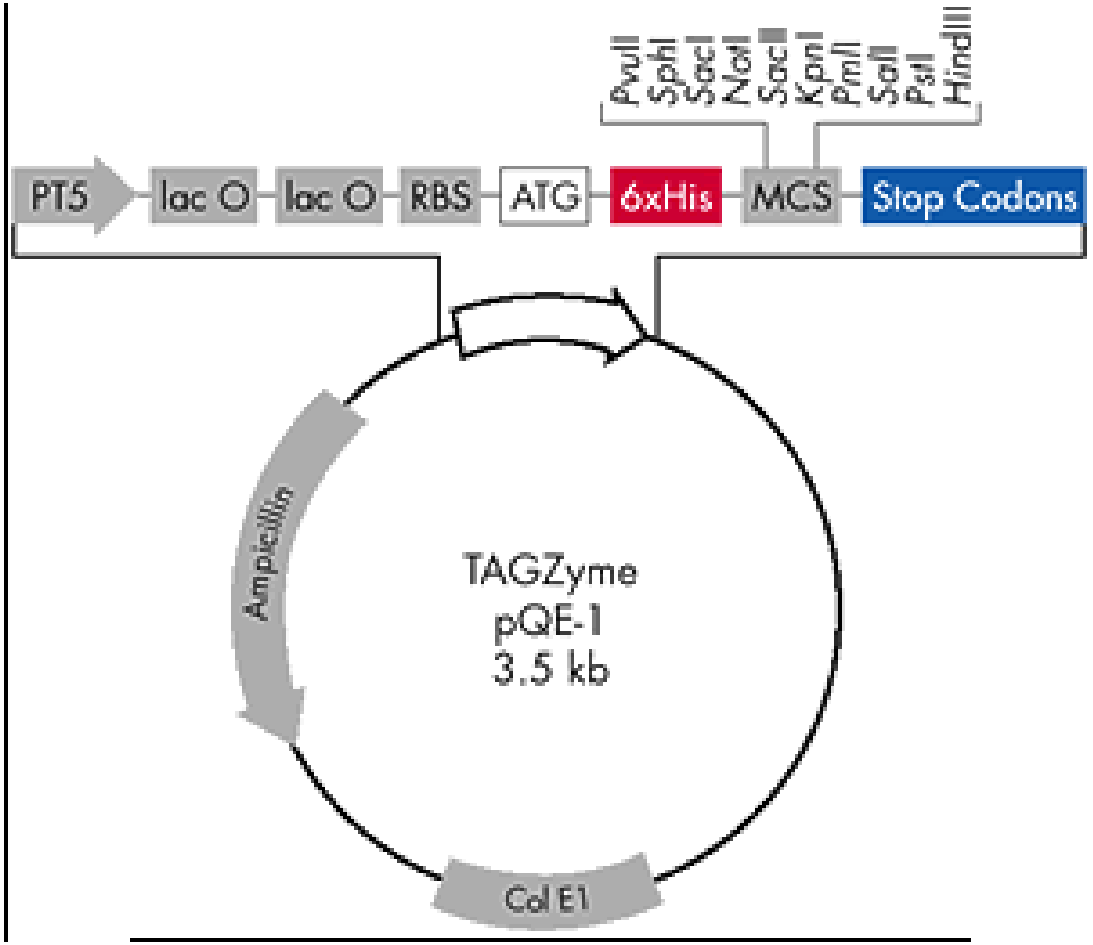
Die Shine-Dalgarno-Sequenz am Anfang der mRNA ist notwendig, um die Ribosomenbindung an die mRNA zu ermöglichen

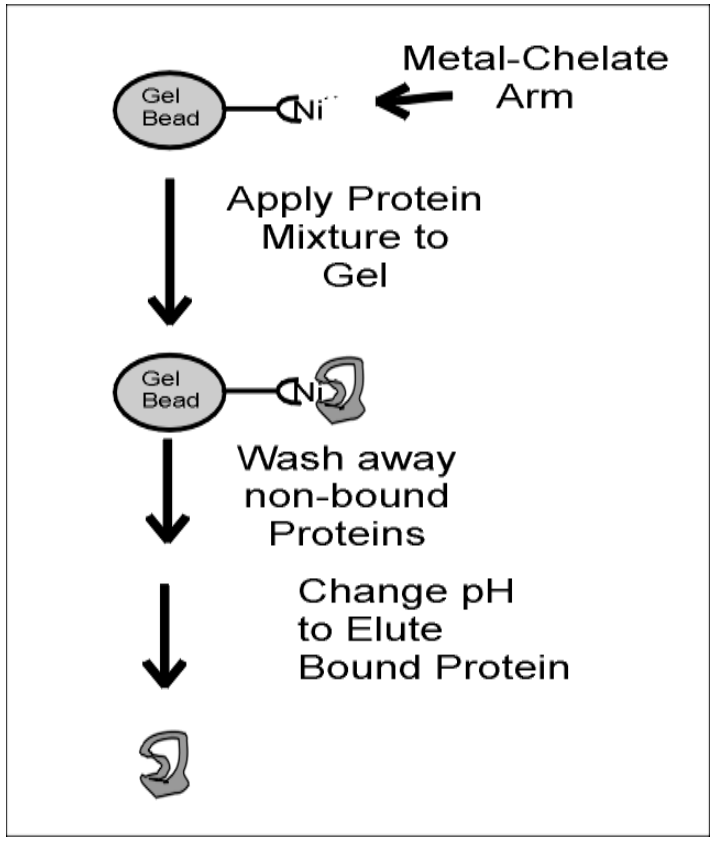
Bakterielle Expression

Zur schnellen Reinigung des exprimierten Proteins dienen Fusionskonstrukte



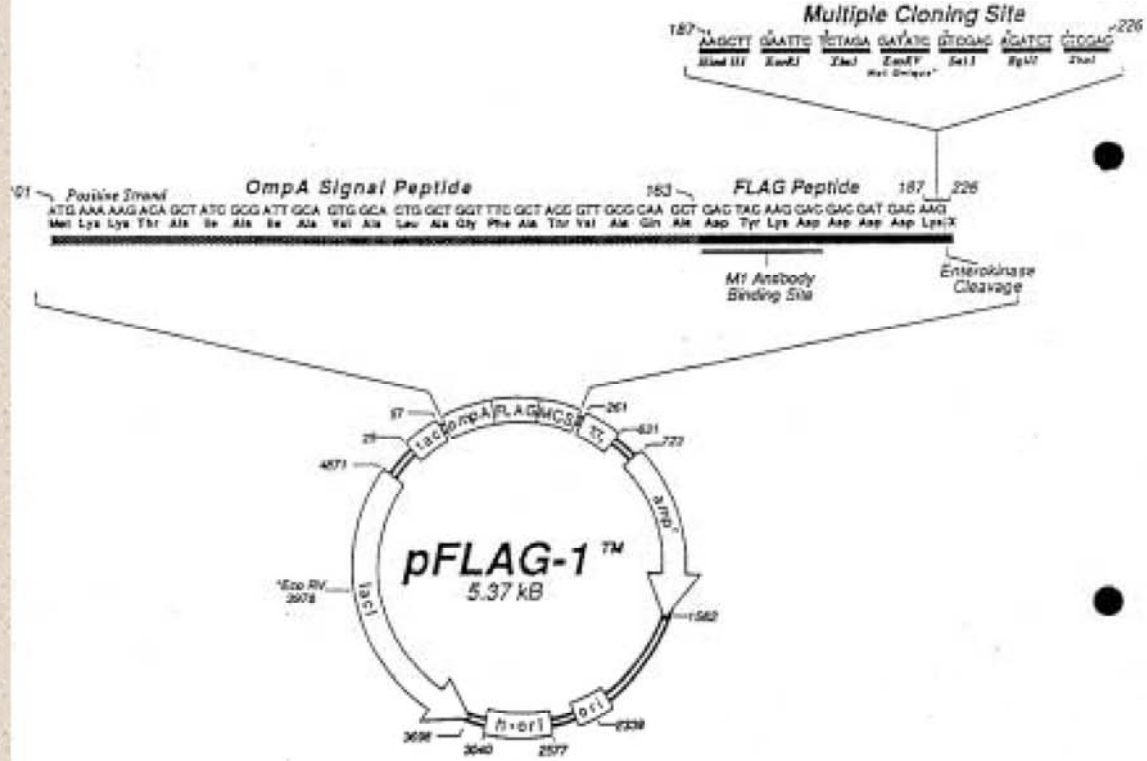
Expression von Fusionsproteinen mit sog. „His-tag“

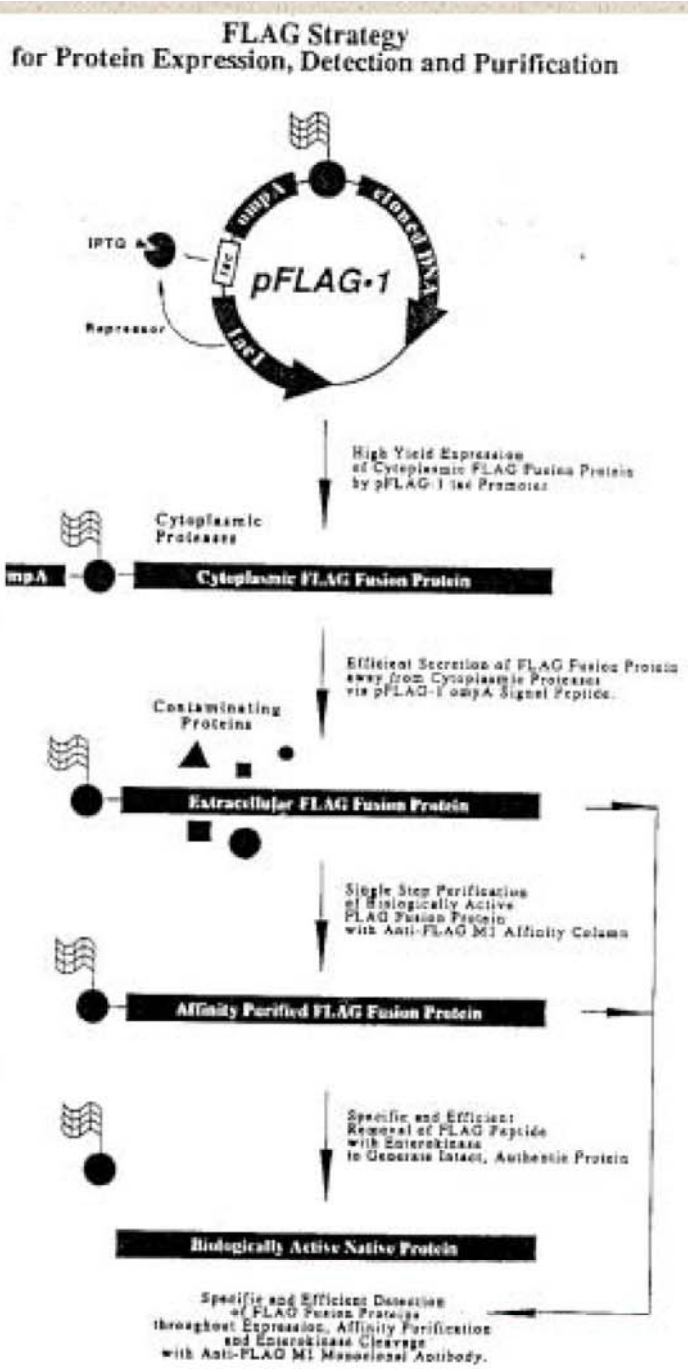




„Flag“-Vektoren dienen der Expression und schnellen Reinigung von Fusions-Proteinen in Bakterien

Figure 5
pFLAG-1 Expression Vector

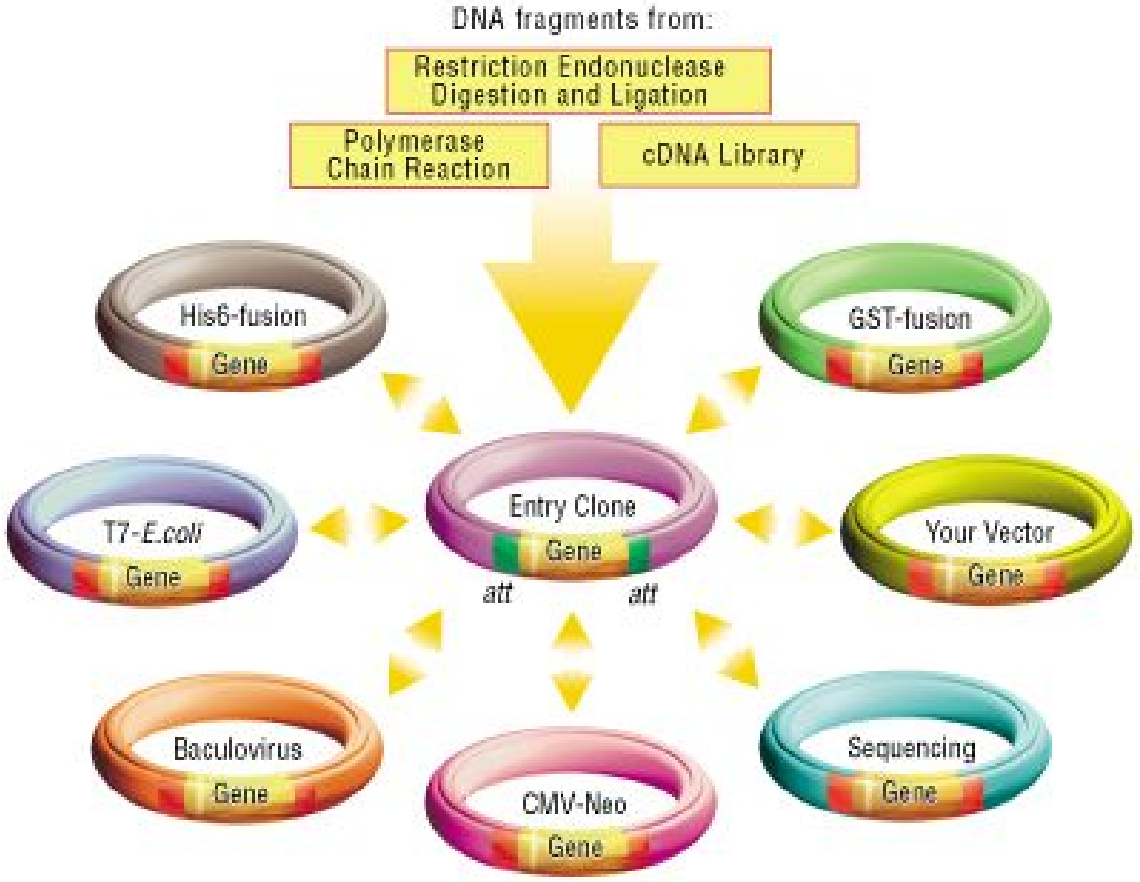




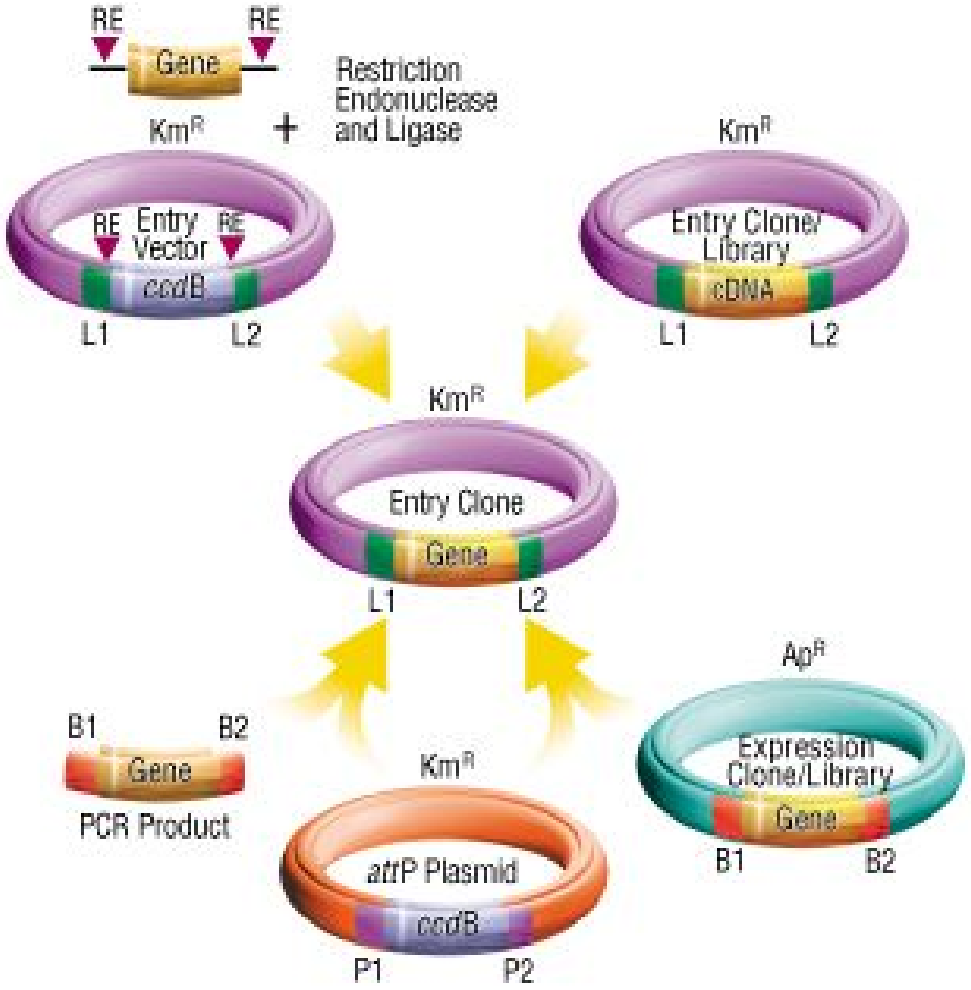
Suizidvektoren bringen alle Zellen um, die nicht gentechnisch verändert sind

ccdB-Gen = controlled cell death
(Inhibition der Ligationsaktivität der Gyrase)

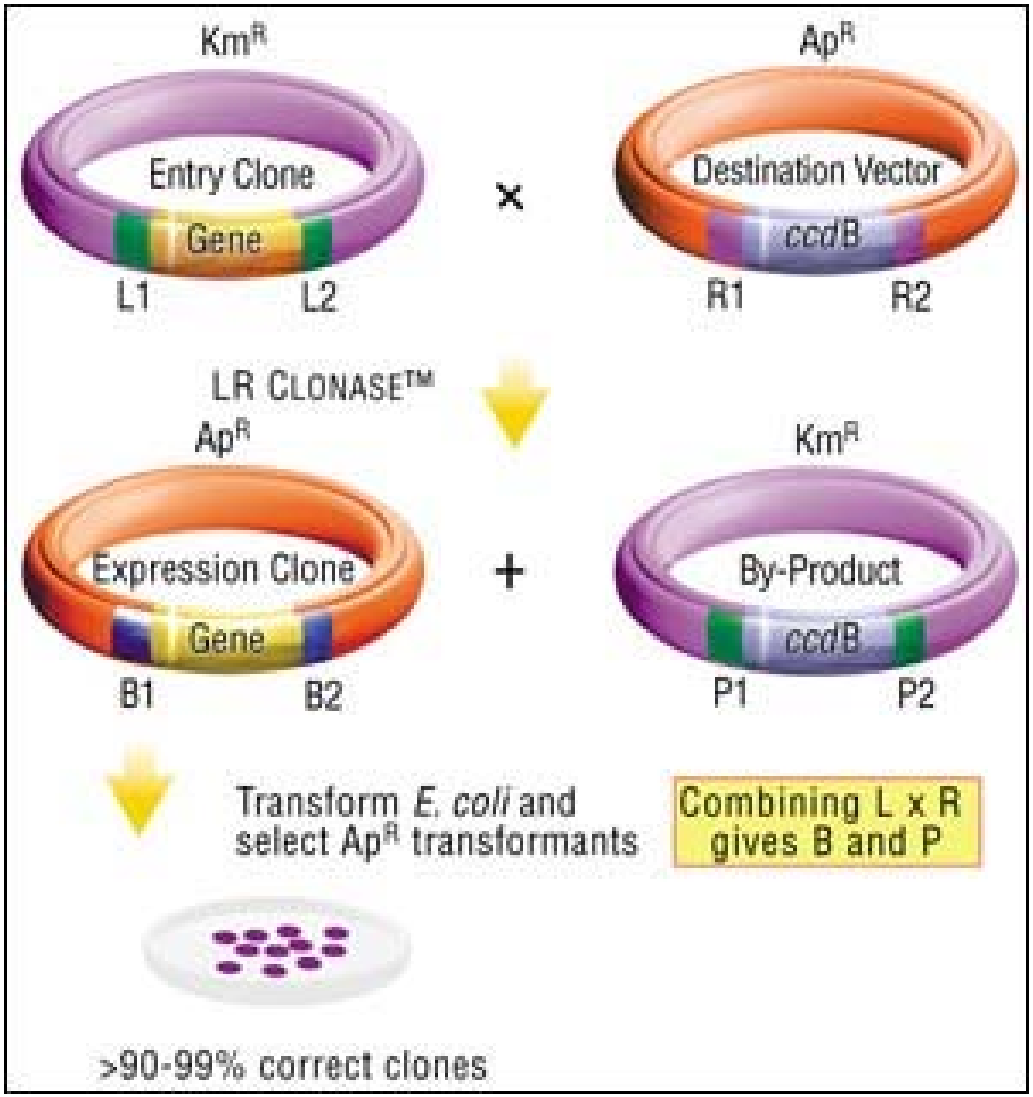
„Gateway“-Klonierungssystem



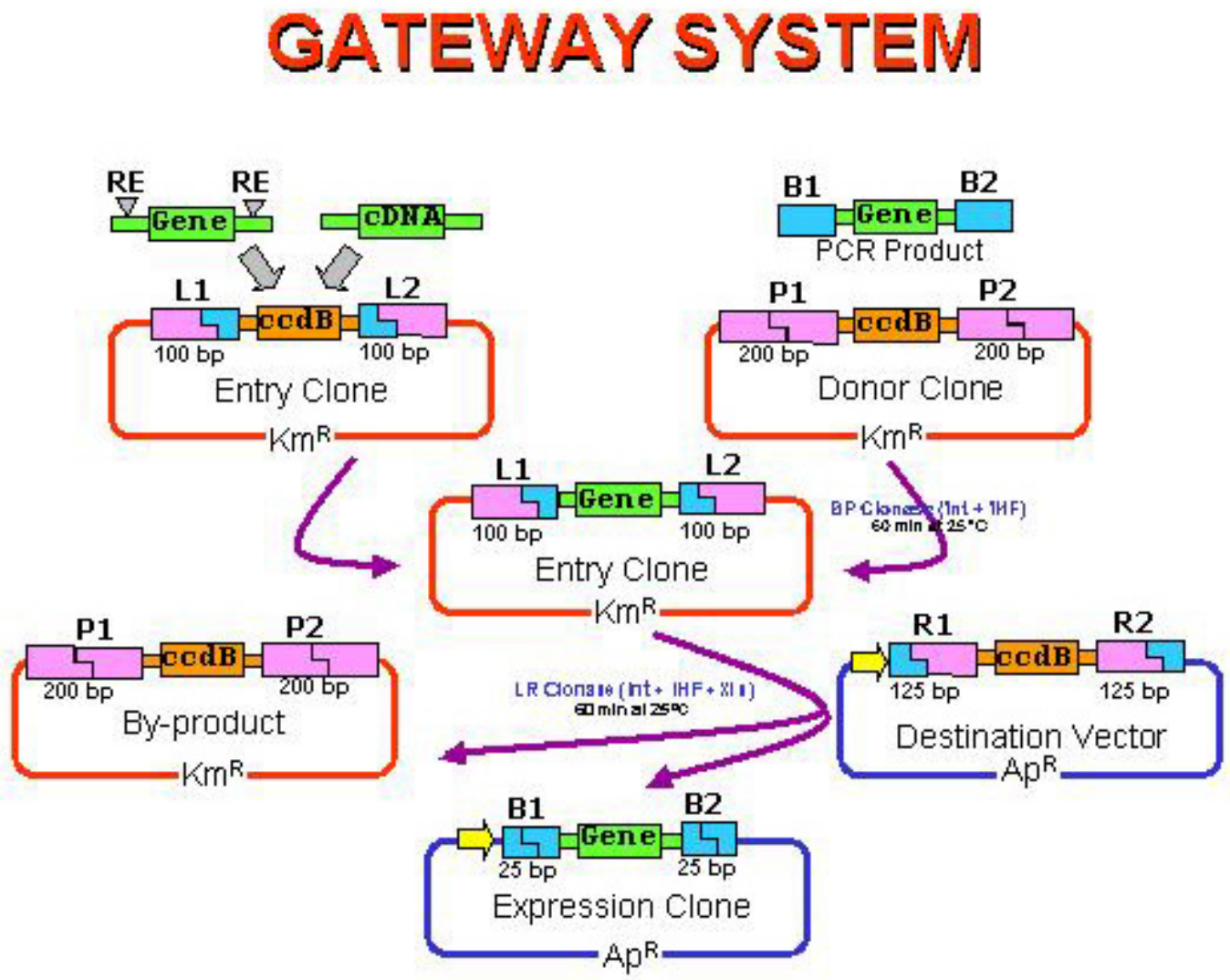
„Gateway“-Klonierungssystem



„Gateway“-Klonierungssystem



„Gateway“-Klonierungssystem



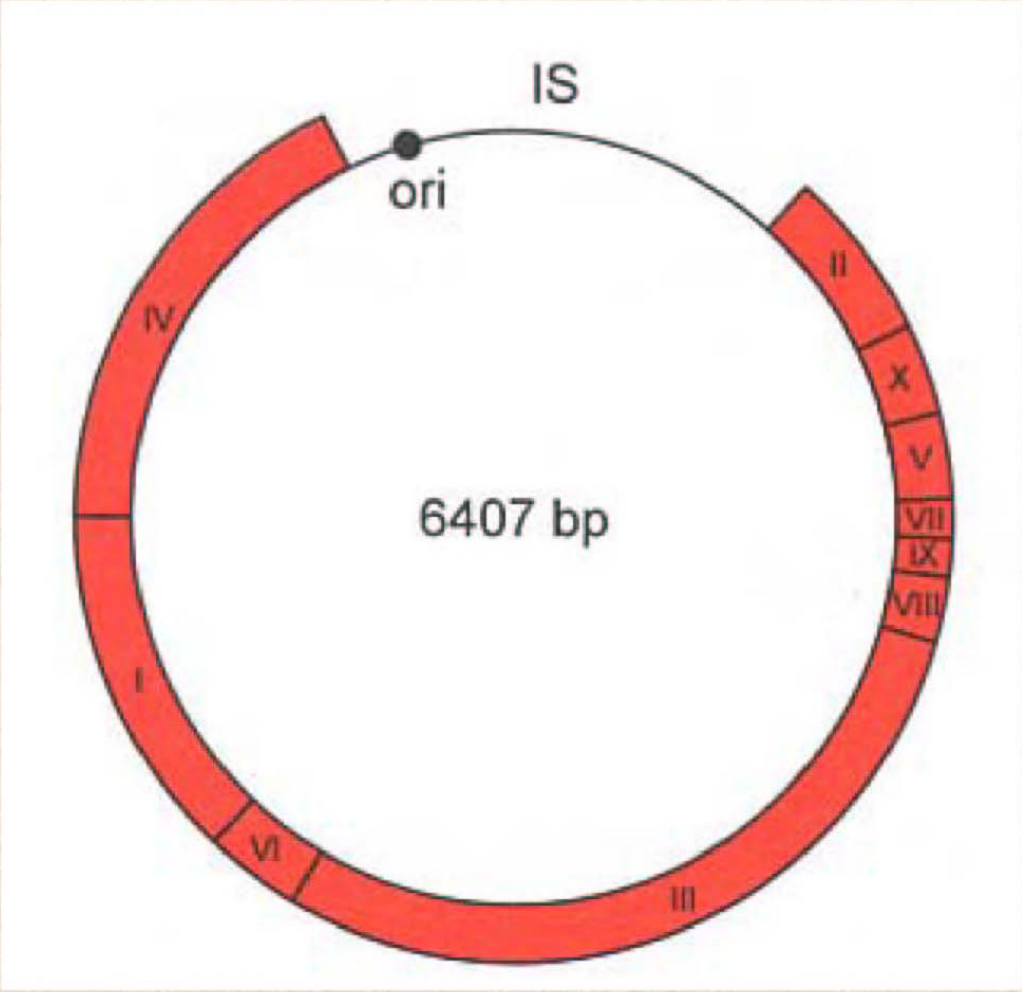
Vektoren auf der Basis von Bakteriophagen

M13/f1-Phage

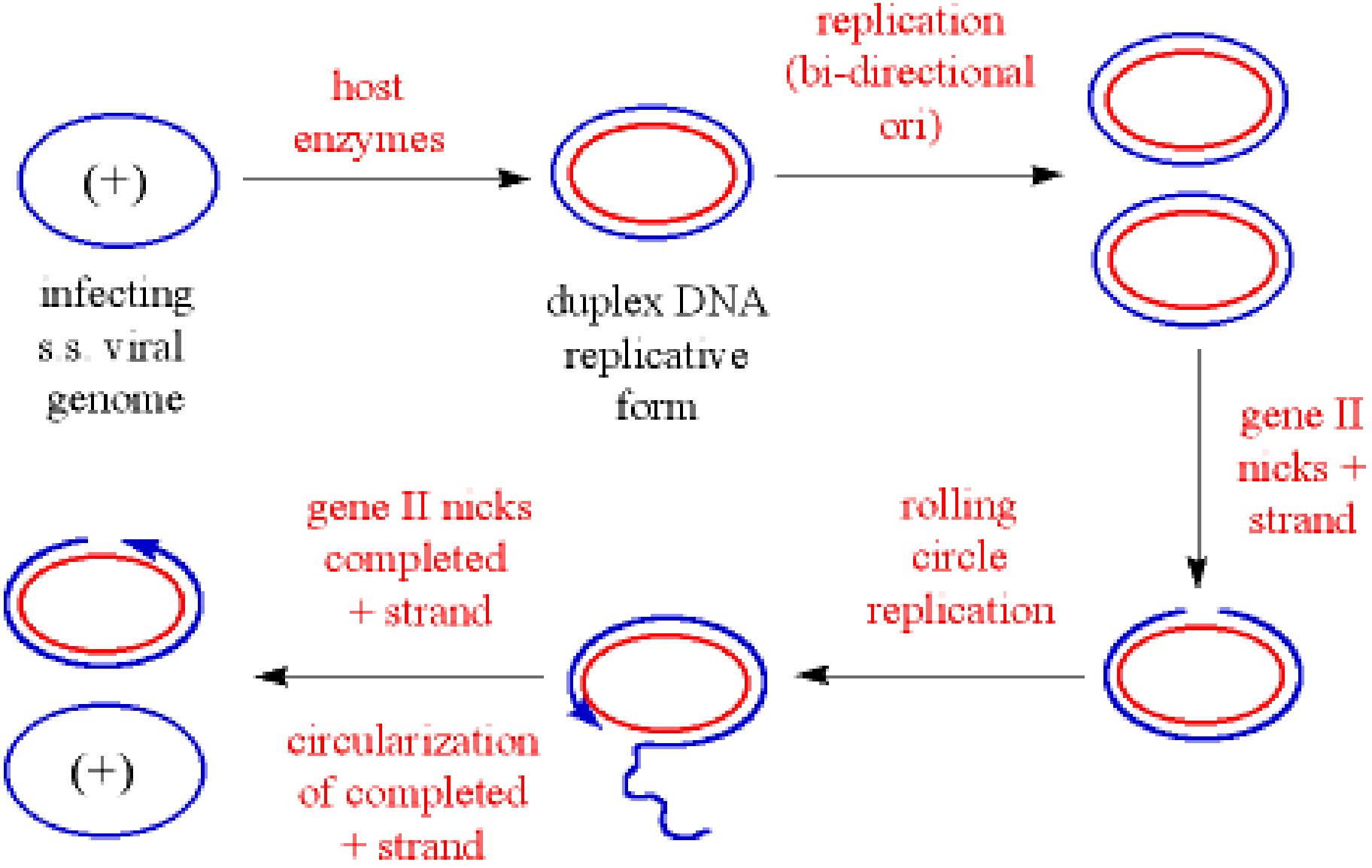
λ–Phage

P1-Phage

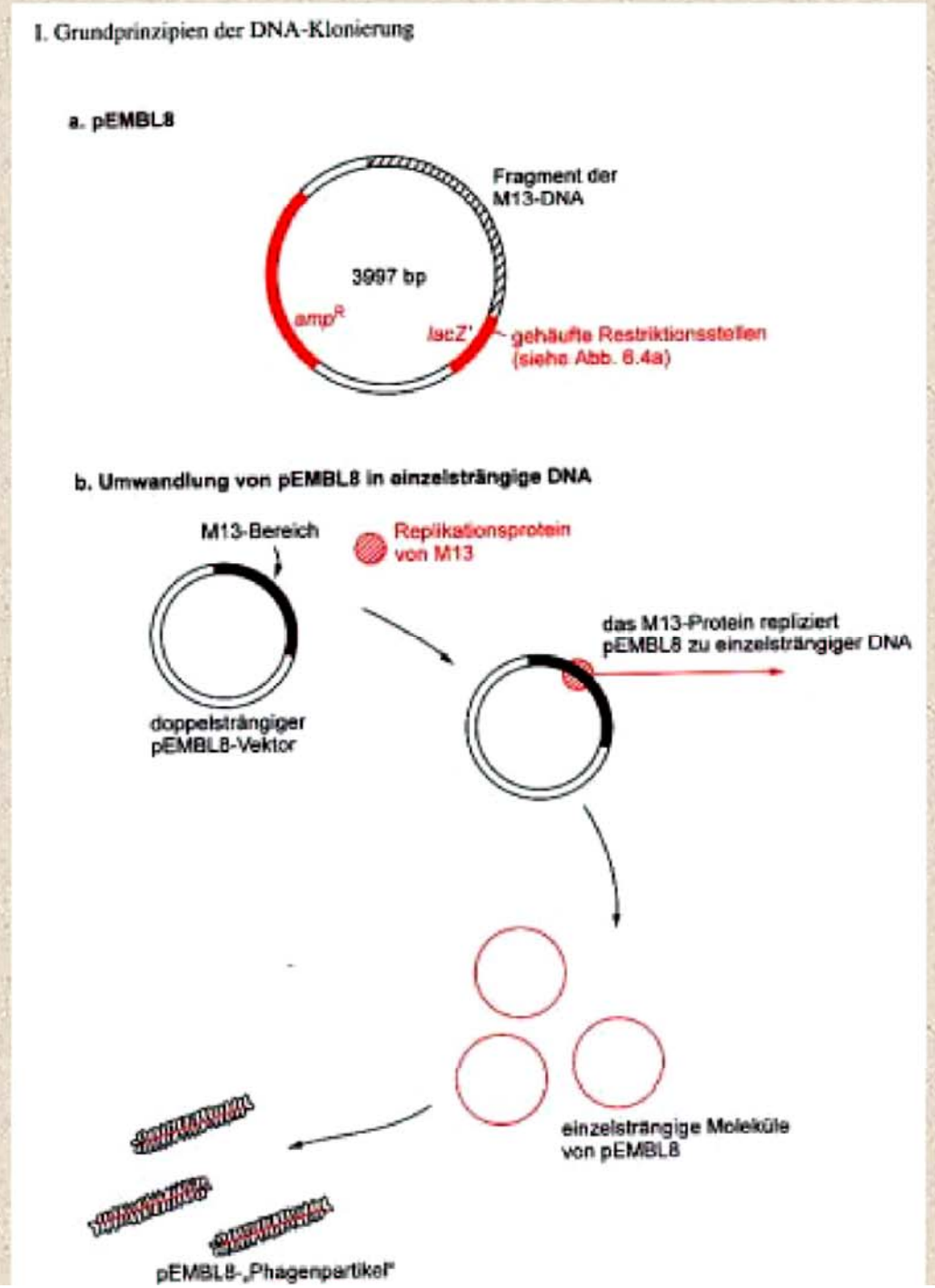
Bakteriophage- M13



Replikation M13-Bakteriophage



Konstruktion eines Phagemids



Bluescript, ein klassisches Phagemid

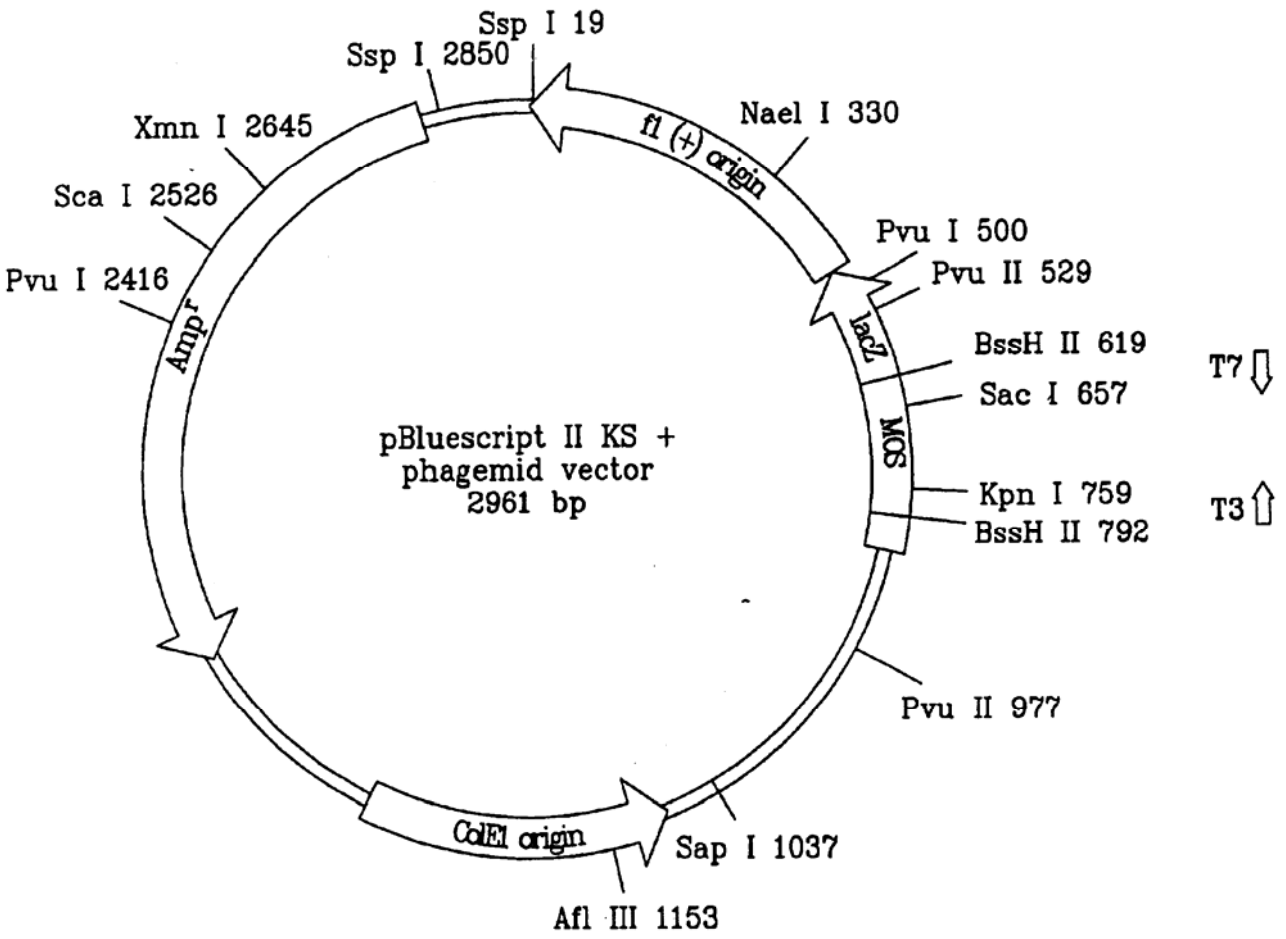
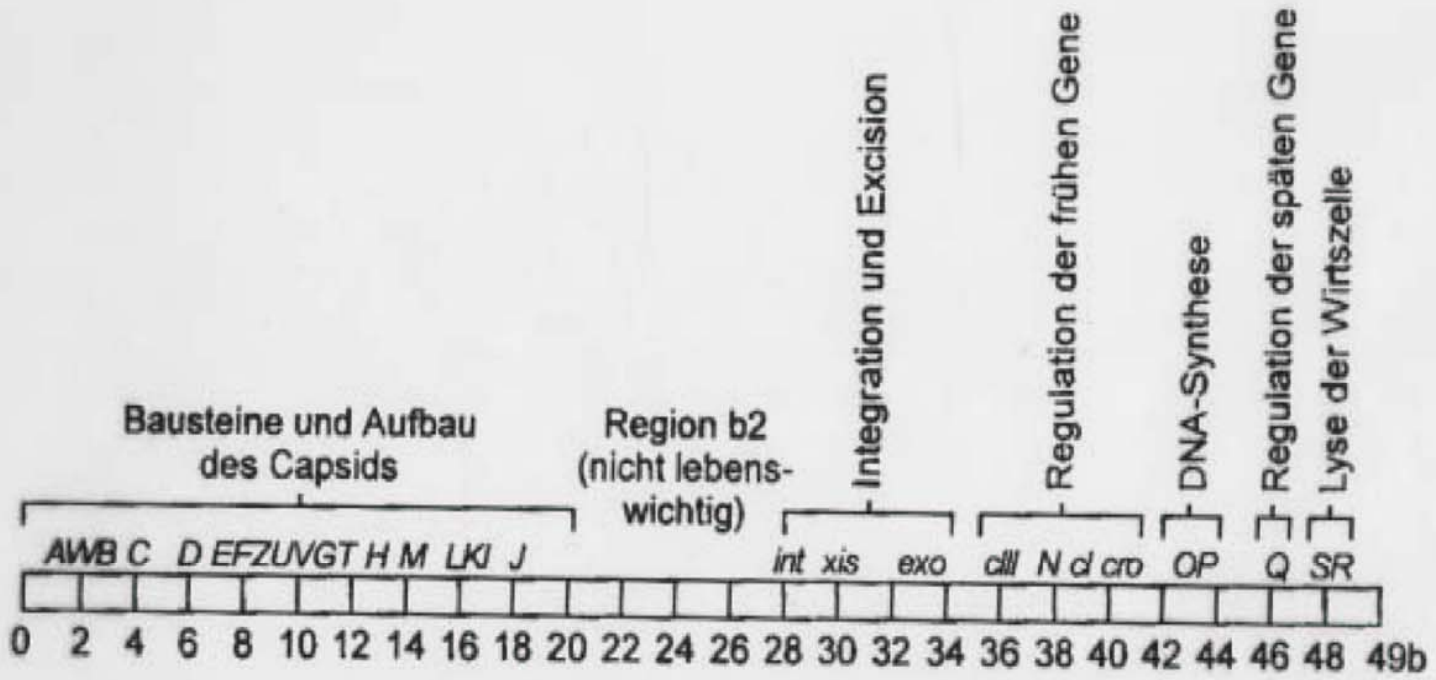


FIG. 1

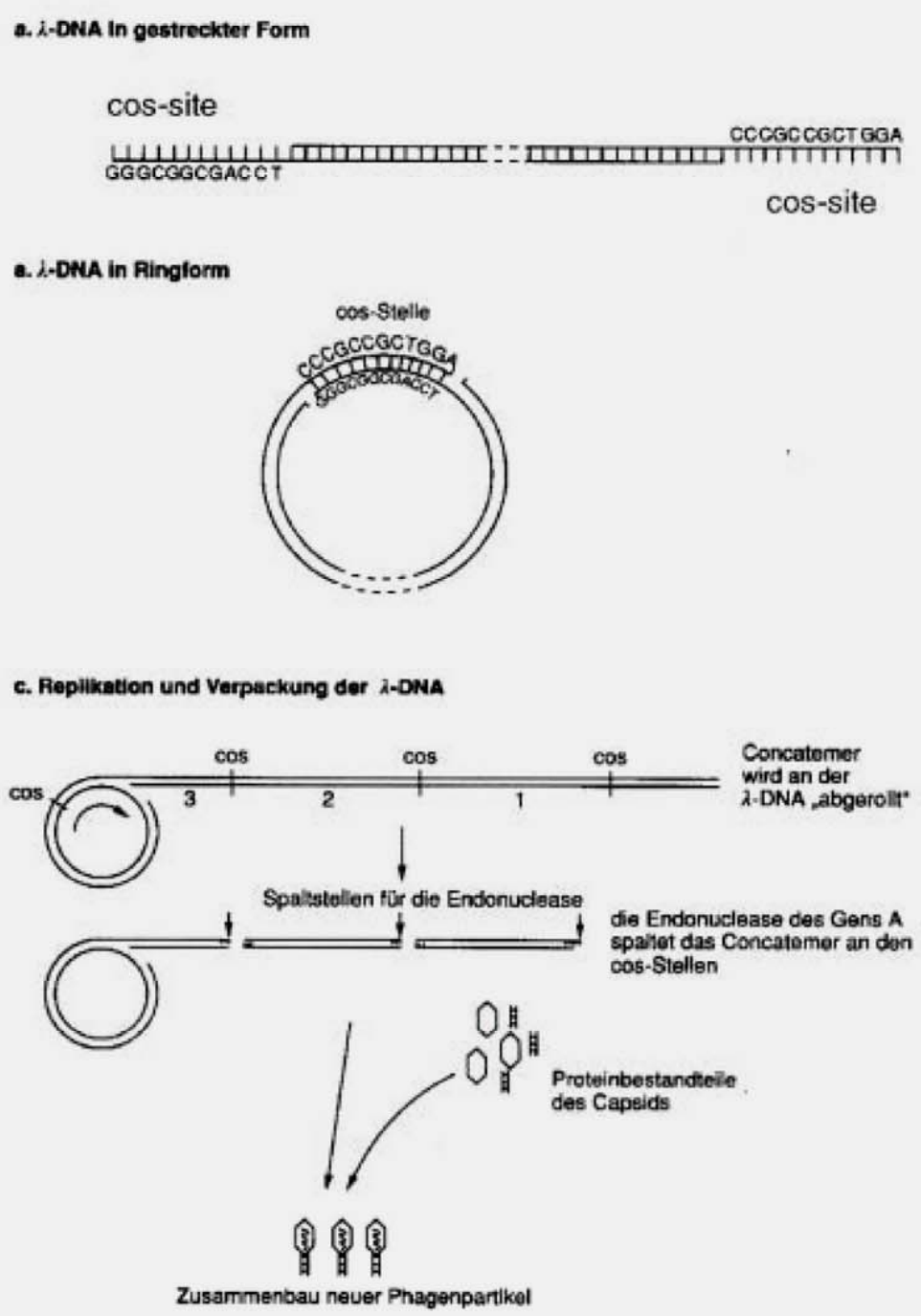
United States Patent 6803230
Inventors:
Bowdish, Katherine S. (Del Mar, CA)
Fredrickson, Shana (Solana Beach, CA)
Wild, Martha (Solana Beach, CA)

Bakteriophage λ
Genkarte



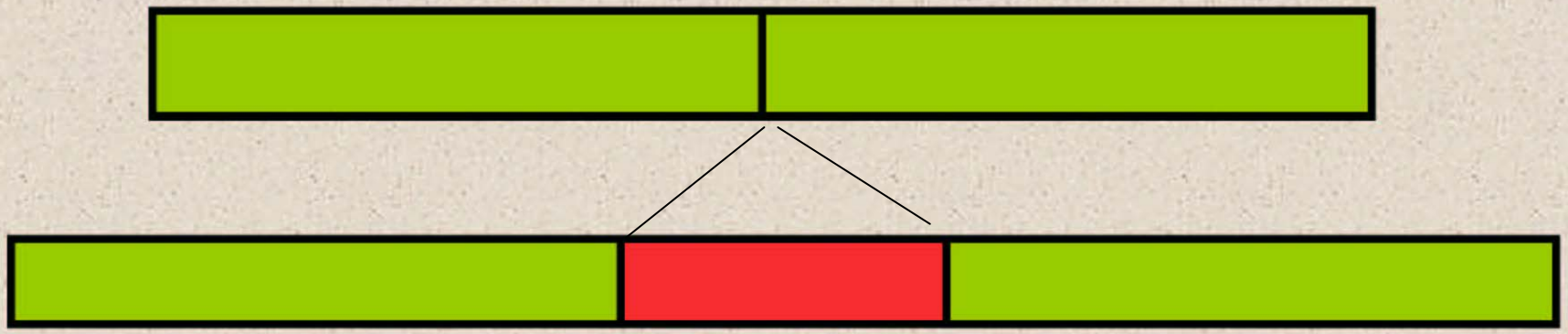
2.9 Die Genkarte von λ. Eingetragen sind die Positionen der wichtigsten Gene und die Funktionen der Gengruppen.

Bakteriophage λ Infektion und Replikation

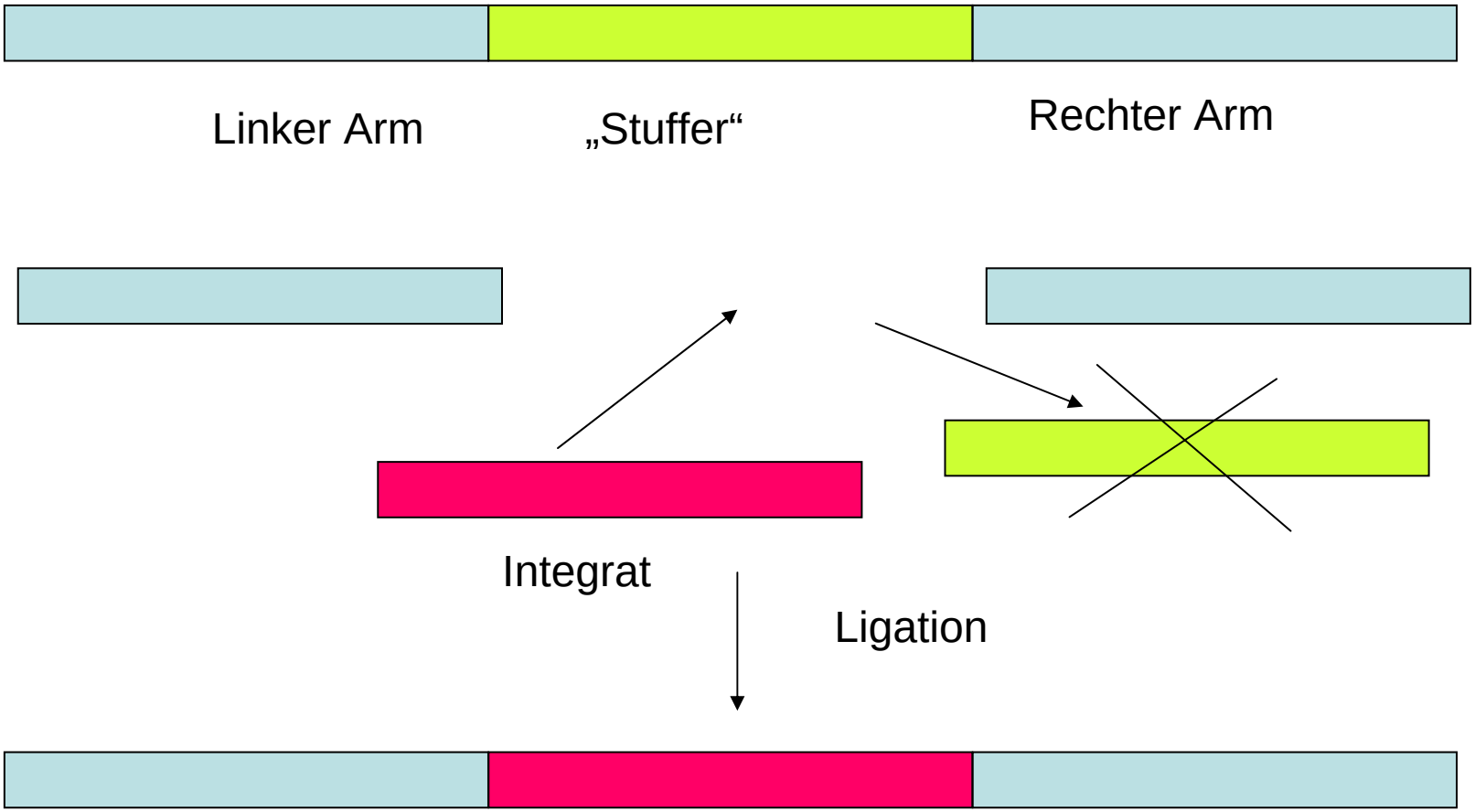


Bakteriophage λ

Integrationsvektoren



Substitutionsvektoren



Lambda gt10/11

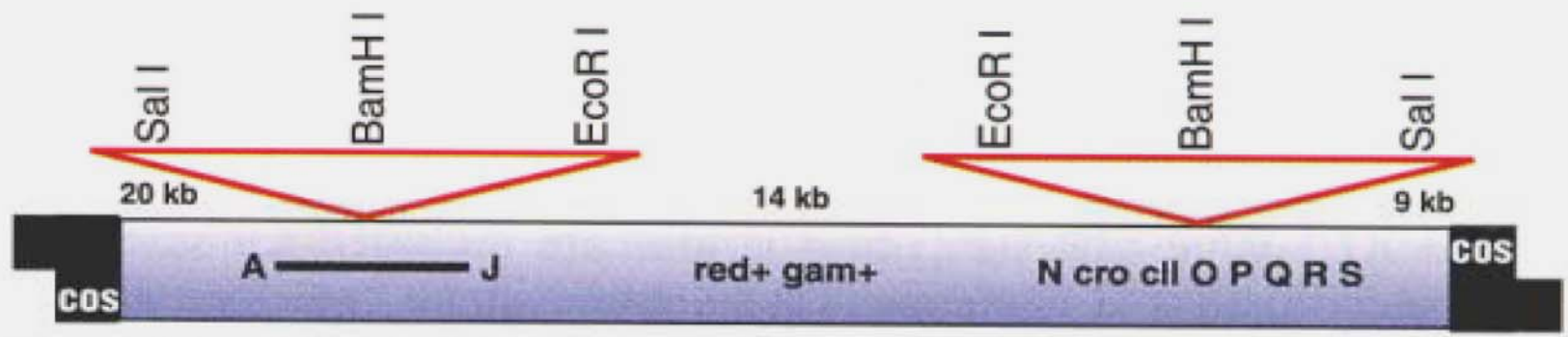
The Lambda gt11 Vector

- Predigested with *EcoR* I
- For construction of cDNA libraries

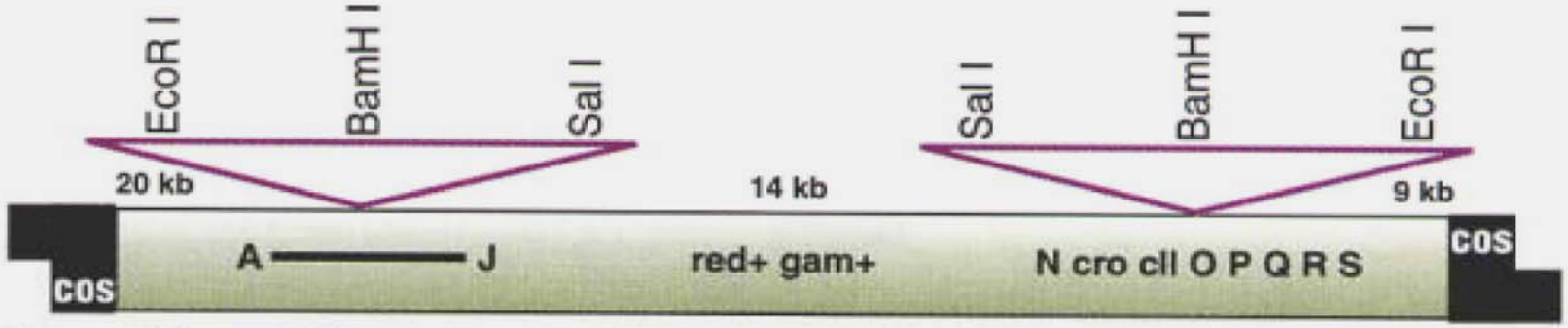
Map of Lambda gt11 Vector

CLONING CAPACITY Capacity of 7.2 kb

Lambda EMBL3/4



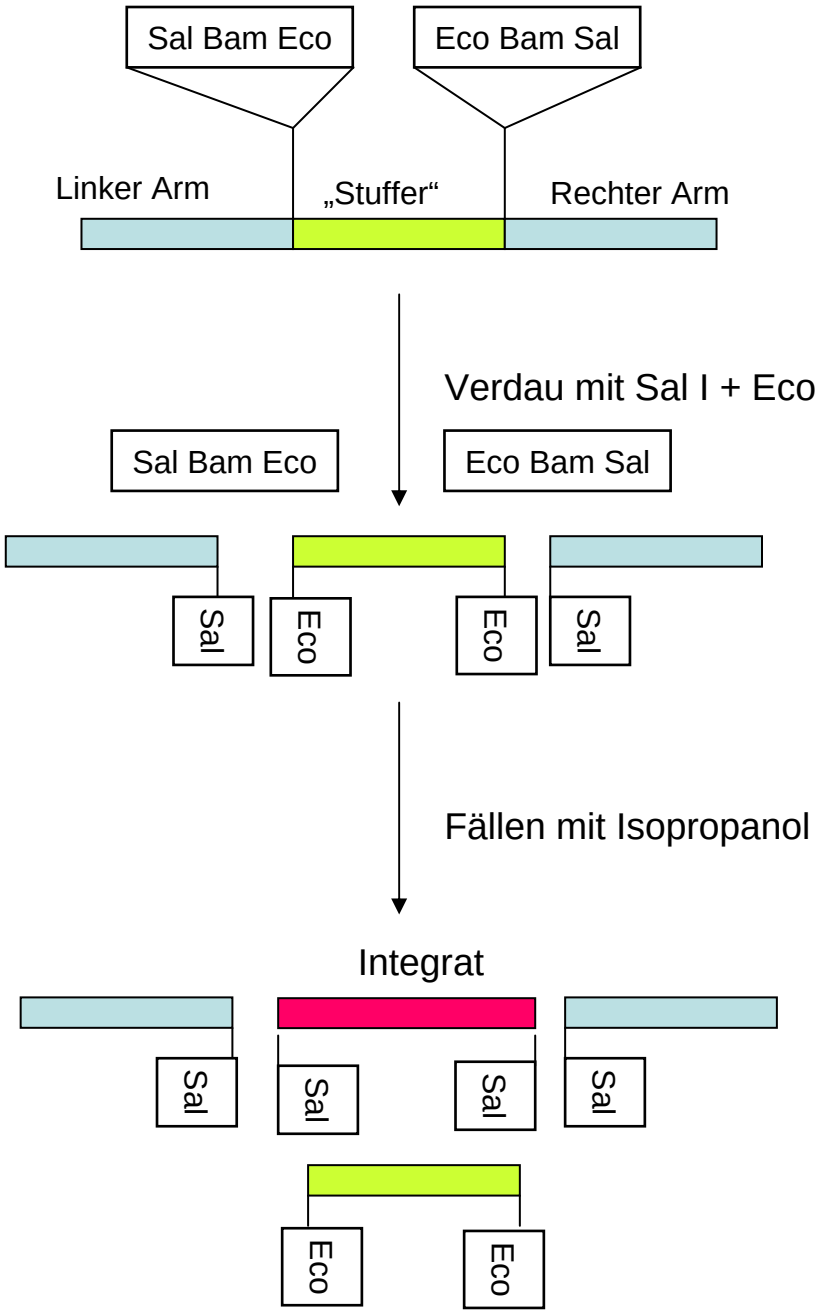
Map of Lambda EMBL3



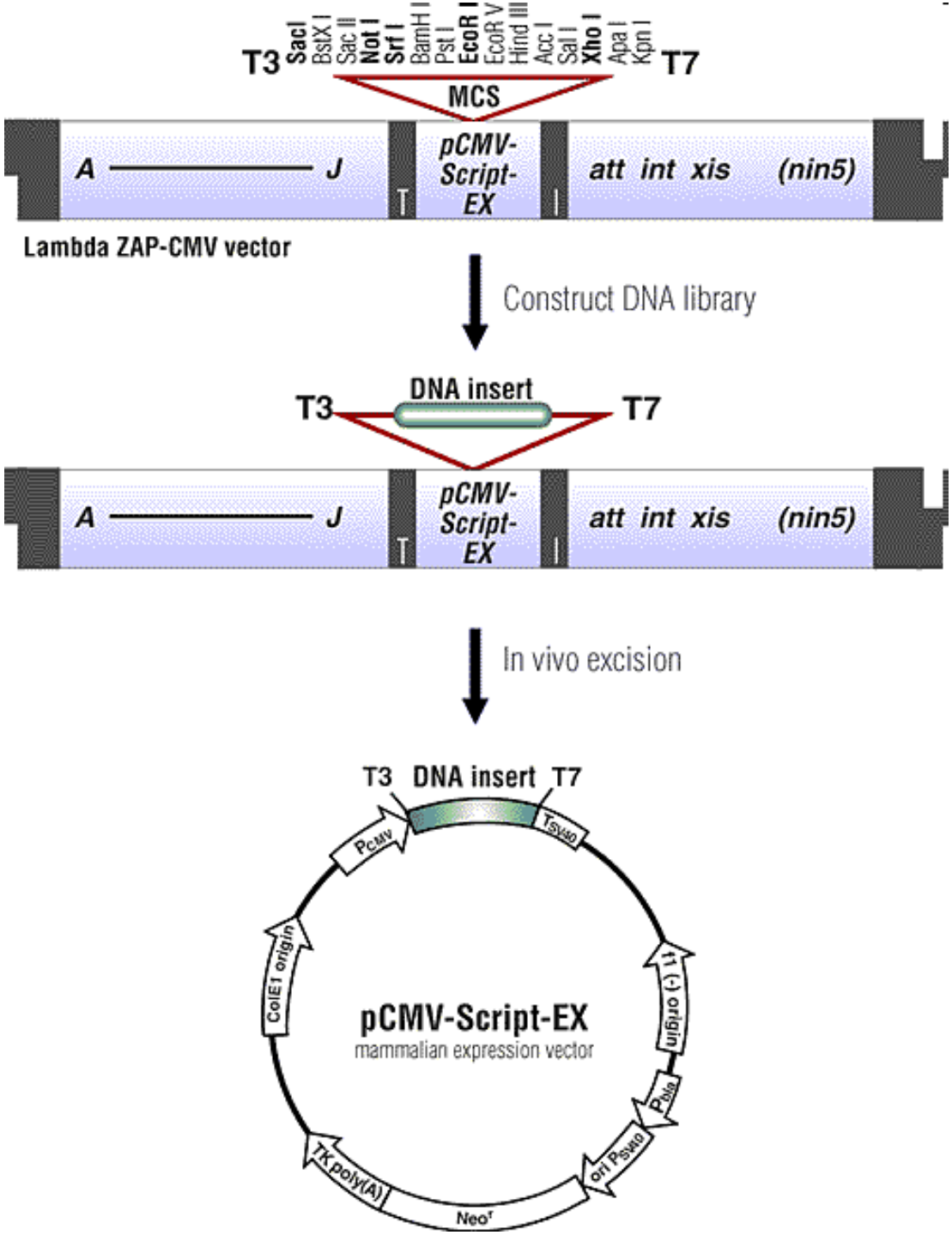
Map of Lambda EMBL4

Lambda EMBL3/4

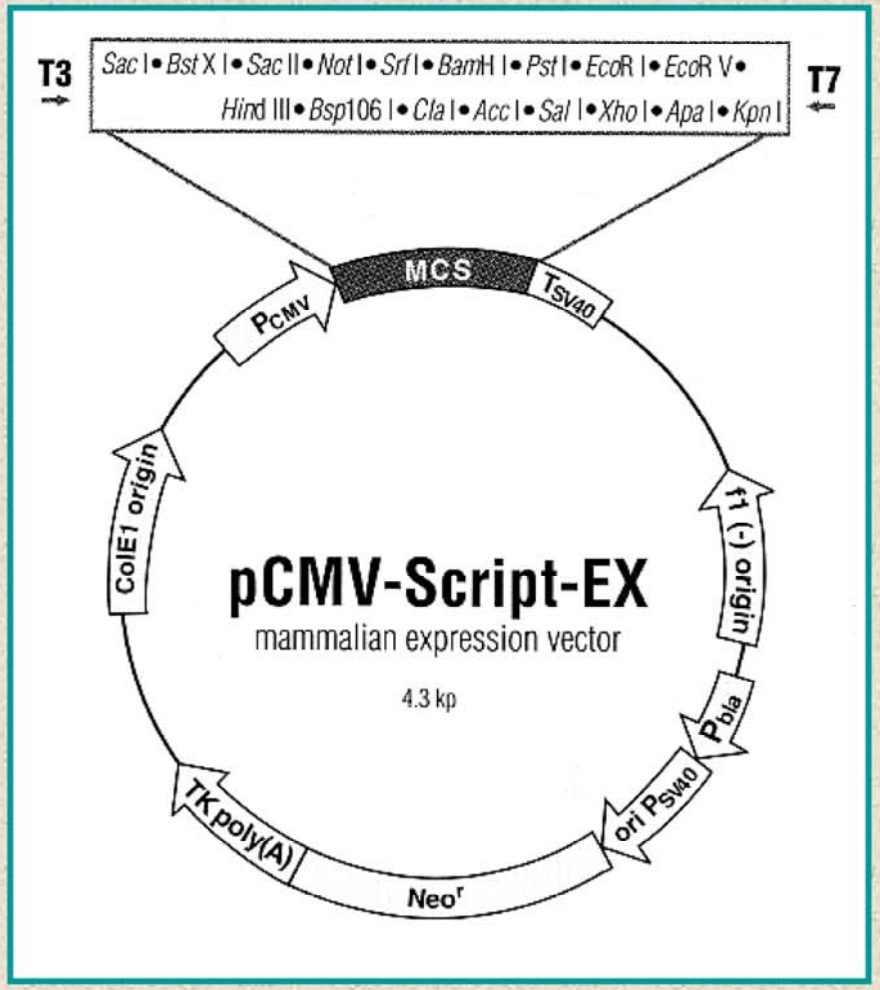
Durch Doppelverdau mit SalI und EcoRI erhalten die Arme andere Enden als das zentrale Fragment („stuffer“), so dass eine Religation des Vektors sehr unwahrscheinlich ist, insbesondere wenn die Linker durch Fällung aus dem Gemisch entfernt werden

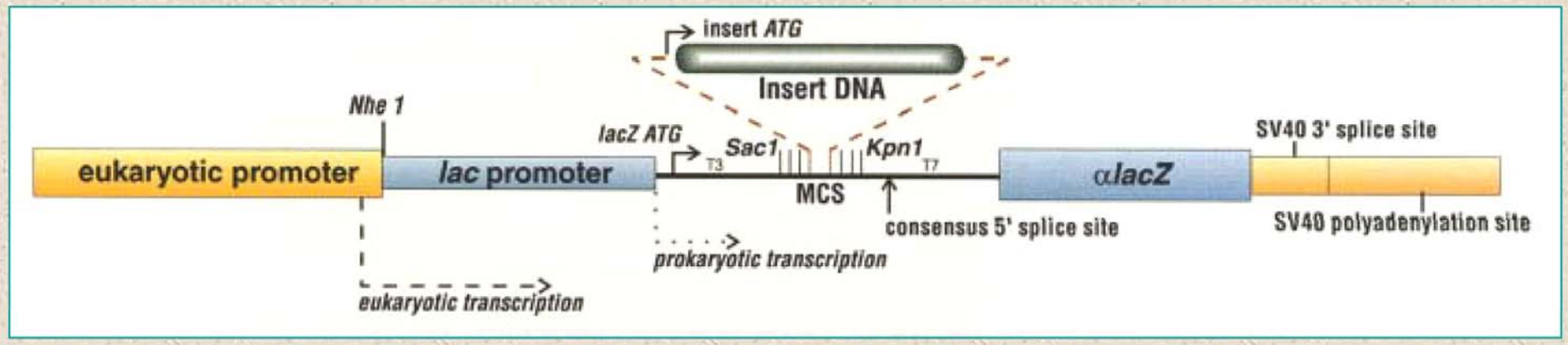


Lambda Zap:
Moderne Vektoren
Mit Phagen und Plasmid-eigenschaften

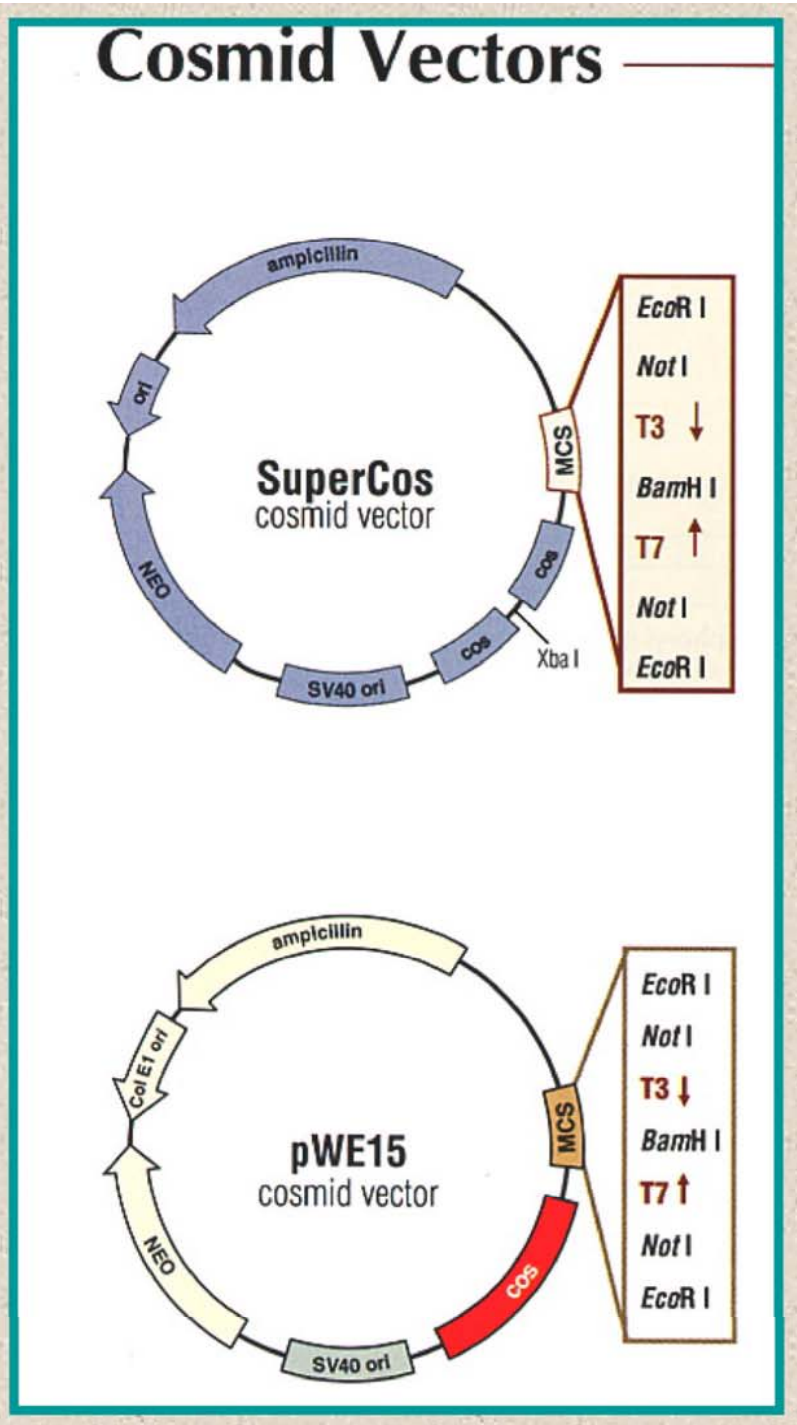


Lambda - „shuttle“ Expressions vektoren

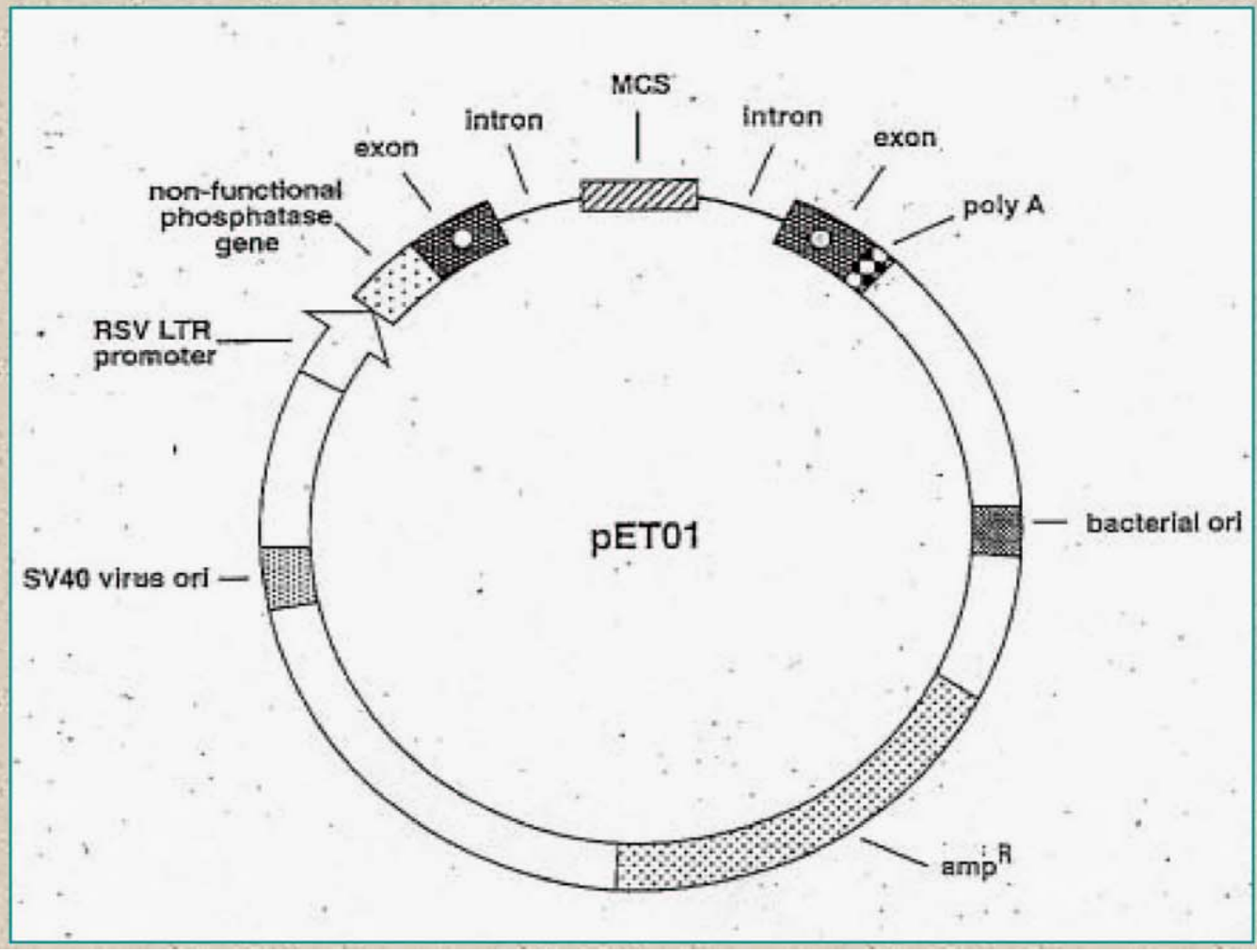




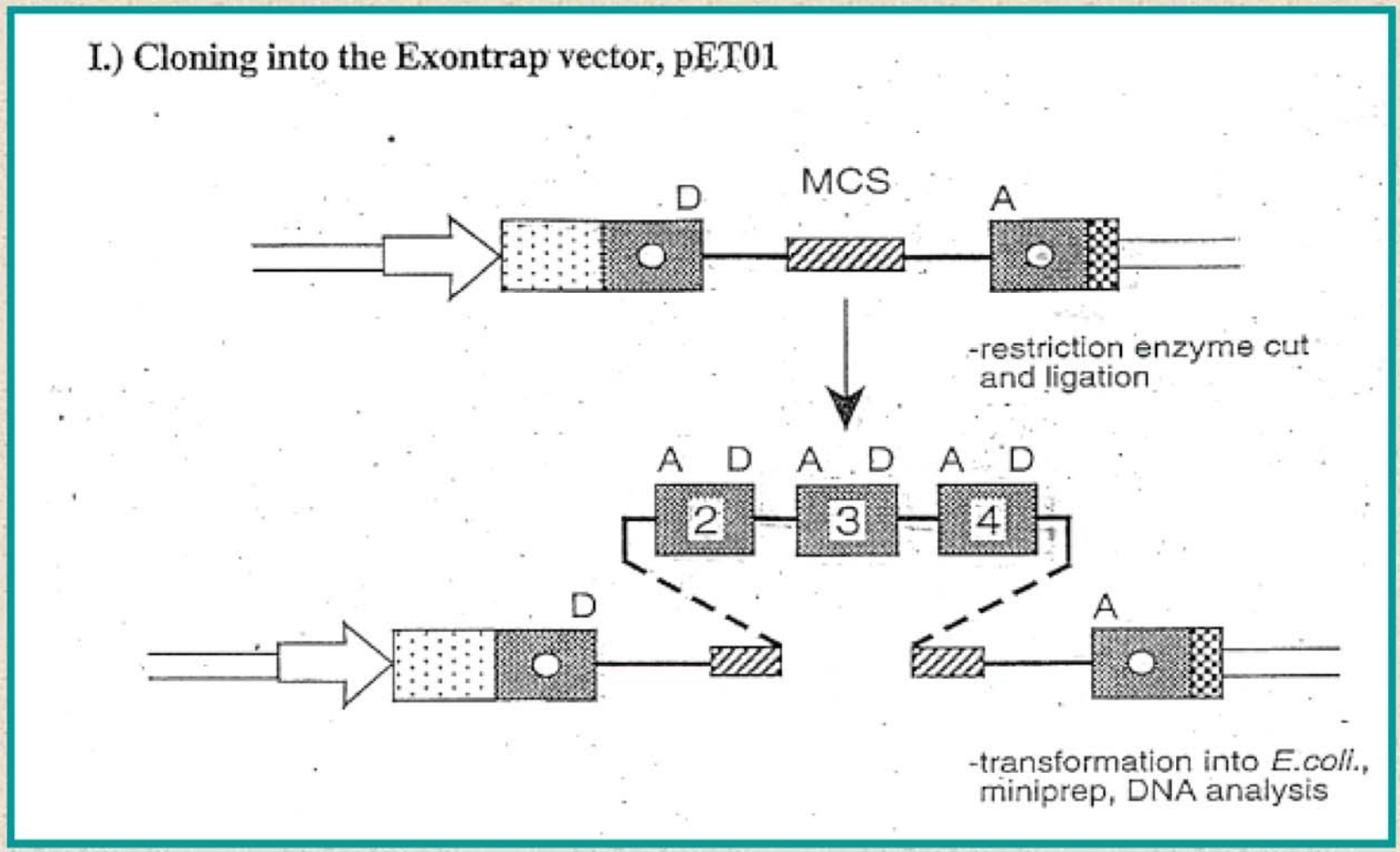
Cosmide,
Plasmide, die wie Bakteriophagen in die Zellen eingeschleust werden



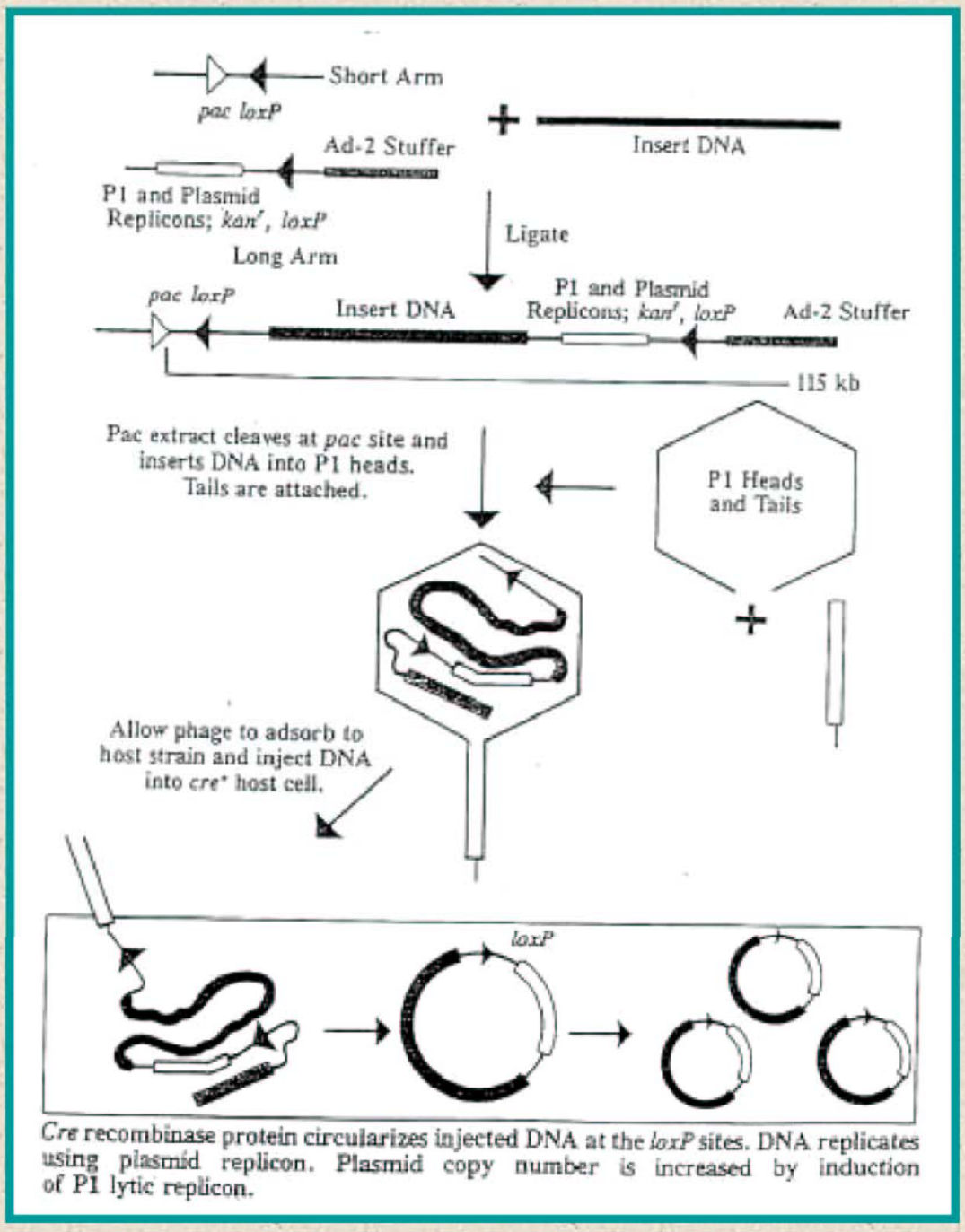
Spezialvektoren für „Exontrapping“



Spezialvektoren für „Exontrapping“

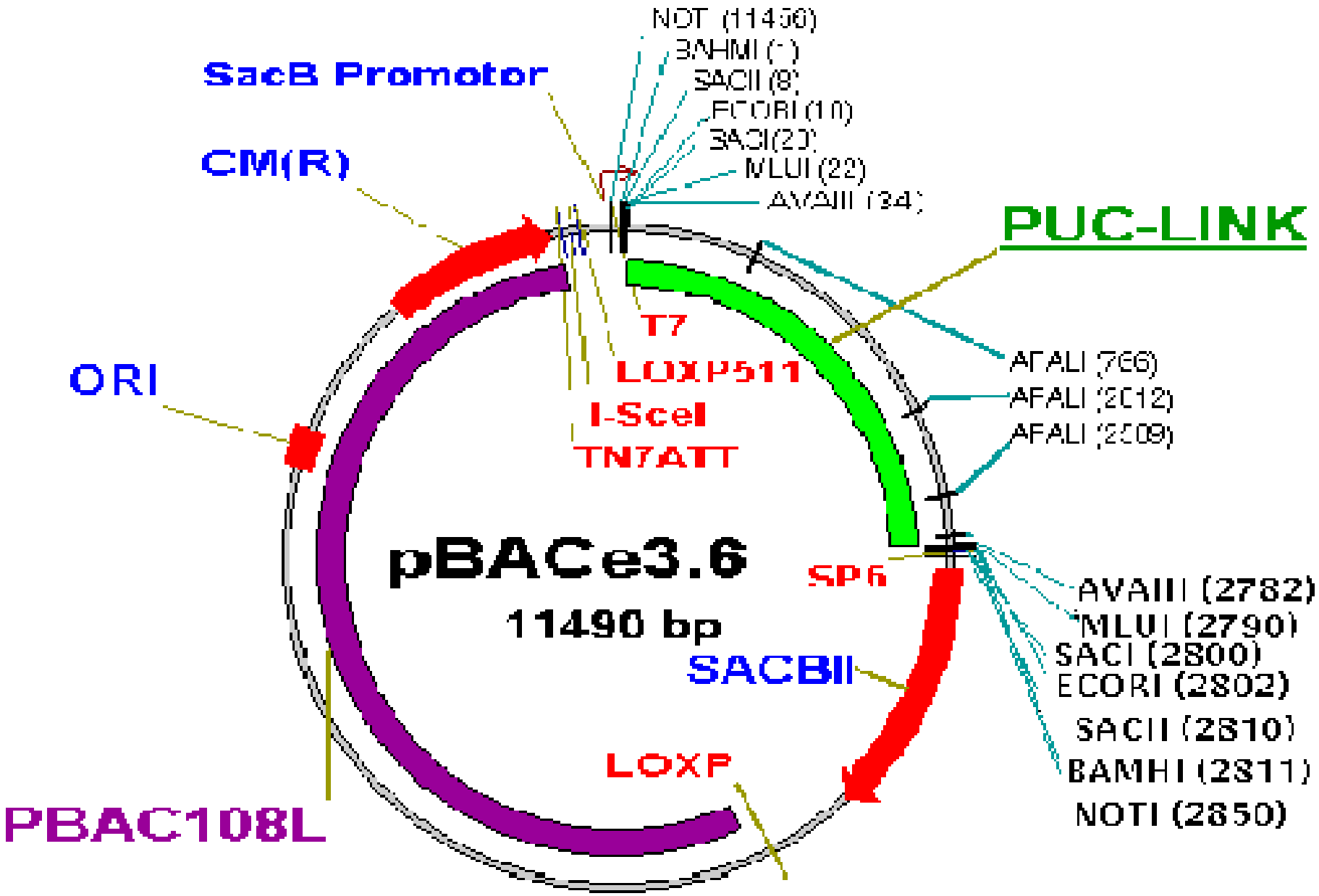


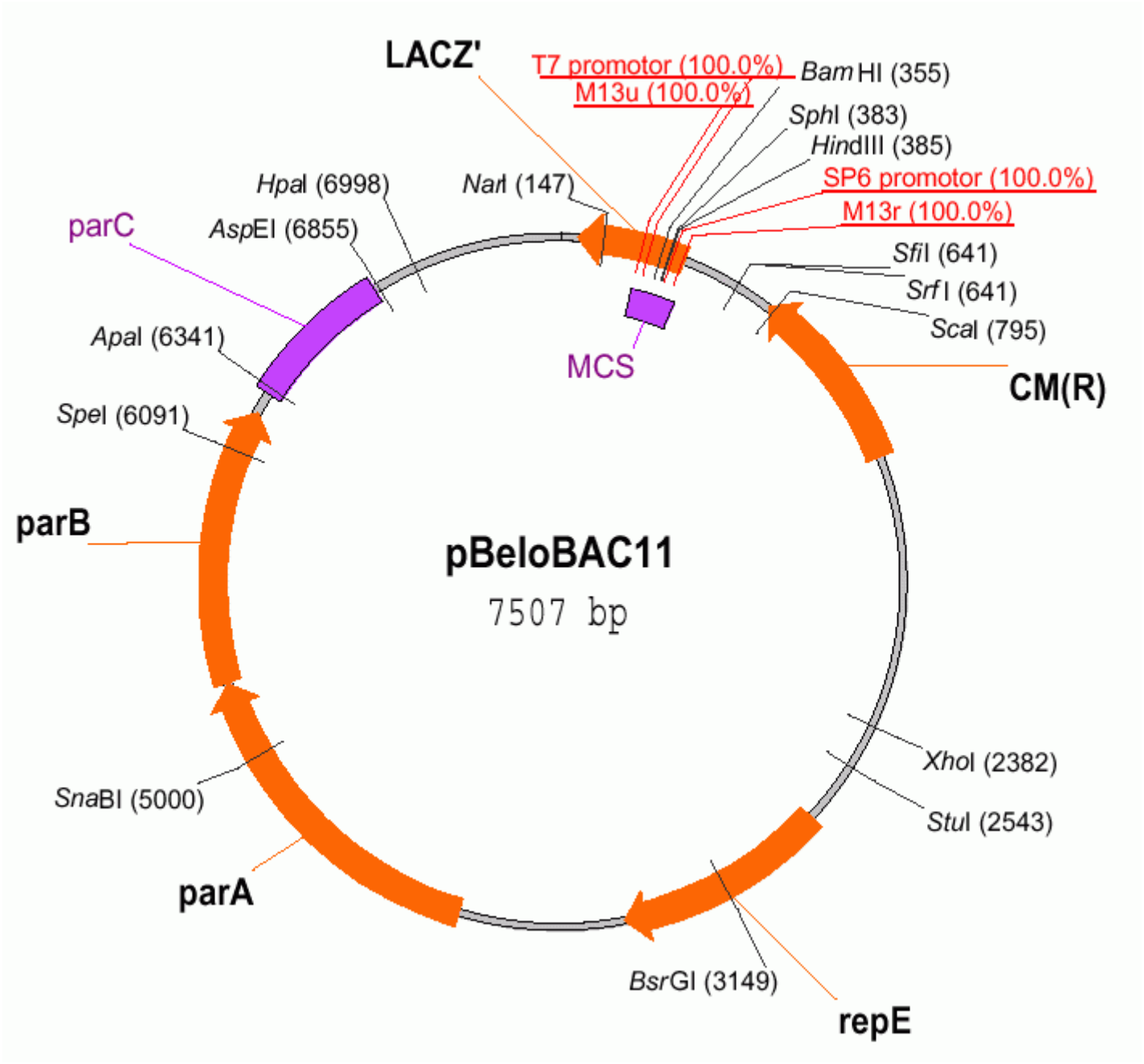
P1-Phage als Vektor: PAC: P1 artificial chromosome



Cre recombinase protein circularizes injected DNA at the *loxP* sites. DNA replicates using plasmid replicon. Plasmid copy number is increased by induction of P1 lytic replicon.

BAC-Vektoren
„bacterial artificial chromosomes“


NOT (11456)
BAHMI (1)
SACII (8)
ECORI (10)
SACI (20)
MLUI (22)
AVAIII (34)
SacB Promotor
CM(R)
PUC-LINK
T7
LOXP511
I-SceI
TN7ATT
ORI
AFALI (766)
AFALI (2012)
AFALI (2509)
pBACe3.6
11490 bp
SP6
AVAIII (2782)
MLUI (2790)
SACI (2800)
ECORI (2802)
SACII (2810)
BAMHI (2811)
NOTI (2850)
SACBII
LOXP
PBAC108L


LACZ'
T7 promotor (100.0%)
M13u (100.0%)
BamHI (355)
SphI (383)
HindIII (385)
SP6 promotor (100.0%)
M13r (100.0%)
SfiI (641)
SrfI (641)
ScaI (795)
CM(R)
XhoI (2382)
StuI (2543)
repE
BsrGI (3149)
parA
SnaBI (5000)
parB
SpeI (6091)
ApaI (6341)
parC
AspEI (6855)
HpaI (6998)
NarI (147)
MCS
pBeloBAC11
7507 bp

Künstliche Chromosomen für Eukaryoten

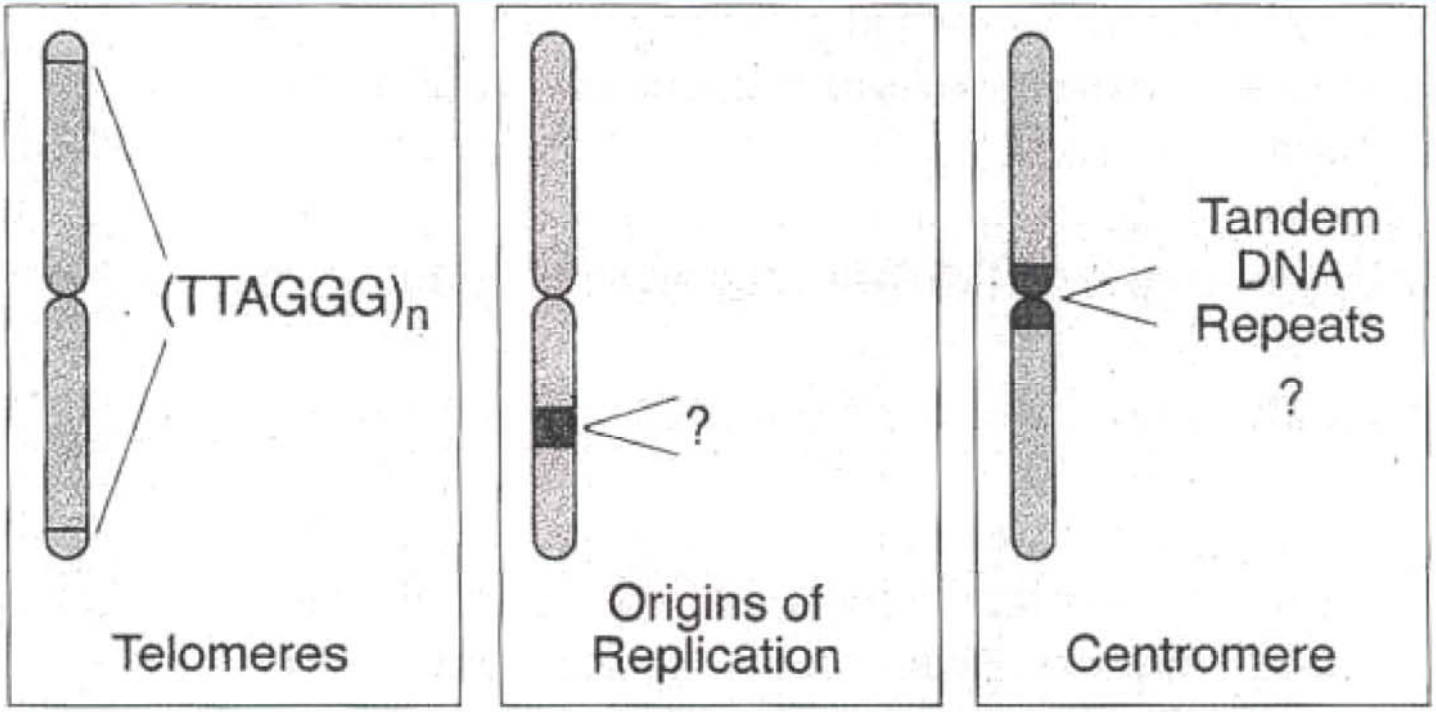
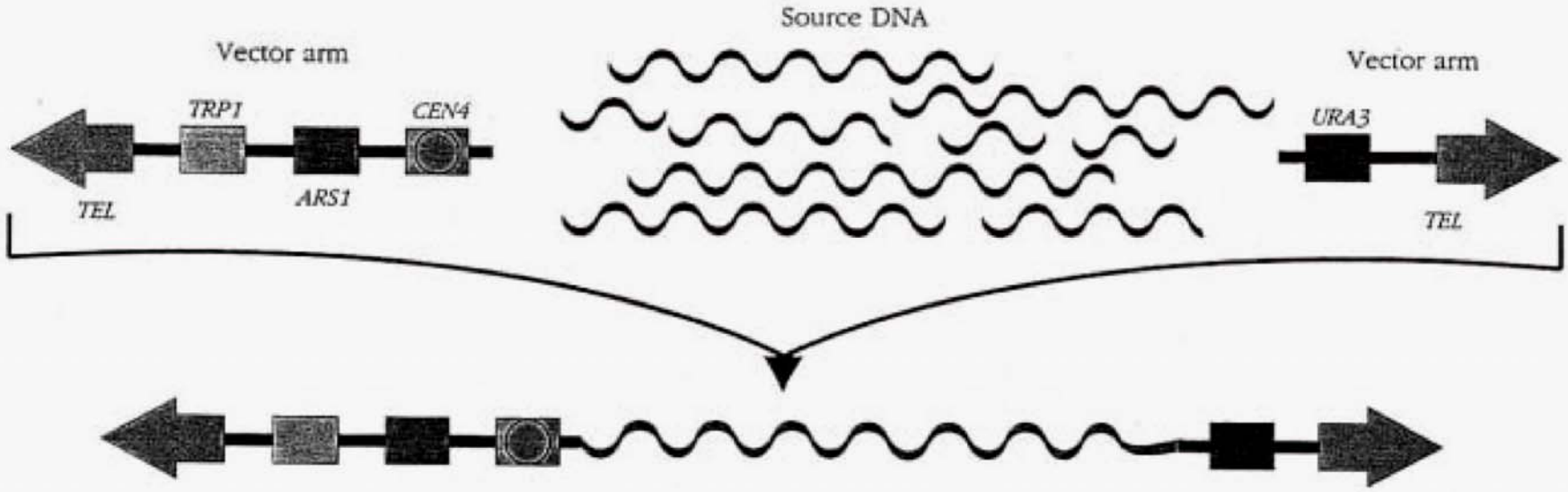


Fig. 1 Basic functional elements of linear chromosomes.

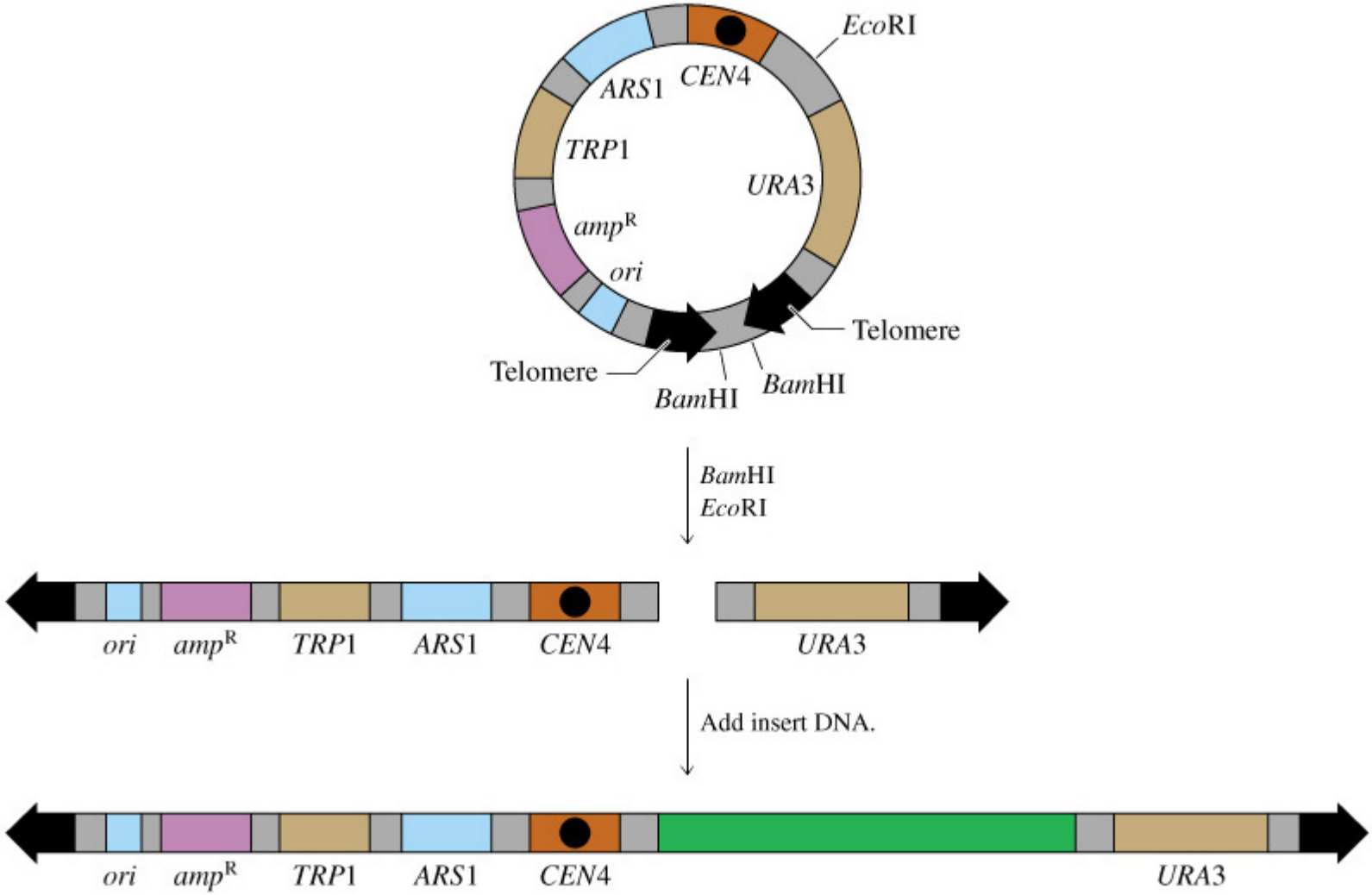
aus: Rosenfeld, M.: Human artificial chromosomes get real; Nature Genetics 15(4), pp 333-335

YACs = Yeast Artificial Chromosomes

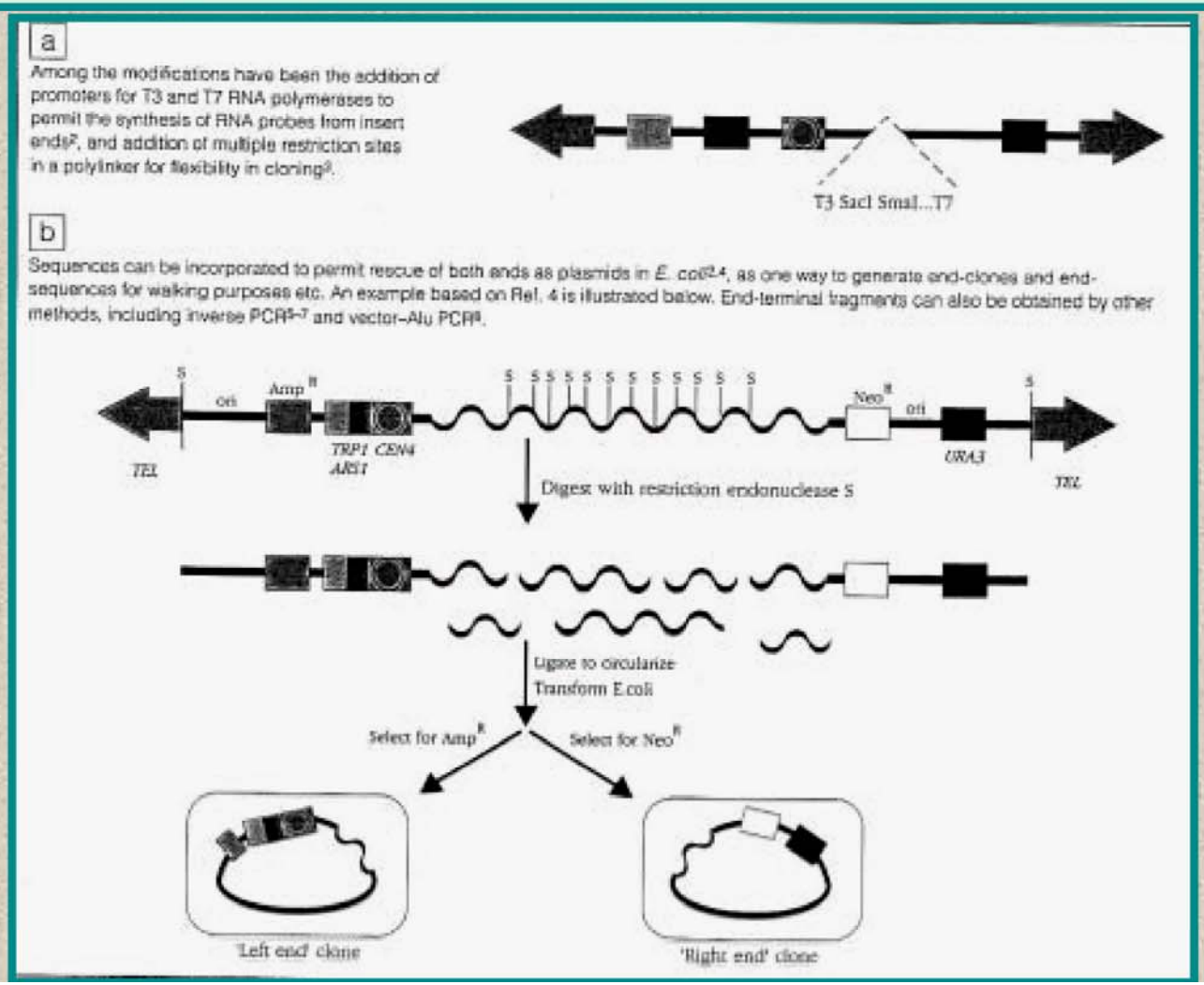


Large inserts of DNA are ligated between vector arms to form a YAC (from Ref. 1). The arms end in telomere sequences and contain centromeres (*CEN*), replication origins (*ARS*) and selectable markers (here, *TRP1* and *URA3*) to stabilize the YACs in yeast. A source DNA fragment is ligated between two arms to form a YAC. The original vectors[1] also contained a suppressor tRNA (which, because it was split by the insert, allowed YAC colonies to be recognized) and pBR322 sequences to facilitate mapping and recovery of one end of the insert as a plasmid in *E. coli* (see Panel 2b). Note that this and other diagrams here are not to scale: the arms total about 10 kb, the insert hundreds of kb.

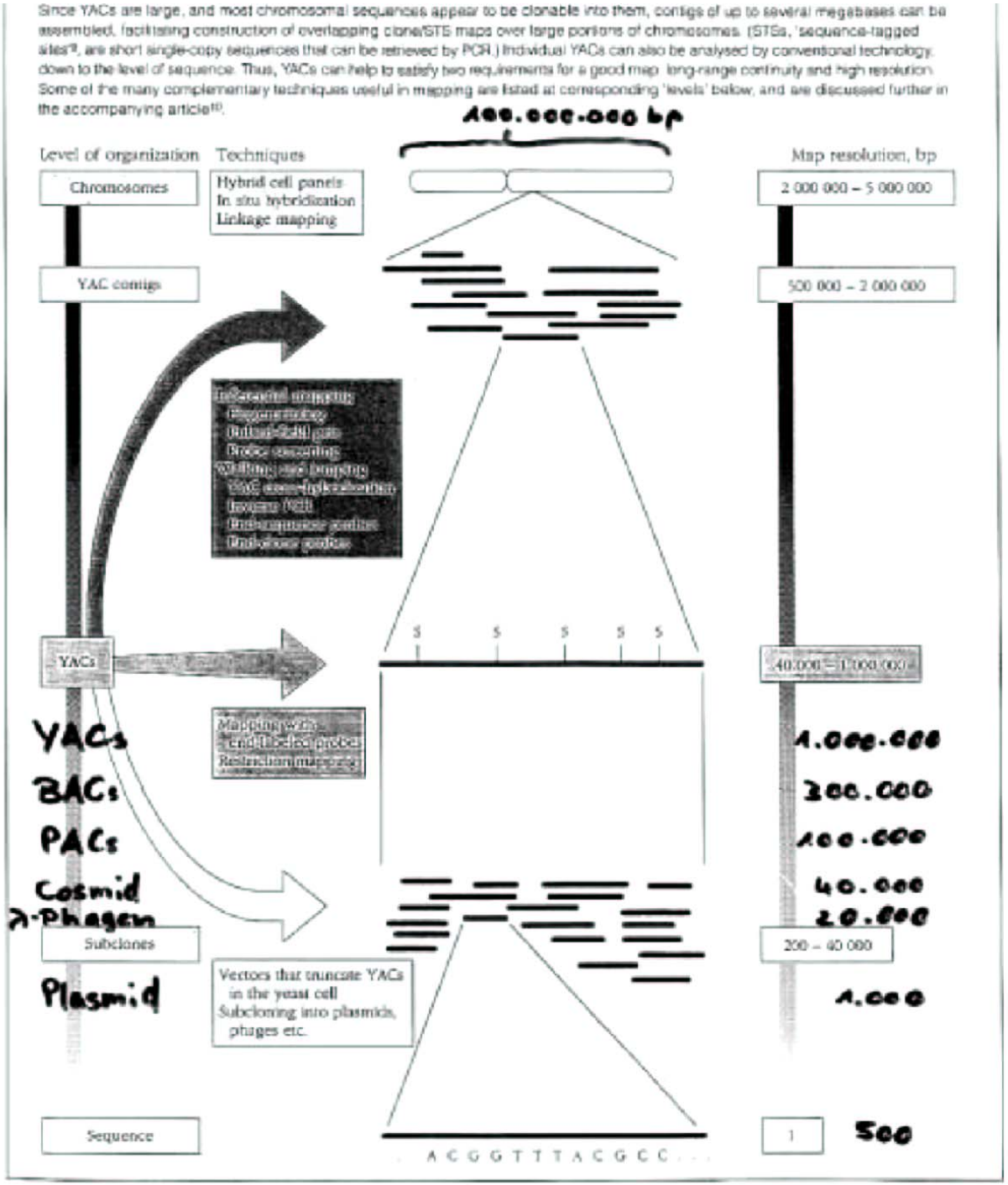
YACs = Yeast Artificial Chromosomes



Mit YACs kann man schnell die Endpunkte großer DNA-Fragmente klonieren

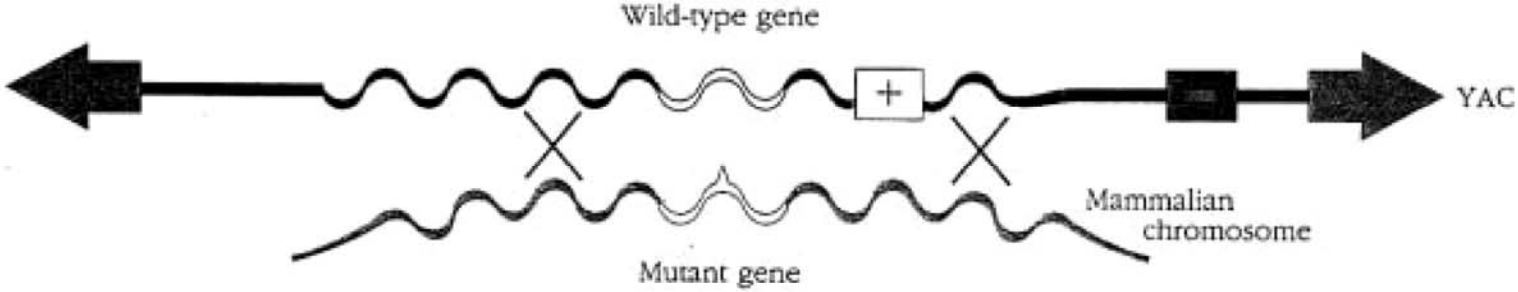


YACs sind besonders für große DNA Fragmente geeignet



YACs können für homologe Rekombination verwendet werden

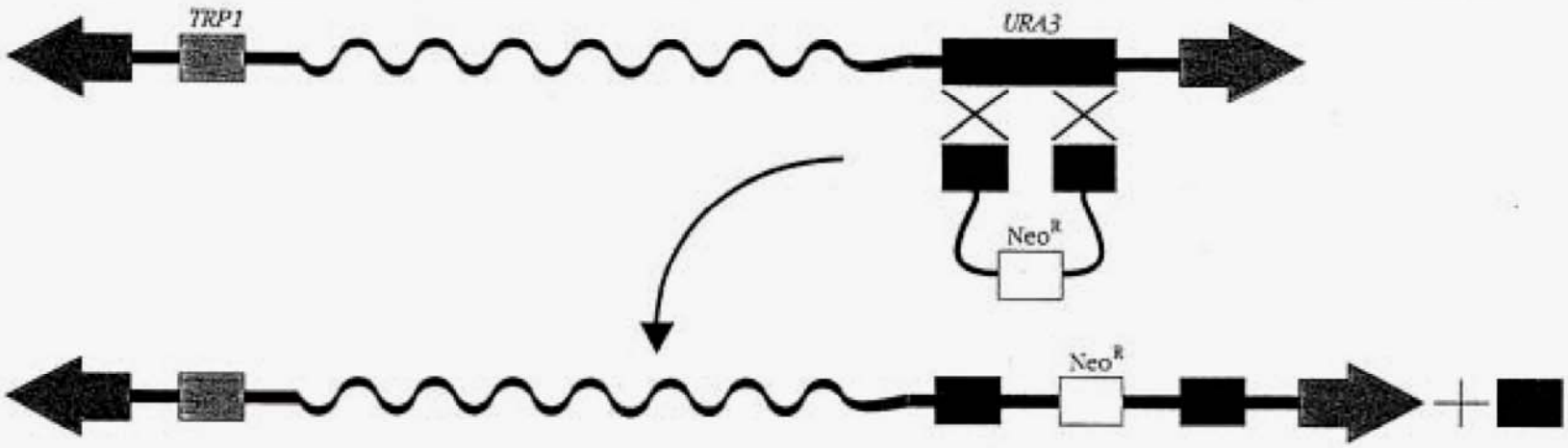
6 GENE THERAPY/HOMOLOGOUS RECOMBINATION



In principle, YACs could be used to provide an unaffected homologue to cells carrying a mutant gene associated with a disease. Repair of the mutant allele by homologous recombination remains a distant possibility, but might be favored by the large size of the homologous region and possibly by selectable markers, positive in the insert DNA (+) and negative in the vector arm (-) (see accompanying article[10] and Ref. 15).

YACs können für eukaryotische Expression eingesetzt werden

5 GENE EXPRESSION STUDIES



YACs can be large enough to contain genes longer than 200 kb, intact and in their normal context. When introduced into mammalian cells, for example by spheroplast fusion they can be expressed into products seen after 1–2 days (transient expression) or after selection of cloned stable transformants. YAC vectors may be designed so that they contain markers selectable in mammalian cells (e.g. the Neo^R gene conferring G418 resistance), or existing YACs may be fitted with such markers by homologous recombination: in the example shown above, a DNA fragment carrying the Neo^R gene and portions of the *URA3* gene recombines with an existing YAC, yielding a new YAC that has a split *URA3* gene and has gained Neo^R (adapted from B. Eliceiri *et al.*, work in progress).

Vektoren, die fremde DNA in die Chromosomen der Keimzellen einschleusen können, gibt es nur wenige

Transposons und **Retroviren** sind genetische Elemente, die sich gezielt in die chromosomale DNA der Wirtszelle einschleichen können

P-Elemente sind aktive Transposons bei Drosophila melanogaster und verursachen „Hybrid-dysgenesis“

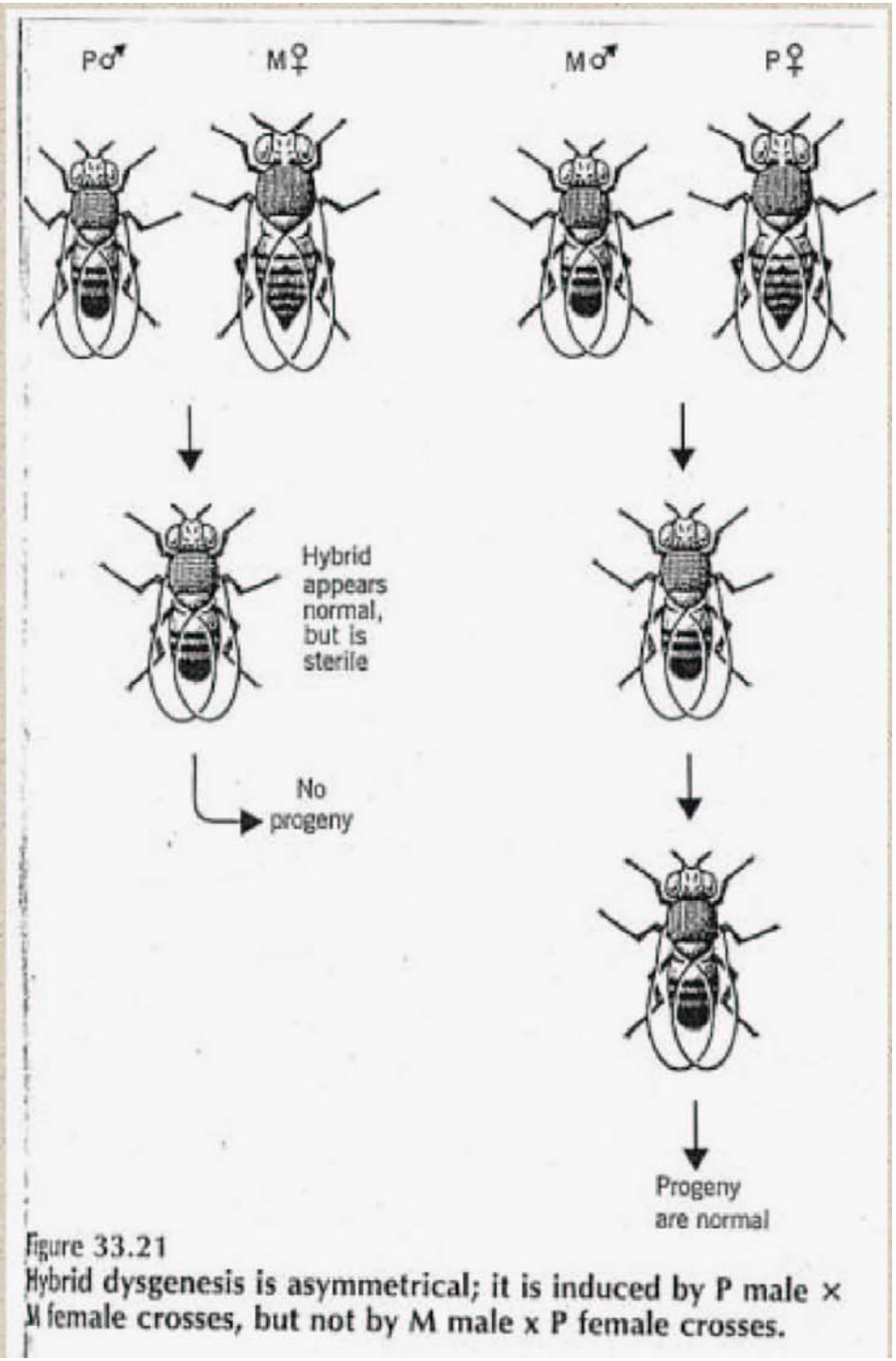
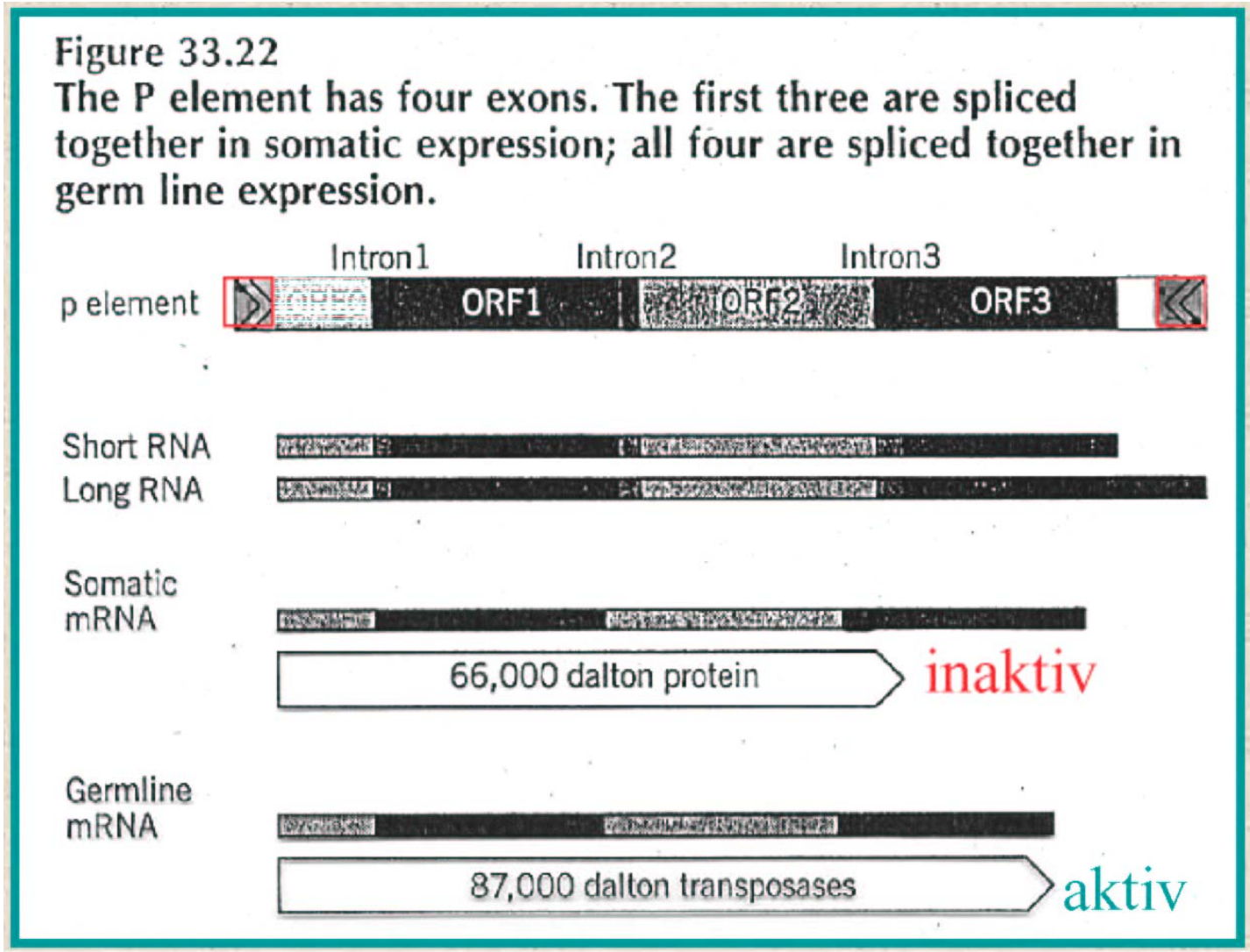
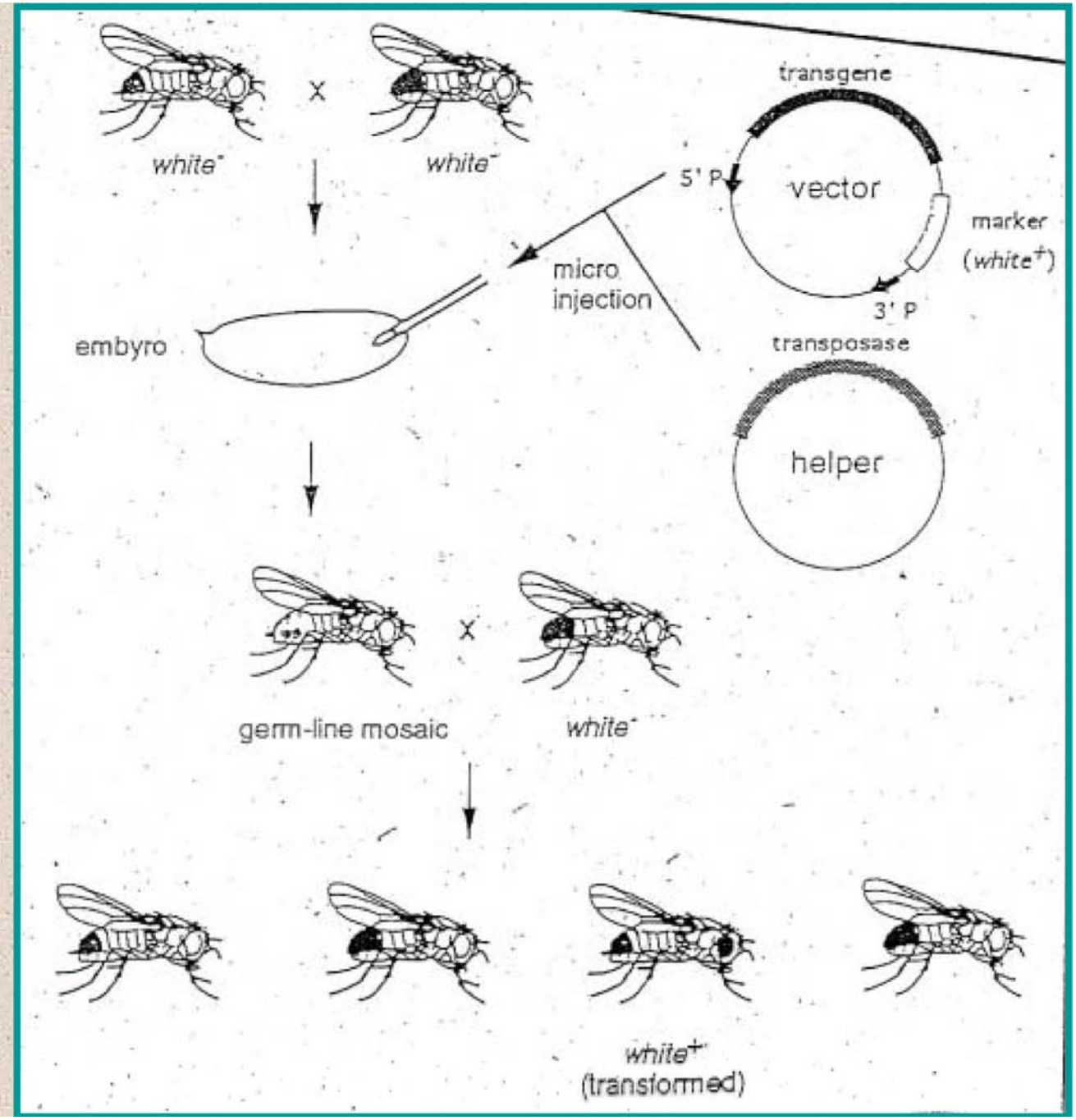


Figure 33.21
Hybrid dysgenesis is asymmetrical; it is induced by P male × M female crosses, but not by M male x P female crosses.

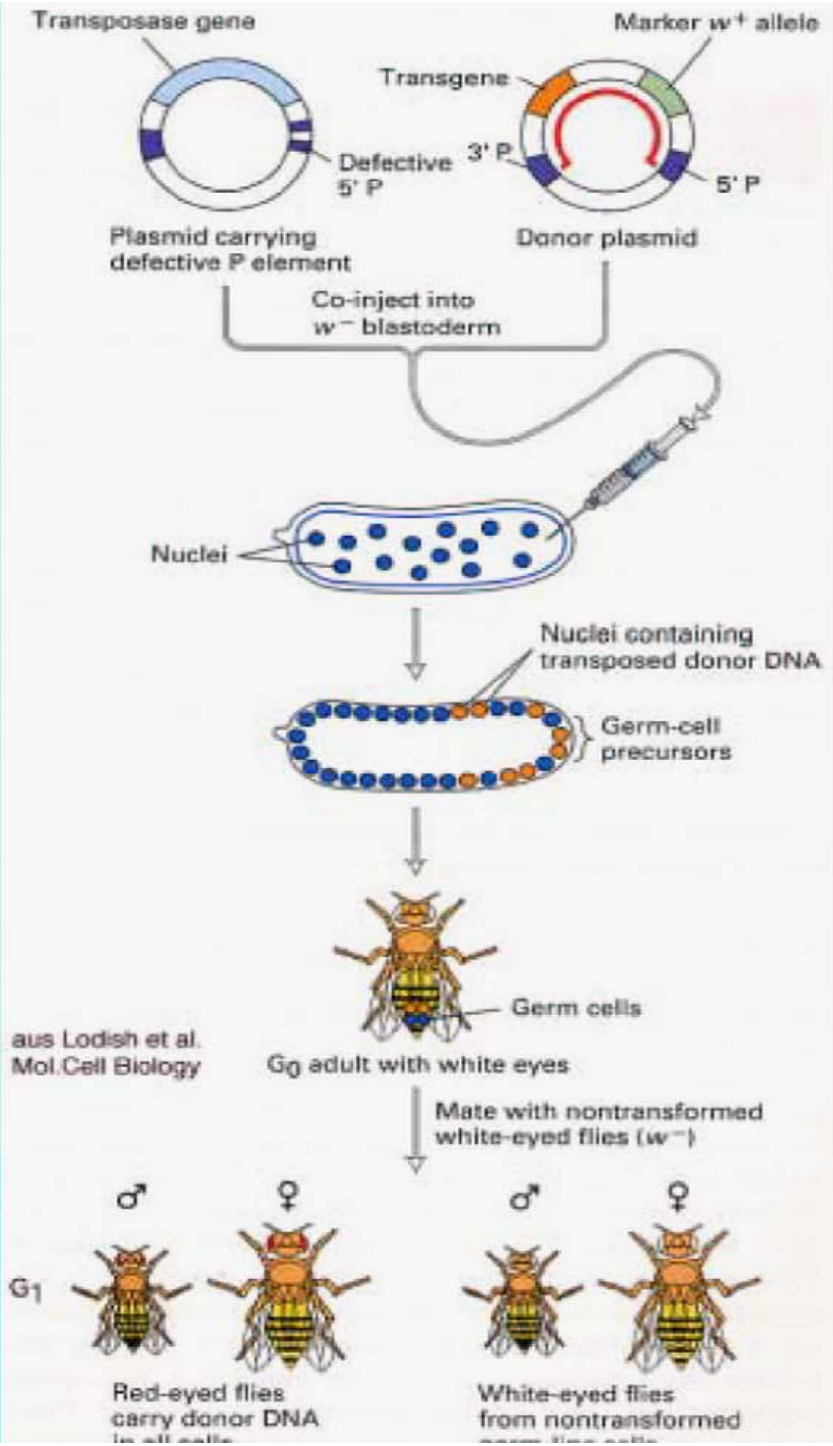
P-Elemente sind Transposons bei D. melanogaster,
die eine Keimbahn-spezifisch aktive Transposase exprimieren



P-Element vermittelte Keimbahn-trans-formation ist effizient und zuverlässig

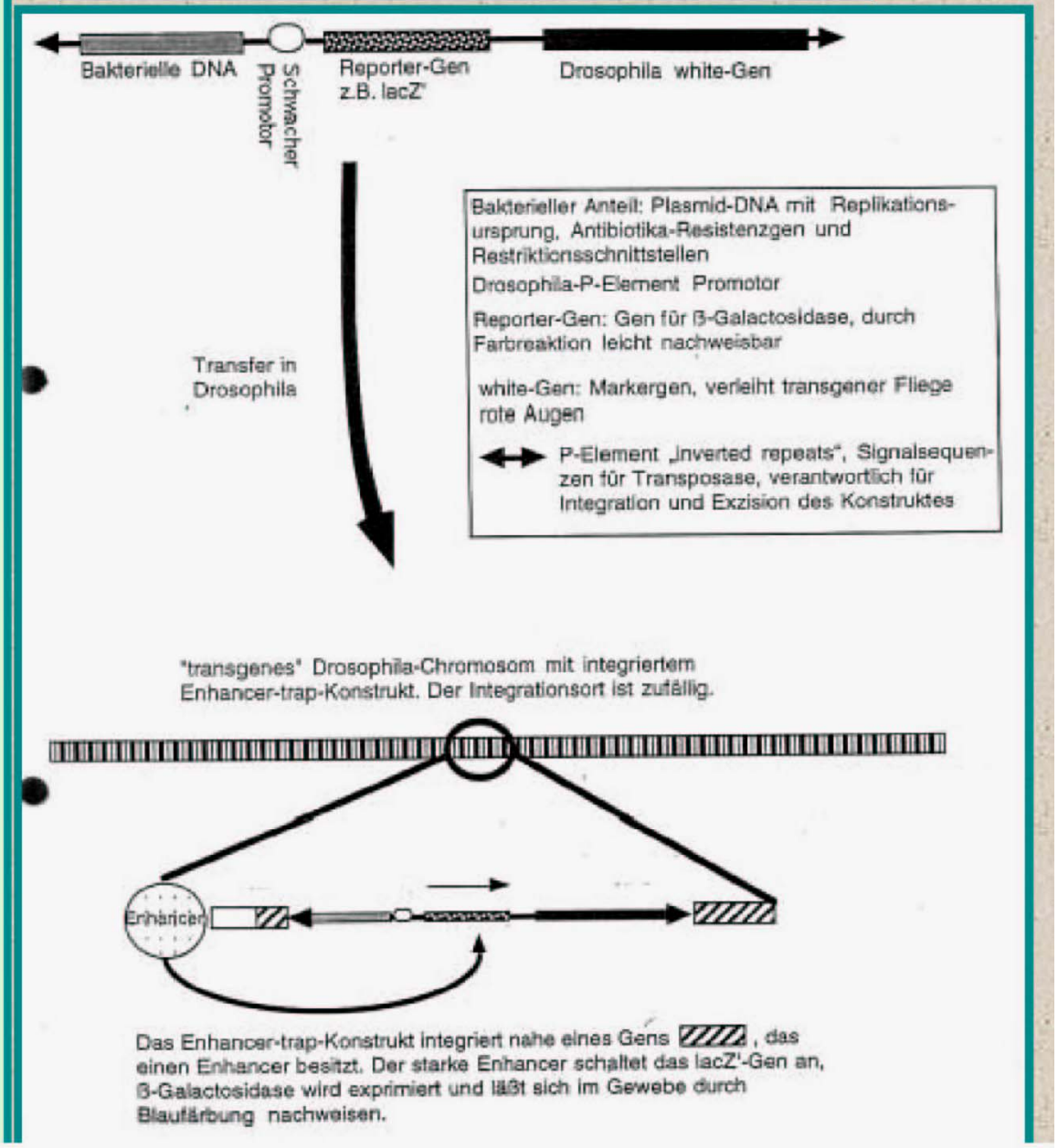


P-Element vermittelte Keimbahn-trans-formation ist effizient und zuverlässig

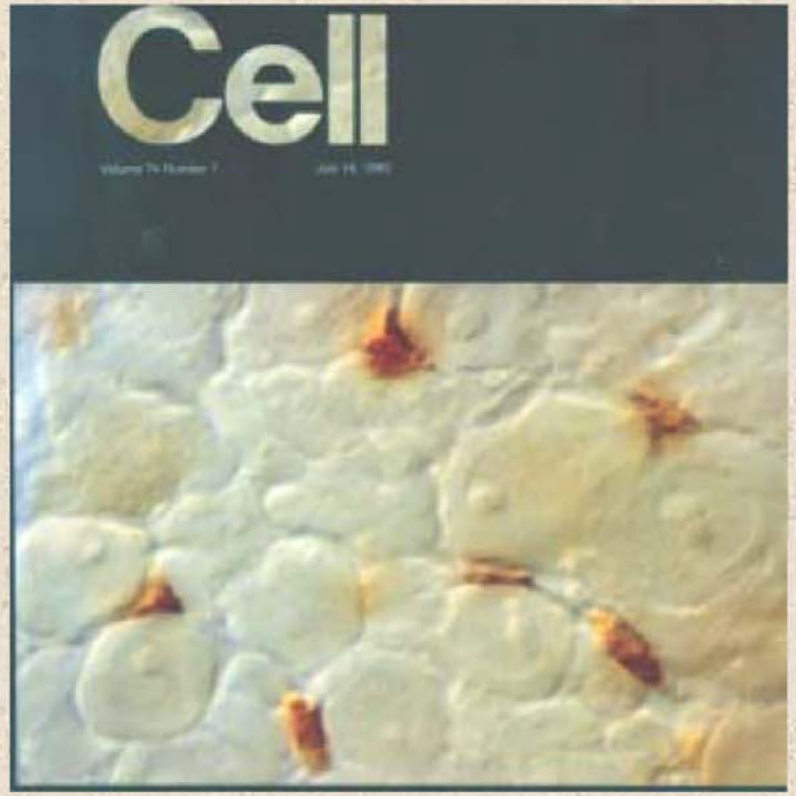


P-Elemente sind extrem nützlich zum Studium der Genfunktion z. B. beim Nachweis von Enhancer-Elementen

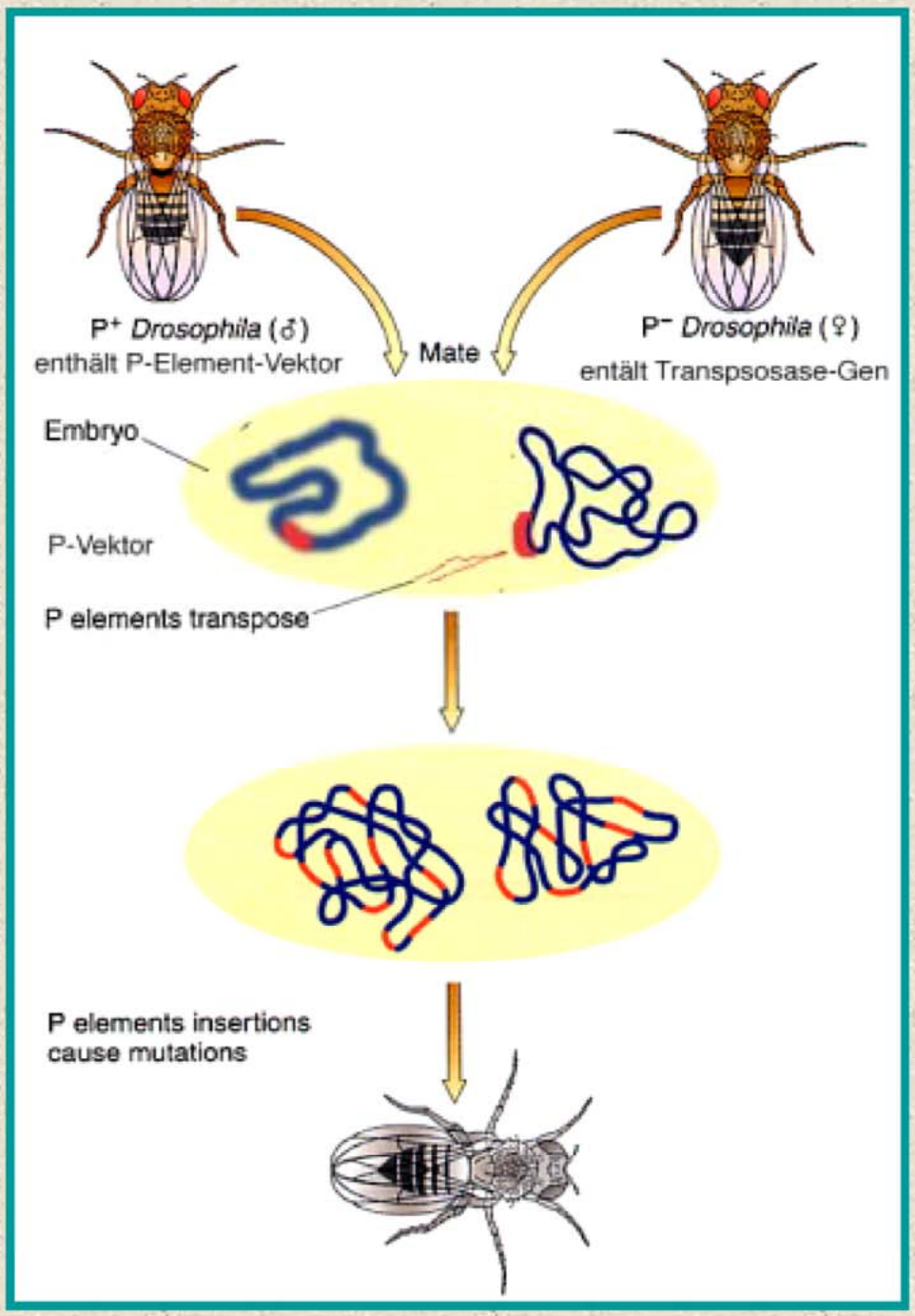
„Enhancertrap“-Methode



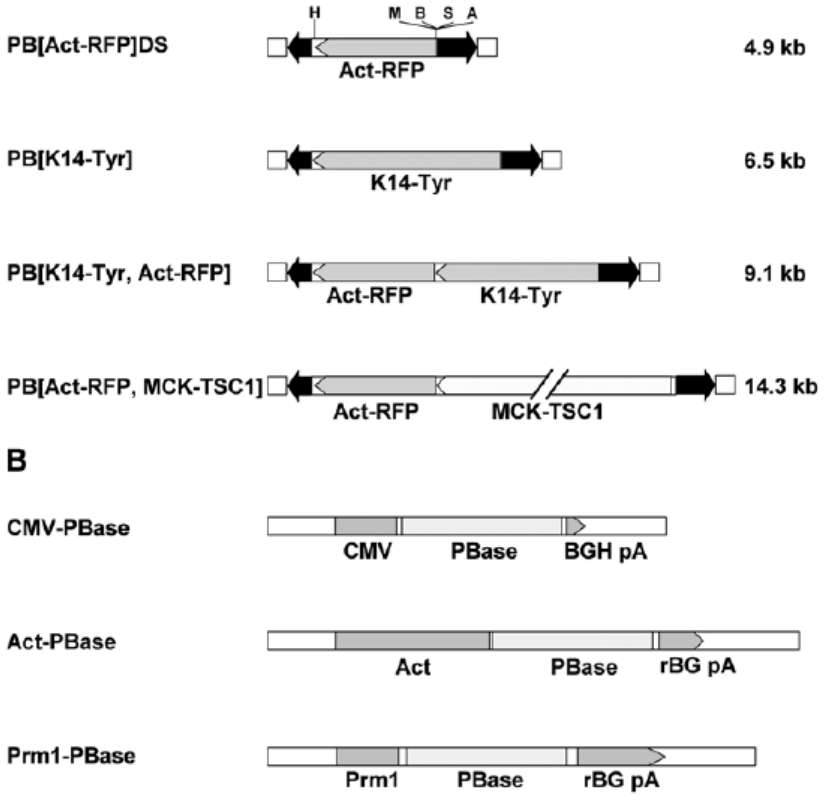
Mit der Enhancer-Trap-Methode lassen sich sehr spezifisch exprimierte Gene erkennen



Künstlich eingeführte P-Elemente können zur Mutagenese verwendet werden



PiggyBac-Transposon aus *Trichoplusia ni* (Schmetterling) funktioniert in einer großen Bandbreite von Organismen inklusive der Maus



(A) *PB* donor constructs. Marker or endogenous genes (shaded boxes with arrows denoting transcription direction) driven by various promoters were placed between a pair of *PB* repeat termini (PBL and PBR, black arrows). Arrowheads above the termini show the relative positions of primers used for inverse PCR. Total lengths of the transposons are also indicated. Open boxes represent the plasmid backbone sequences. M: MfeI, B: BamHI, S: SwaI, A: AscI, H: HindIII.

(B) *PB* transposase helper constructs. The *piggyBac* transposase gene (*PBase*) driven by cytomegalovirus (*CMV*), β-actin (*Act*), or Protamine 1 (*Prm1*) promoters were followed by either bovine growth hormone polyA (*BGH pA*) or rabbit β-globin polyA (*rBG pA*).

Cell Immediate Early Publication

Published Online July 21, 2005
DOI: 10.1016/j.cell.2005.07.013

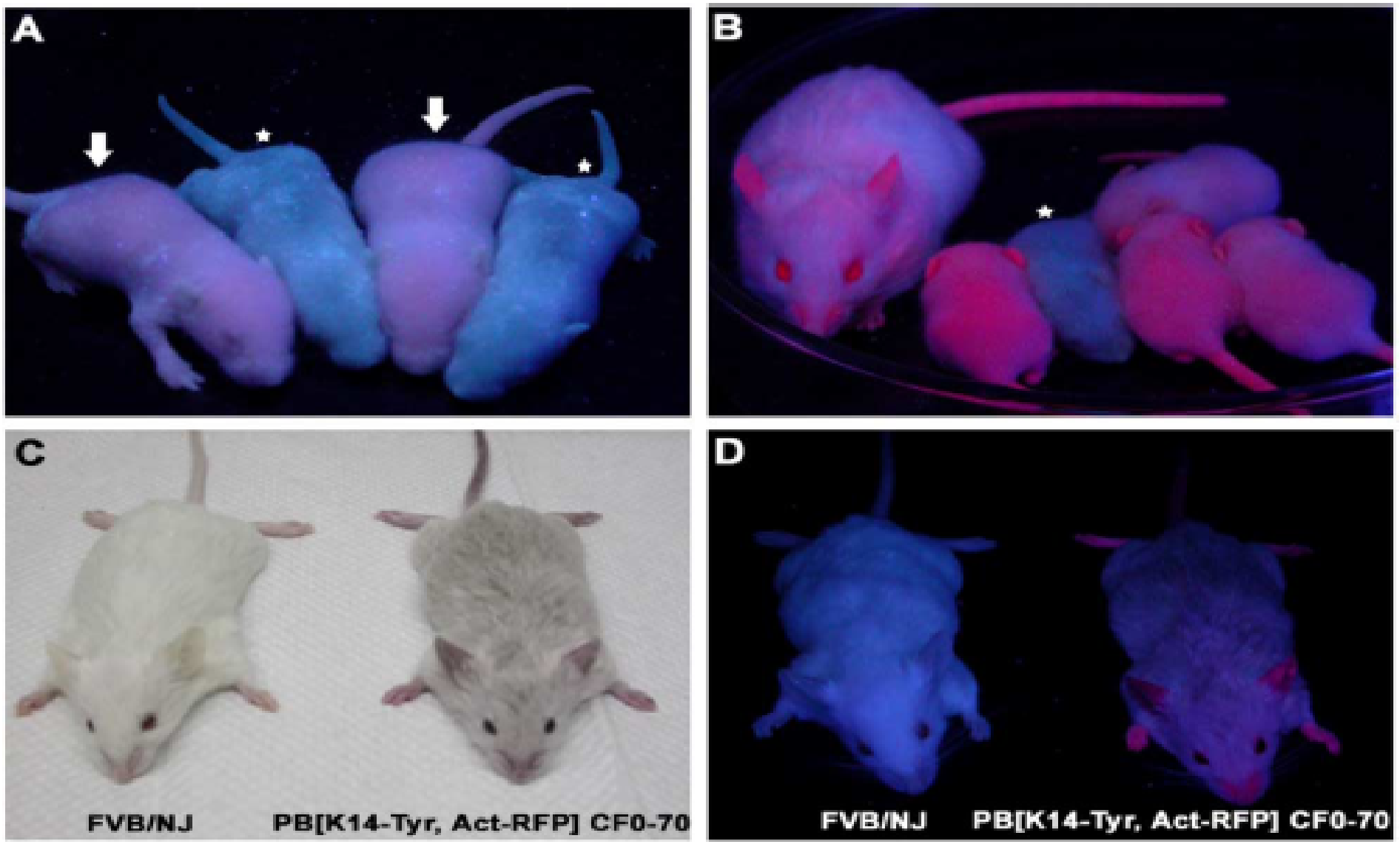


Figure 5. Expression of Transgenes in *piggyBac* Vectors