Transfert de gènes dans des cellules en culture

Christian Diot

TRANSFERT DE GENES DANS DES CELLULES EN CULTURE

C. DIOT UMR PEGASE, 2016


BAH MINCE ALORS !
IL PARAIT QU'ON VA TRANSFERER DES GENES DANS LES CELLULES

TRANSFERT DE GENES DANS DES CELLULES EN CULTURE

- **Pourquoi ?**
  - ✓ produire une protéine (à forte valeur ajoutée)
  - ✓ comprendre la fonction d'une protéine
  - ✓ comprendre la régulation de l'expression d'un gène
  - ✓ ...

- **Comment ?**
  - ✓ ajouter une (nouvelle) information génétique
  - ✓ modifier qualitativement ou quantitativement l'expression d'un gène
  - ✓ ajouter la séquence modifiée d'un promoteur, ou des fragments, dirigeant l'expression d'un gène (rapporteur)
  - ✓ …

TRANSFERT DE GENES DANS DES CELLULES EN CULTURE

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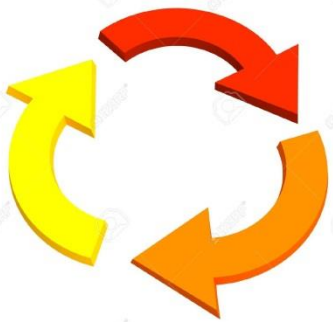
  - ✓ **...**

Approches haut-débit (*omiques)
⇨ découverte de « nouveaux » et nombreux gènes dont la fonction est inconnue, ou partiellement connue

TRANSFERT DE GENES DANS DES CELLULES EN CULTURE



- **Comment ?**

Trois facteurs (transfert, gène, cellule) interdépendants :

Orchestration coordonnée de ces paramètres en fonction de l'objectif à atteindre

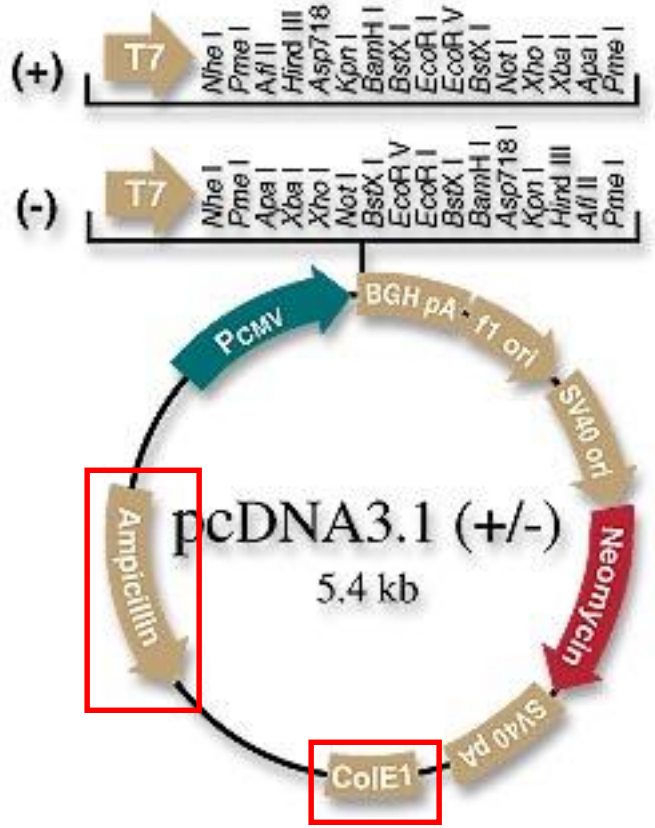
⇨ le transfert doit être fonctionnellement efficace
(le transgène doit s'exprimer dans les cellules)

TRANSFERT DE GENES DANS DES CELLULES EN CULTURE

- **Gène est… générique : séquence d'acide nucléique**
  - ✓ **transgène : gène, exogène à la cellule**
  - ✓ **« modulateur » : outil de modification de l'expression d'un gène endogène à la cellule**

TRANSFERT DE GENES DANS DES CELLULES EN CULTURE

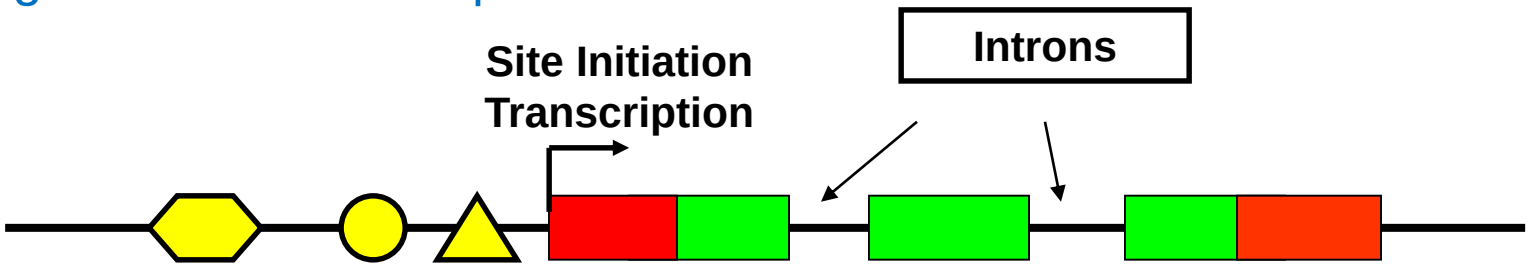
- **le transgène doit s'exprimer dans les cellules**
  = structure d'un gène eucaryote



- **le transgène doit s'amplifier facilement, en quantité importante**
  = Plasmide bactérien
  - ⇨ Origine autonome de réplication
  - ⇨ Gène de sélection (Résistance ABio)

TRANSFERT DE GENES DANS DES CELLULES EN CULTURE

- **le transgène doit s'exprimer dans les cellules**
  = structure d'un gène eucaryote
  - ⇨ Promoteur (+ séquences de régulation)
  - ⇨ Séquence codante
  - ⇨ Introns
  - ⇨ signaux arrêt transcription



Promoteur
TATA-box
CAAT-box
Elt(s) Régul.

Séquences transcrites Exons

traduites (ATG, stop)

non traduites
5 ', 3 ' arrêt Trscr, poly(A)+

TRANSFERT DE GENES DANS DES CELLULES EN CULTURE

- **le transgène doit s'exprimer dans les cellules**
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  - ⇨ Séquence codante -> site de clonage
  - ⇨ Introns
  - ⇨ signaux arrêt transcription

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  = Plasmide bactérien
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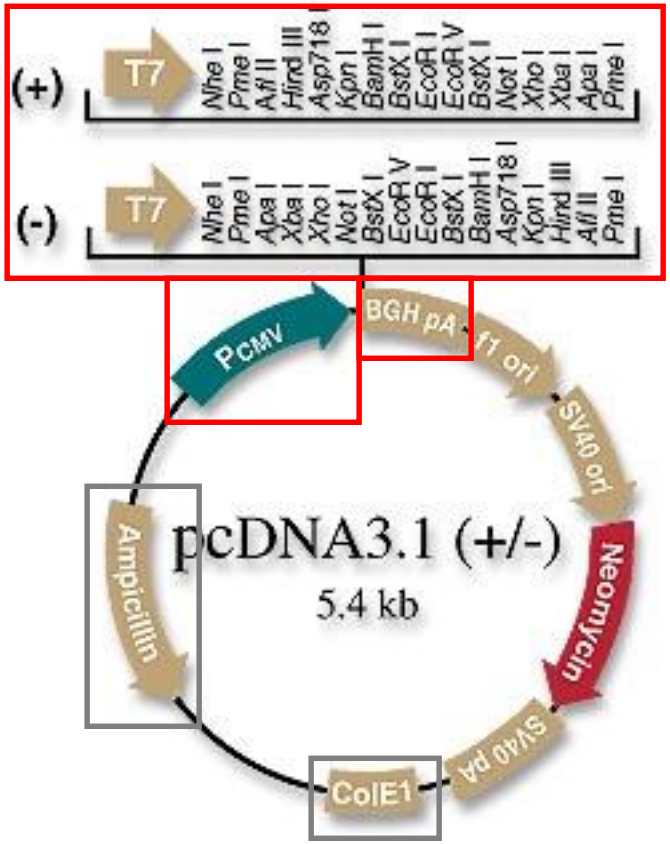

TRANSFERT DE GENES DANS DES CELLULES EN CULTURE

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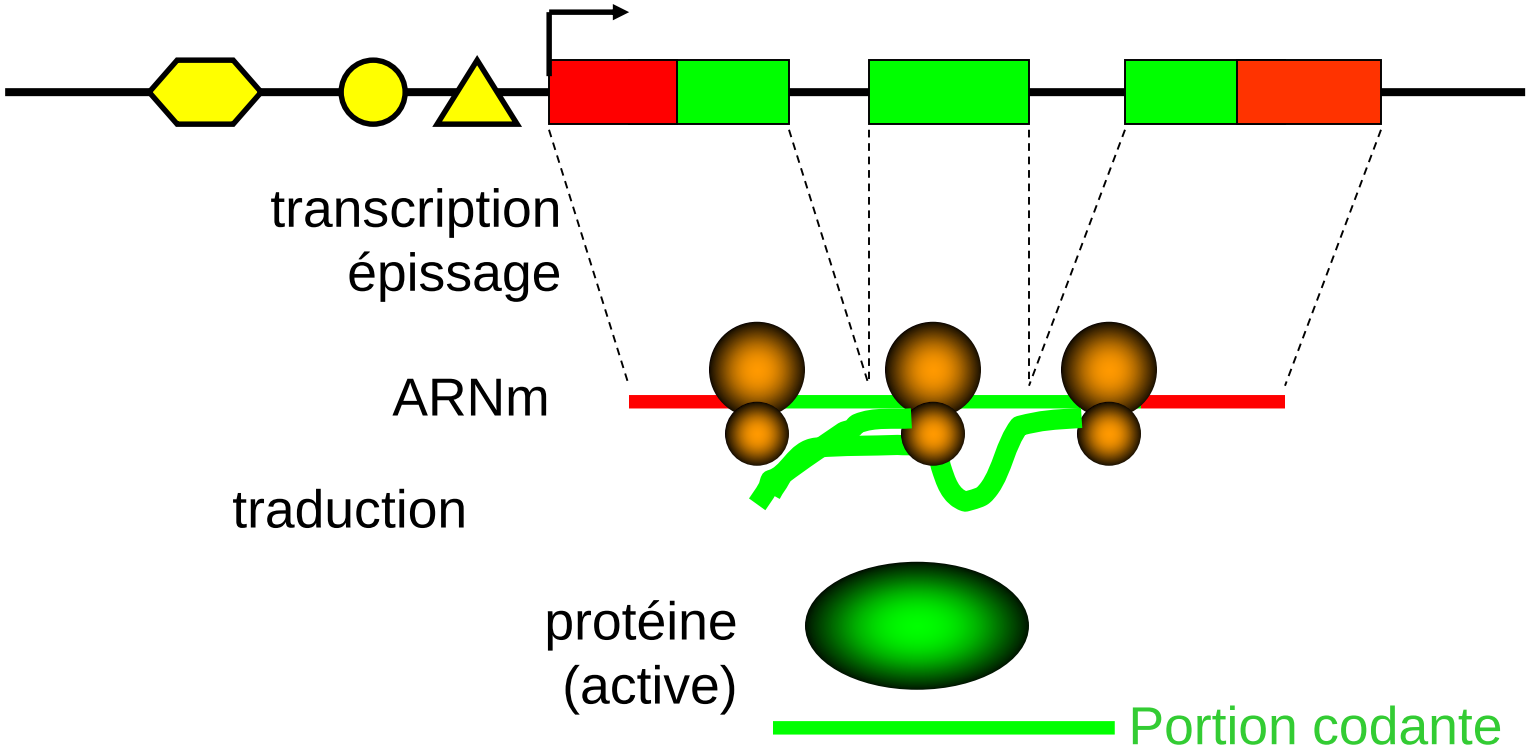

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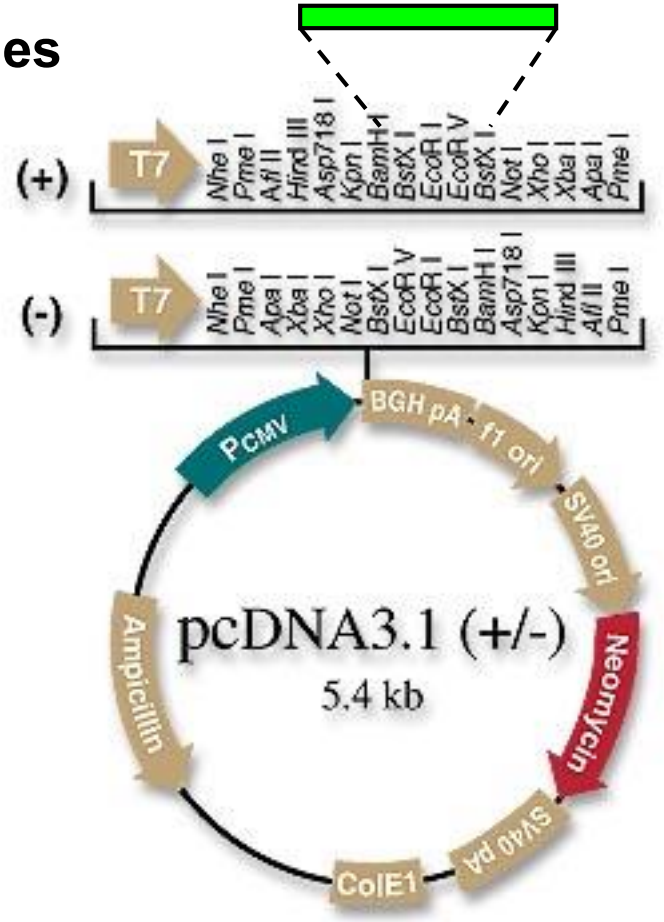
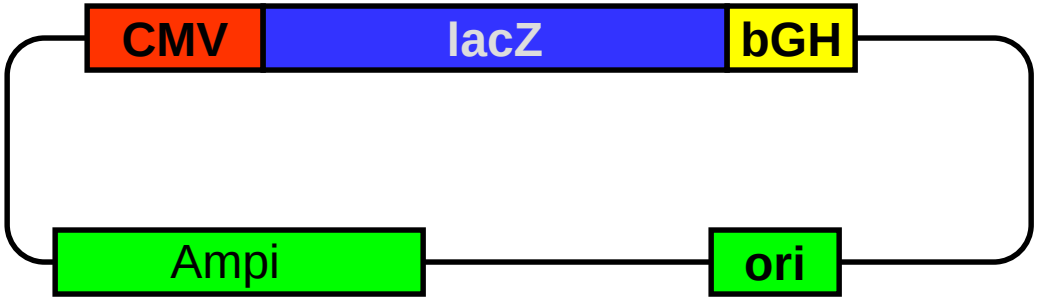


TRANSFERT DE GENES DANS DES CELLULES EN CULTURE

Plasmide pCMV-lacZ



lacZ ➔ beta galactosidase

lactose (Glc-Gal) → Glc + Gal

TRANSFERT DE GENES DANS DES CELLULES EN CULTURE

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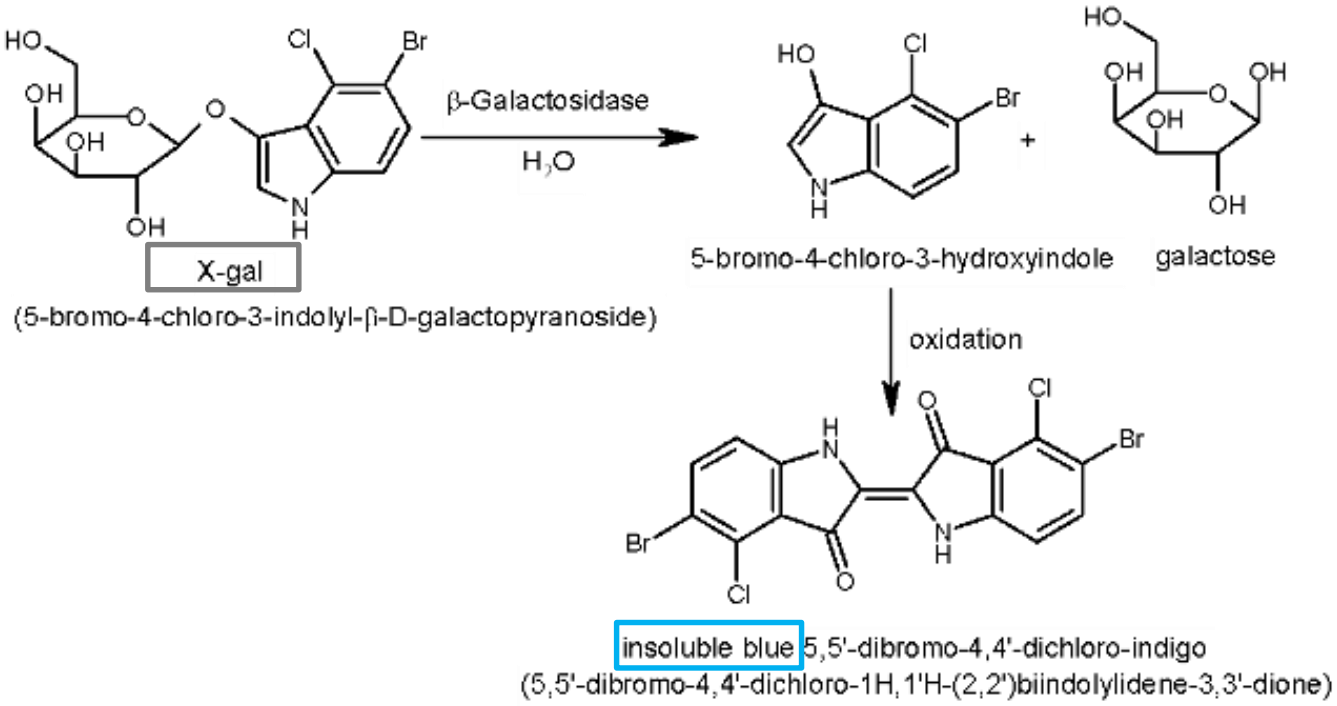

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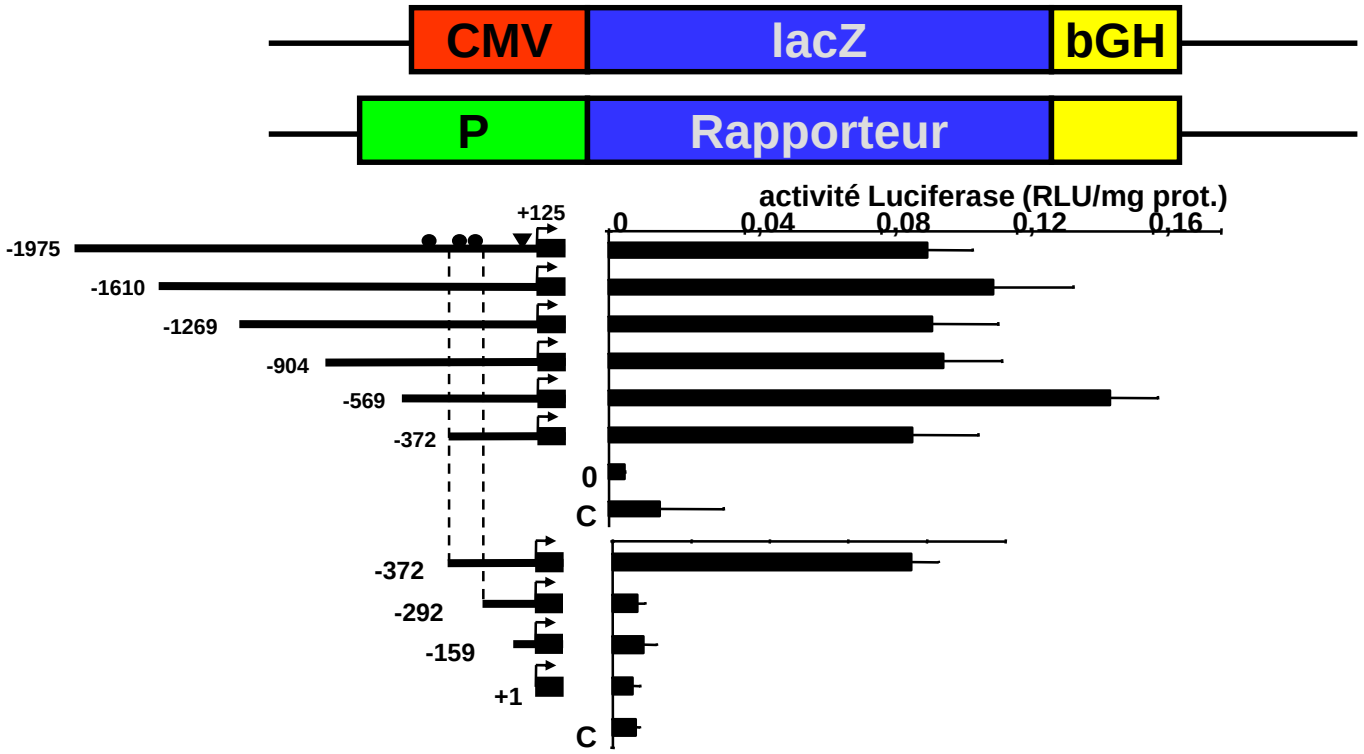
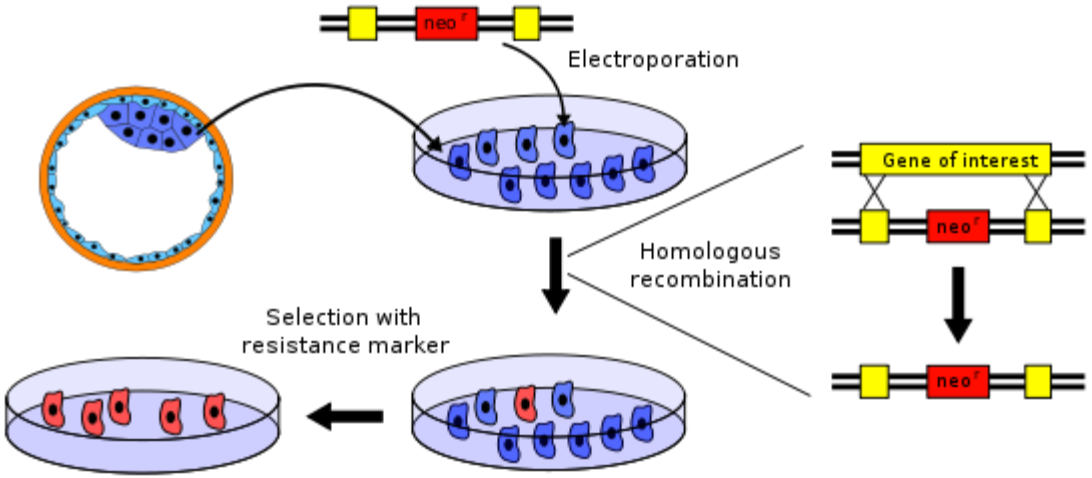


TRANSFERT DE GENES DANS DES CELLULES EN CULTURE

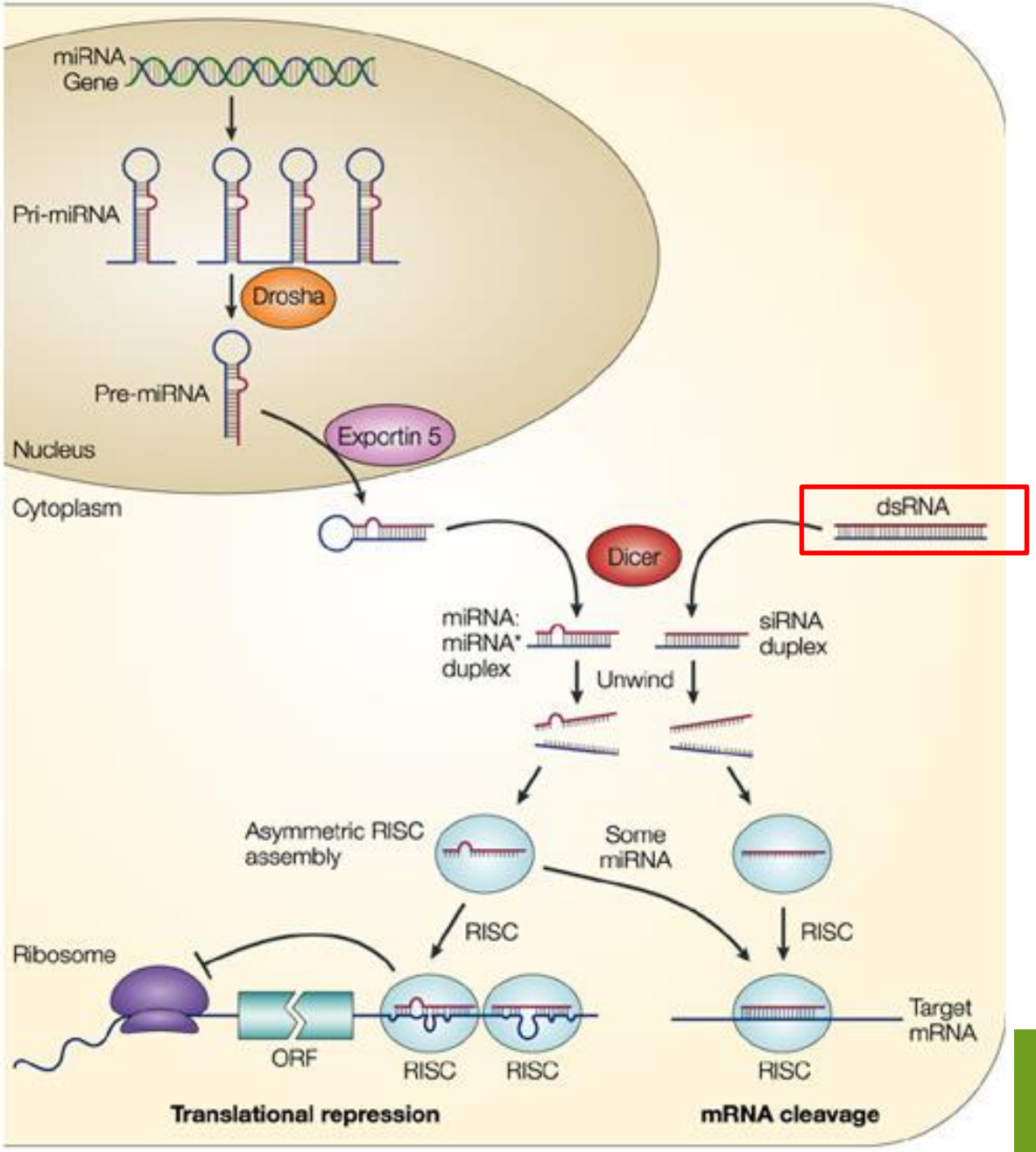
- **modifier l'expression d'un gène**
  - ✓ **Ajouter un gène exogène**
  - ✓ **Invalider un gène : Knock-Out**



TRANSFERT DE GENES DANS DES CELLULES EN CULTURE

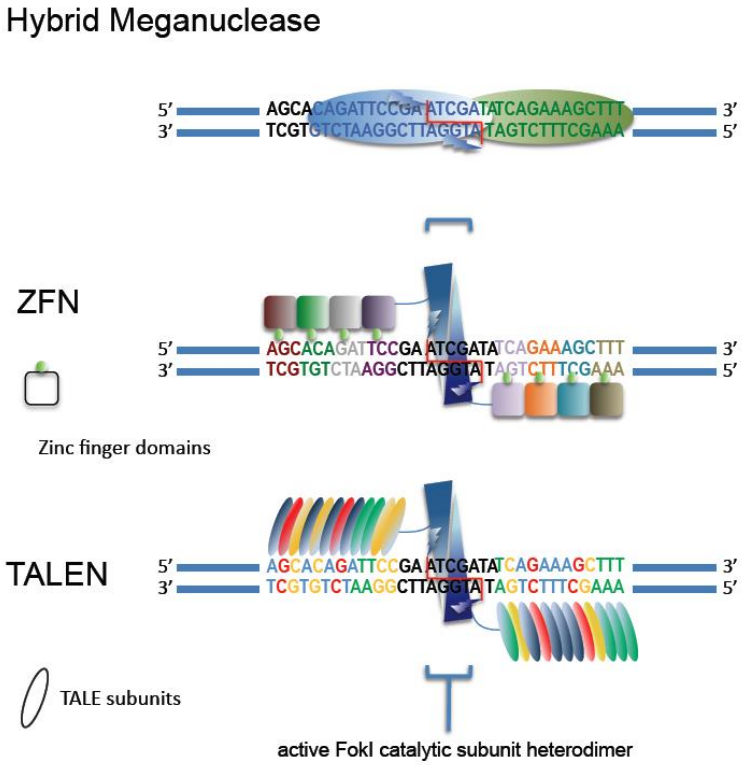
- **modifier l'expression d'un gène**
  - ✓ **Ajouter un gène exogène**
  - ✓ **Invalider un gène : Knock-Out**
  - ✓ **Moduler l'expression : interférence à ARN**

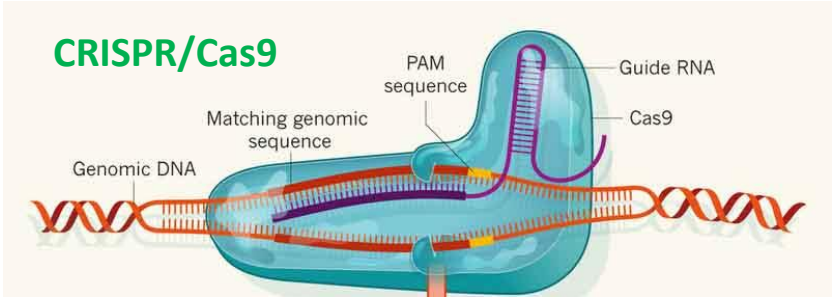
RNA Interference, d'après He and Hannon. 2004. Nature Reviews Genetics, 5:522-531.



Genome editing : modification du génome

Implique une nucléase ciblant spécifiquement un locus
genome editing with engineered nuclease





- the **quickest and cheapest method** (less than two hundred dollars and a few days of time)
- **requires the least amount of expertise** in molecular biology as the design lays in the guide RNA instead of the proteins.

TRANSFERT DE GENES DANS DES CELLULES EN CULTURE

- **Gène est… générique : séquence d'acide nucléique**
  - ✓ **transgène : gène, exogène à la cellule**
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⇨ plein d'autres choses finalement

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- Virus packaging
- Library construction
- BAC modification
- and more...

Retrieve Vector Information » Retrieve Service Proposal »

98,650 »
Total Vectors Delivered

Mammalian CRISPR Vectors

• Regular plasmid (single gRNA)	Guide »	From $99.00	Go to Design ▶
• Regular plasmid (dual gRNA)		From $289.00	Go to Design ▶
• Lentivirus (single gRNA)	Guide »	From $99.00	Go to Design ▶
• Lentivirus (dual gRNA)		From $289.00	Go to Design ▶
• Adenovirus (single gRNA)		From $239.00	Go to Design ▶
• Adenovirus (dual gRNA)		From $389.00	Go to Design ▶
• PiggyBac (single gRNA)		From $99.00	Go to Design ▶
• PiggyBac (dual gRNA)		From $289.00	Go to Design ▶
• Regular plasmid gRNA sensor vector (for testing gRNA specificity)			Coming soon

Mammalian TALEN Vectors

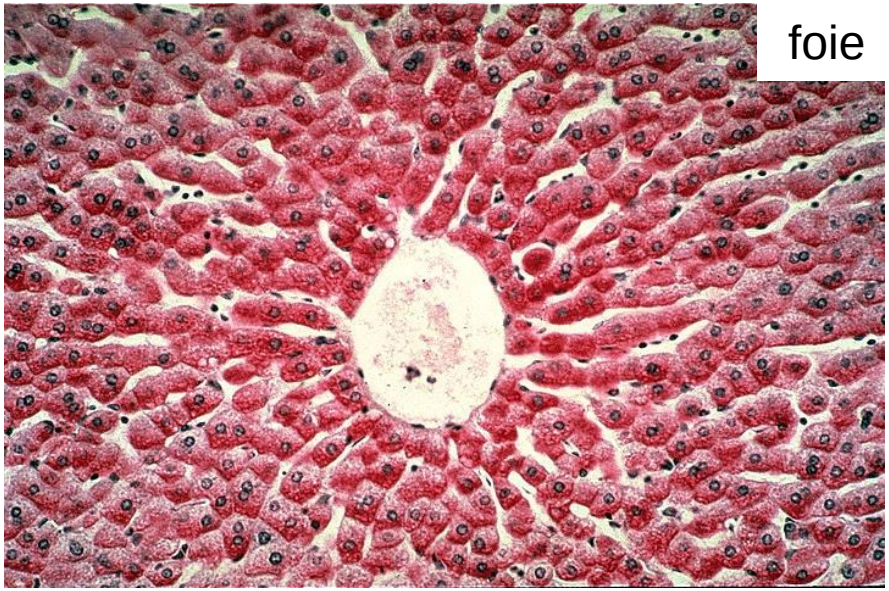
• Regular plasmid	Coming soon

TRANSFERT DE GENES DANS DES CELLULES EN CULTURE

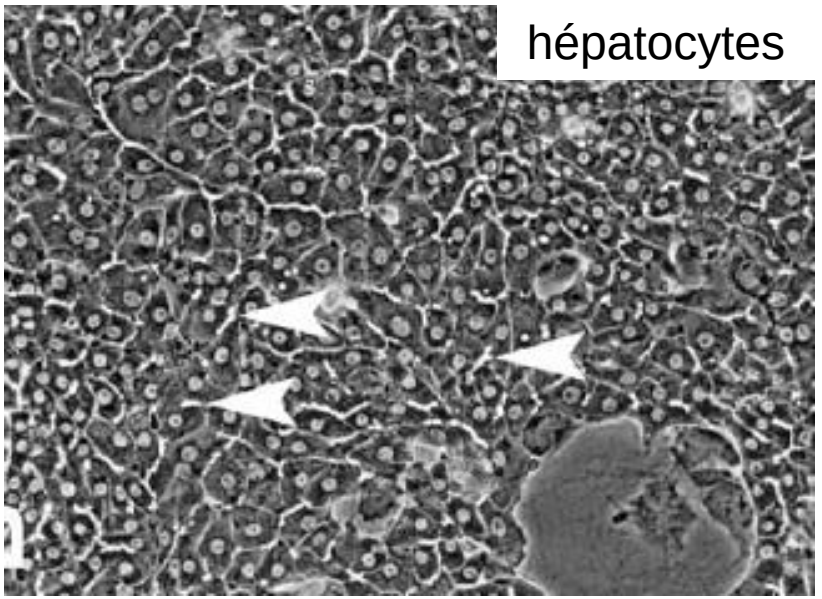
Rappel : le transfert doit être fonctionnellement efficace
(le transgène doit s'exprimer dans les cellules)

cellules « souches » :	œuf (microinjection) cellules ES souris (électroporation) cellules germinales (autres espèces)
cellules normales :	prolifératives ou non différenciées ou non
cellules transformées	

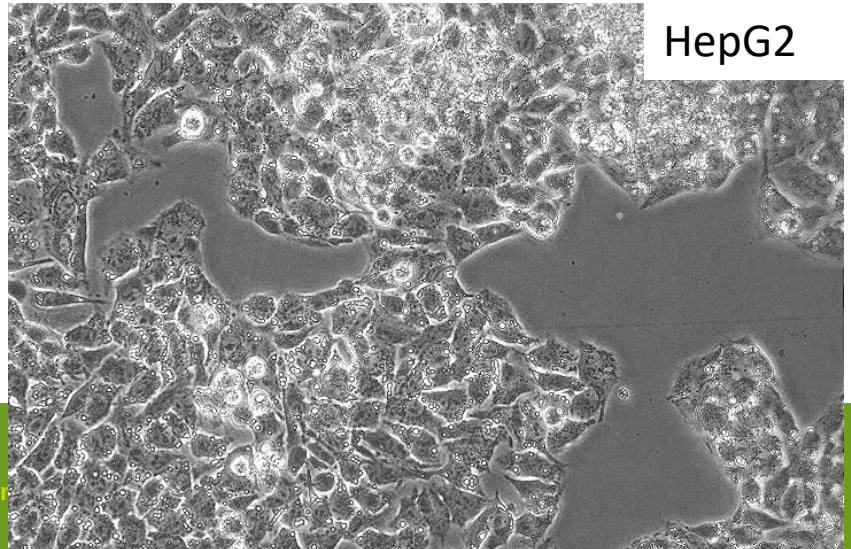
TRANSFERT DE GENES DANS DES CELLULES EN CULTURE



foie

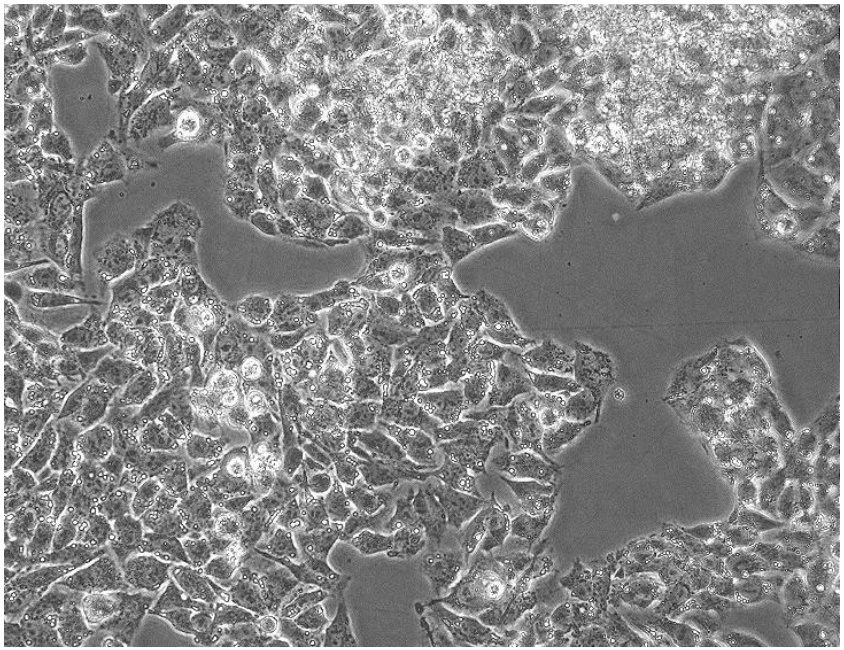


hépatocytes



HepG2

TRANSFERT DE GENES DANS DES CELLULES EN CULTURE



- moins différenciées
- plus transformées
    - aspect d'ensemble
        - canalicule like
        - 3D/inhibition contact
    - aspect cellulaire
        - forme
        - nucléole

OBLIGATION DE TESTER L 'ADEQUATION A LA PROBLEMATIQUE

TRANSFERT DE GENES DANS DES CELLULES EN CULTURE

Rappel : le transfert doit être adapté aux cellules
le transgène doit s ’exprimer dans les cellules

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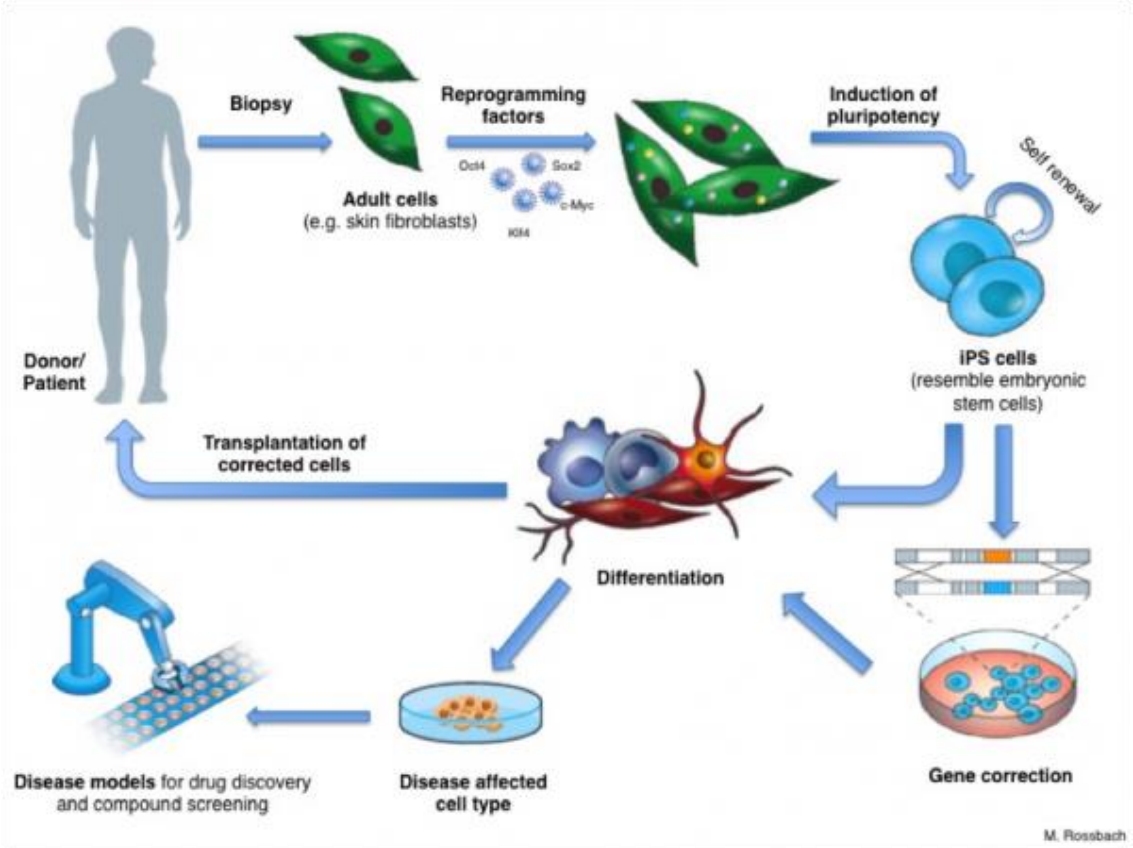
cellules iPS	

TRANSFERT DE GENES DANS DES CELLULES EN CULTURE

Cellules iPSC



TRANSFERT DE GENES DANS DES CELLULES EN CULTURE

Rappel : le transfert doit être adapté aux cellules
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Les cellules doivent être pertinentes pour la problématique

TRANSFERT DE GENES DANS DES CELLULES EN CULTURE

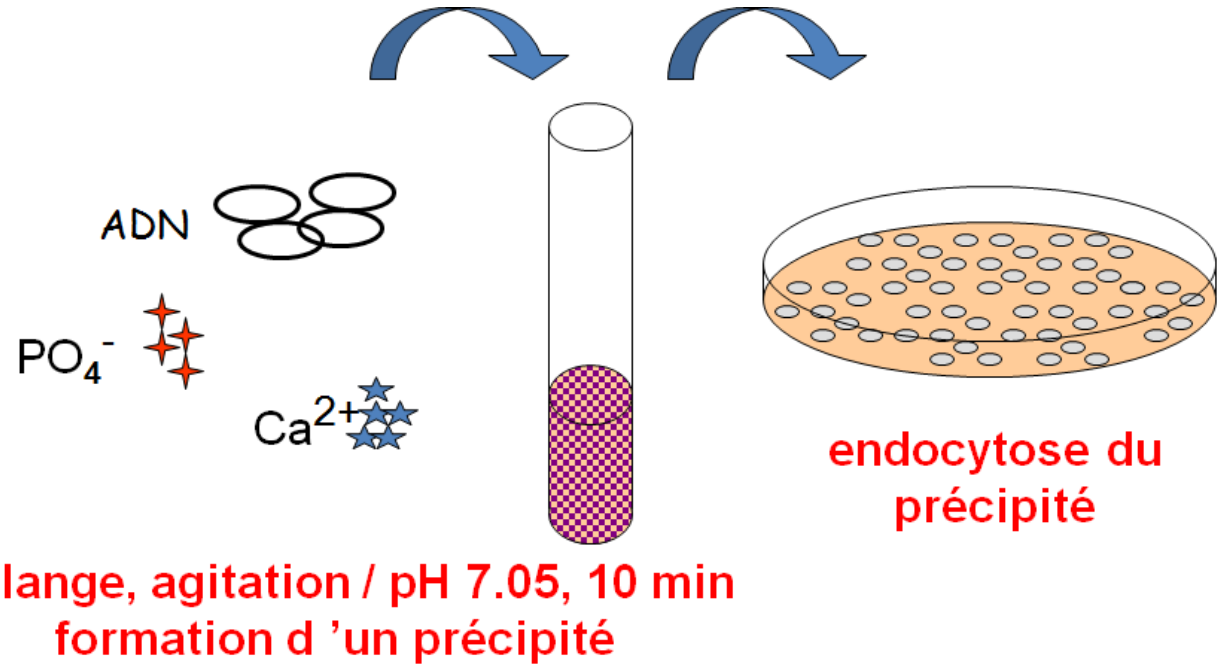
- chimique
  - phosphate de calcium, de strontium
  - DEAE-dextran
  - polybrene
  - …
  - liposomes
- biologique
  - Adenovirus
  - Retrovirus
- physique
  - microinjection
  - électroporation
  - biolistique

TRANSFERT DE GENES DANS DES CELLULES EN CULTURE

Transfert chimique, phosphate de calcium

Graham FL & Van der Eb AJ. **1975**.

= première description d ’une transfection



TRANSFERT DE GENES DANS DES CELLULES EN CULTURE

Transfert chimique, phosphate de calcium

Avantages :

facilité, modicité de prix

l ’ADN est « enrobé » (versus nu) = protégé/DNases

Inconvénients :

Toxicité des composés chimiques

Réactivité des composés chimiques

le calcium est un messager secondaire important

le rajouter en quantité pharmacologique entraîne de graves perturbations des fonctions de la cellule

-> plus adapté à du long terme (stable)

TRANSFERT DE GENES DANS DES CELLULES EN CULTURE

- biologique
  - Adenovirus
  - Adeno-associated virus (AAV)
  - Lentivirus
  - Retrovirus

utiliser les voies d'infection virale
(généralement très efficaces in vivo)

=> thérapie génique somatique

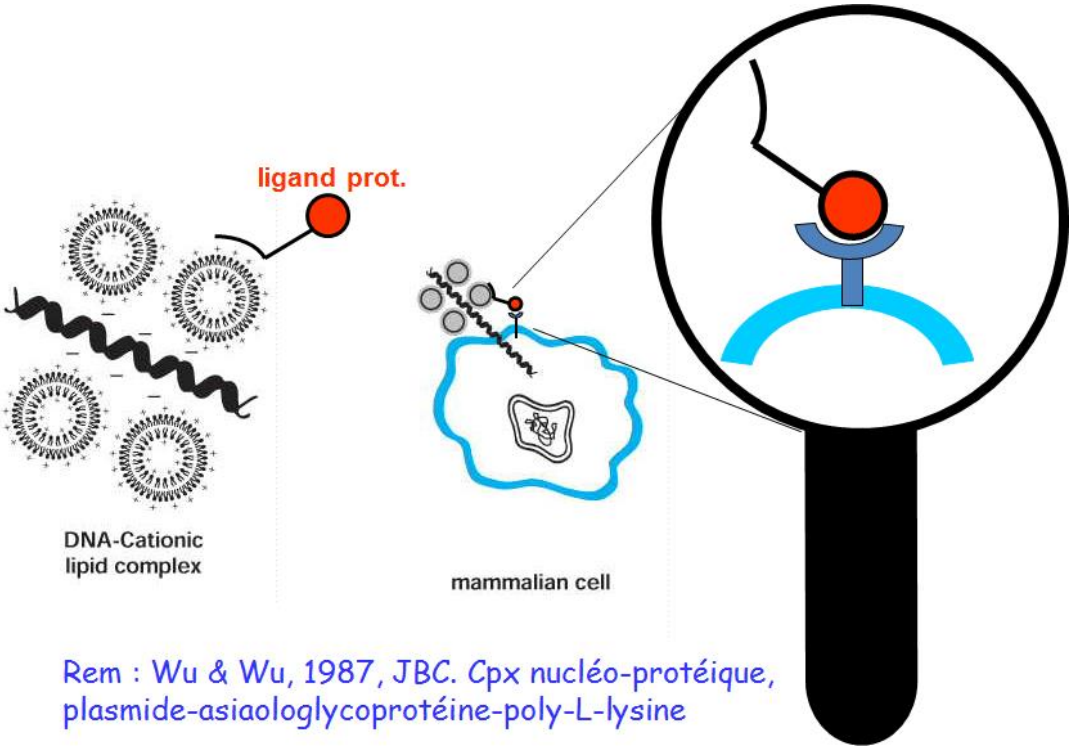
TRANSFERT DE GENES DANS DES CELLULES EN CULTURE

	Advantages	Disadvantages
Retrovirus	Integration into host DNA All viral genes removed Relatively safe	Semi-random integration Transduction requires cell division Relatively low titer
Adenovirus	Higher titer Efficient transduction of nondividing cells	Toxicity Immunological response Prior exposure
Adeno-associated virus	All viral genes removed Safe Transduction of nondividing cells Stable expression	Small genome limits size of foreign DNA Labor-intensive production Status of genome not fully elucidated
Liposomes	Absence of viral components Lack of previous immune recognition	Inefficient gene transfer into the nucleus Lack of persistence of DNA Lack of tissue targeting

TRANSFERT DE GENES DANS DES CELLULES EN CULTURE

- Liposomes : « bio-mimétiques »

Mimer les structures virales

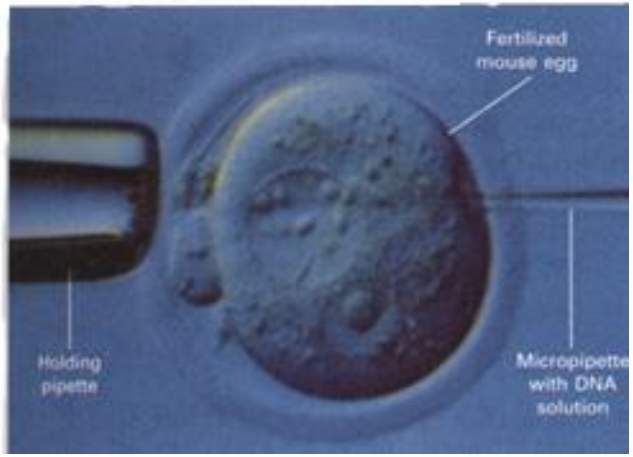


Rem : Wu & Wu, 1987, JBC. Cpx nucléo-protéique, plasmide-asiaologlycoprotéine-poly-L-lysine

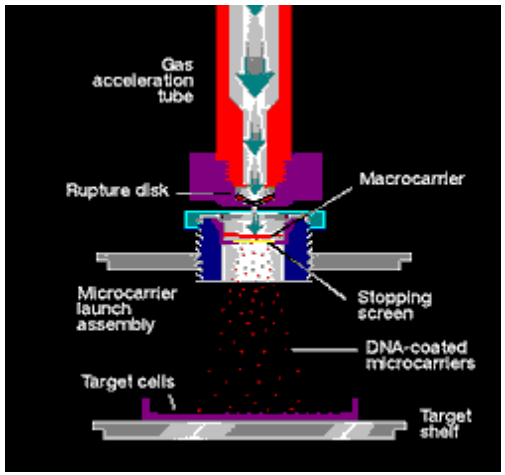
TRANSFERT DE GENES DANS DES CELLULES EN CULTURE

- physique

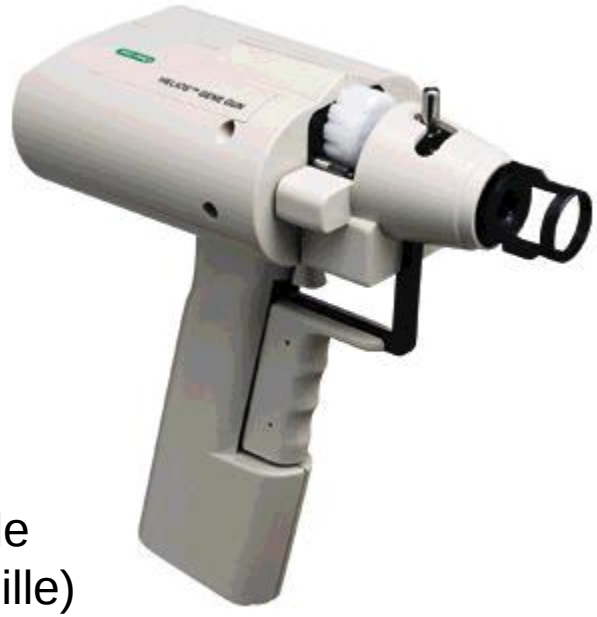
microinjection
électroporation
biolistique



transgenèse animale
« d'addition » sur œuf fécondé
au stade pronucleus

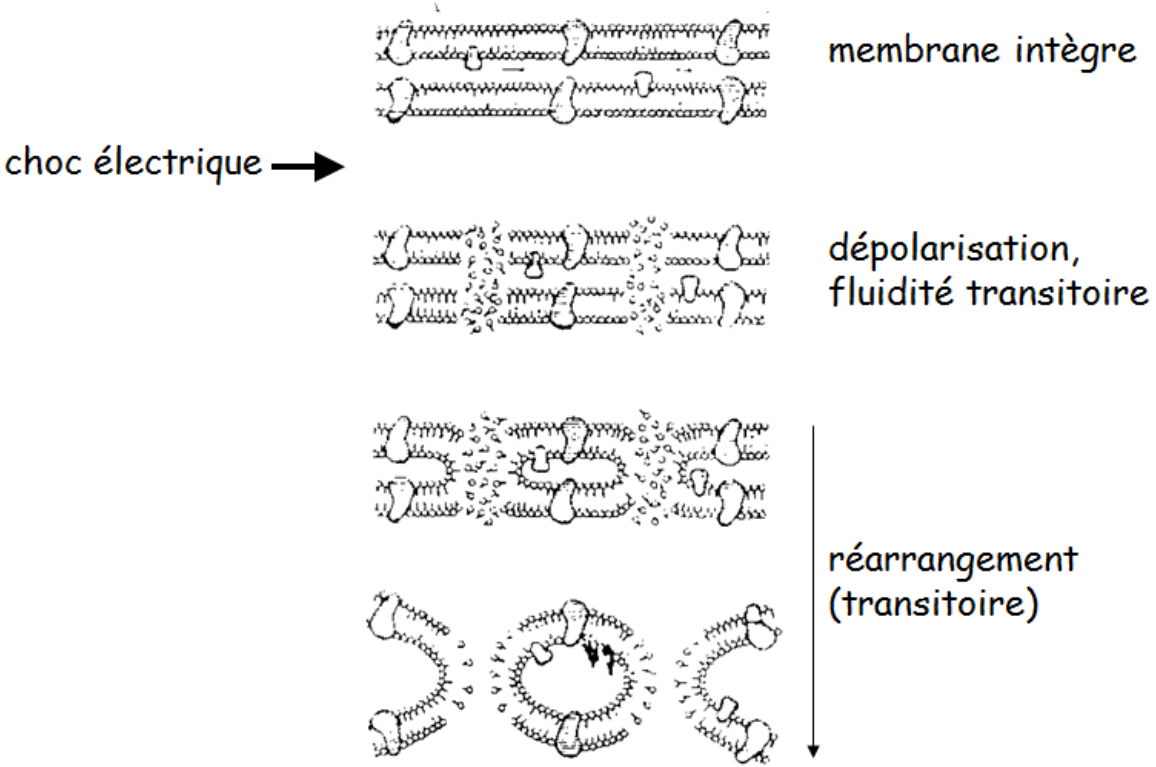


transgenèse végétale
« somatique » (sur feuille)



TRANSFERT DE GENES DANS DES CELLULES EN CULTURE

- physique électroporation



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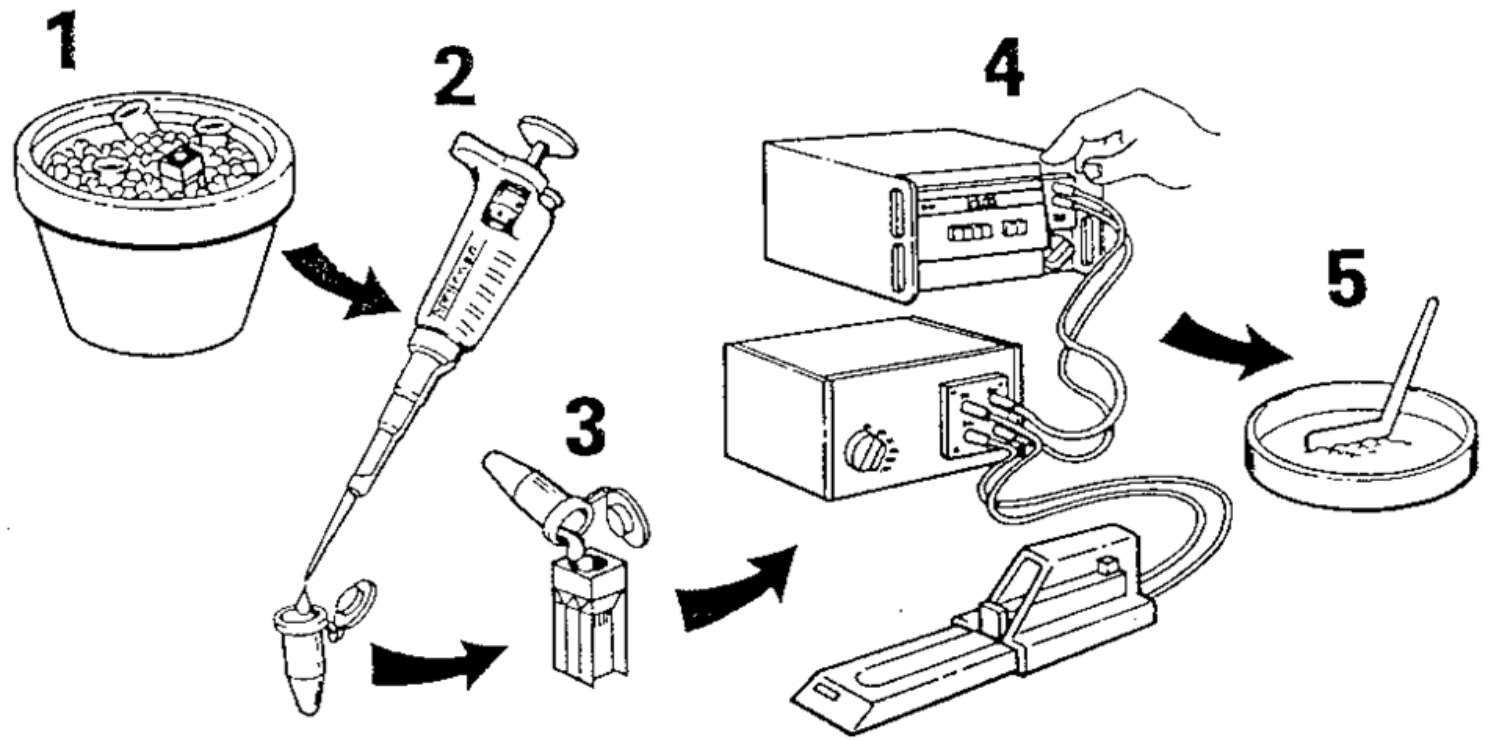

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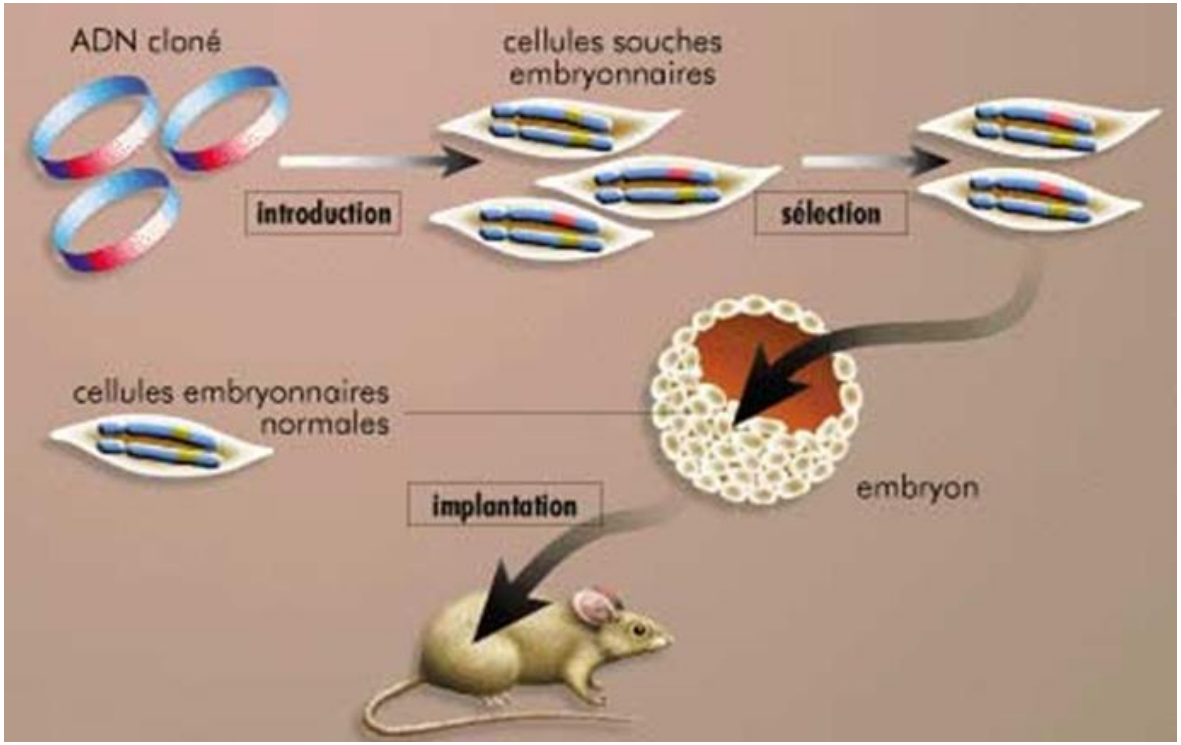

TRANSFERT DE GENES DANS DES CELLULES EN CULTURE

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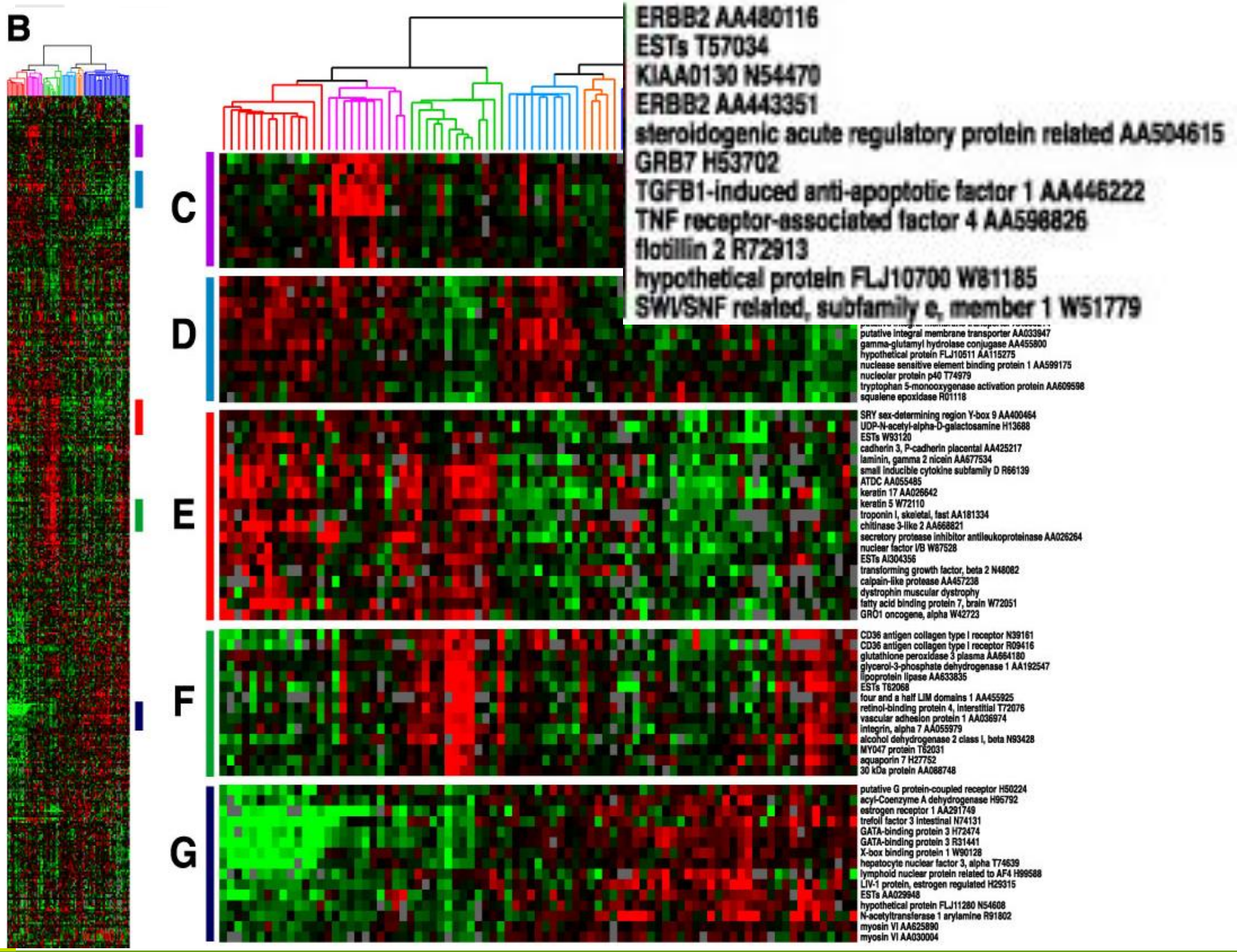
  - ✓ produire une protéine (à forte valeur ajoutée)
  - ✓ comprendre la fonction d'une protéine
  - ✓ comprendre la régulation de l'ex
  - ✓ …
- **Comment ?**
  - ✓ aj… …ormation génétique …vement ou quantitativement …n d'un gène …uter la séquence modifiée d'un promoteur, ou des fragments, dirigeant l'expression d'un gène (rapporteur) …
  - ✓ …

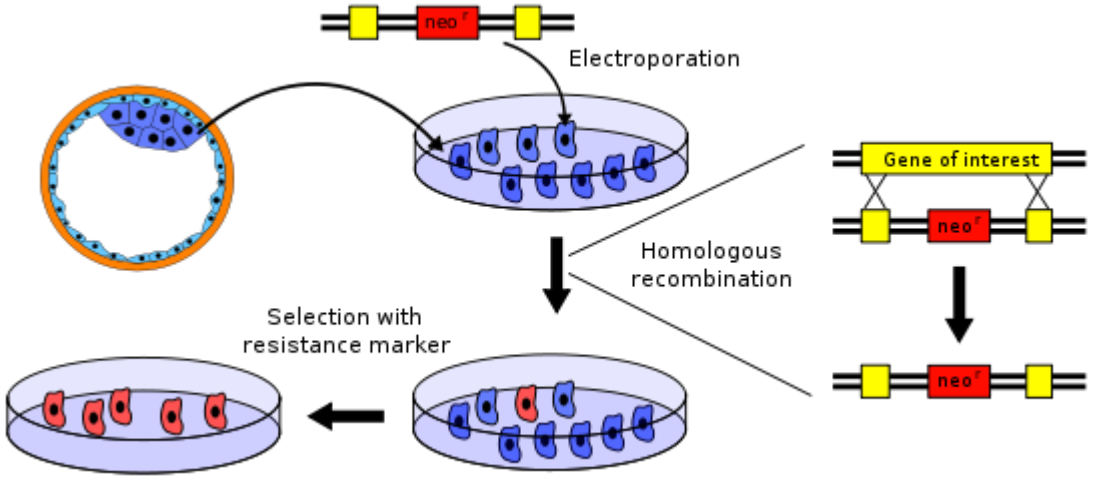
Quid de plusieurs gènes ?
(issus par exemple d 'une analyse « genome wide »)


B
C
D
E
F
G
ERBB2 AA480116
ESTs T57034
KIAA0130 N54470
ERBB2 AA443351
steroidogenic acute regulatory protein related AA504615
GRB7 H53702
TGFB1-induced anti-apoptotic factor 1 AA446222
TNF receptor-associated factor 4 AA596826
flotillin 2 R72913
hypothetical protein FLJ10700 W81185
SWI/SNF related, subfamily e, member 1 W51779
putative integral membrane transporter AA033947
gamma-glutamyl hydrolase conjugase AA455800
hypothetical protein FLJ10511 AA115275
nuclease sensitive element binding protein 1 AA599175
nucleolar protein p40 T74979
tryptophan 5-monooxygenase activation protein AA609598
squalene epoxidase R01118
SRY sex-determining region Y-box 9 AA400464
UDP-N-acetyl-alpha-D-galactosamine H13688
ESTs W93120
cadherin 3, P-cadherin placental AA425217
laminin, gamma 2 nicein AA677534
small inducible cytokine subfamily D R66139
ATDC AA055485
keratin 17 AA026642
keratin 5 W72110
troponin I, skeletal, fast AA181334
chitinase 3-like 2 AA668821
secretory protease inhibitor antileukoproteinase AA026264
nuclear factor I/B W87528
ESTs AI304356
transforming growth factor, beta 2 N48082
calpain-like protease AA457238
dystrophin muscular dystrophy
fatty acid binding protein 7, brain W72051
GRO1 oncogene, alpha W42723
CD36 antigen collagen type I receptor N39161
CD36 antigen collagen type I receptor R09416
glutathione peroxidase 3 plasma AA664180
glycerol-3-phosphate dehydrogenase 1 AA192547
lipoprotein lipase AA633835
ESTs T62068
four and a half LIM domains 1 AA455925
retinol-binding protein 4, interstitial T72076
vascular adhesion protein 1 AA036974
integrin, alpha 7 AA055979
alcohol dehydrogenase 2 class I, beta N93428
MY047 protein T62031
aquaporin 7 H27752
30 kDa protein AA088748
putative G protein-coupled receptor H50224
acyl-Coenzyme A dehydrogenase H95792
estrogen receptor 1 AA291749
trefoil factor 3 intestinal N74131
GATA-binding protein 3 H72474
GATA-binding protein 3 R31441
X-box binding protein 1 W90128
hepatocyte nuclear factor 3, alpha T74639
lymphoid nuclear protein related to AF4 H99588
LIV-1 protein, estrogen regulated H29315
ESTs AA029948
hypothetical protein FLJ11280 N54608
N-acetyltransferase 1 arylamine R91802
myosin VI AA625890
myosin VI AA030004

TRANSFERT DE GENES DANS DES CELLULES EN CULTURE

- **Comment modifier qualitativement ou quantitativement l'expression de nombreux gènes ?**

Knock-Out ?



TRANSFERT DE GENES DANS DES CELLULES EN CULTURE

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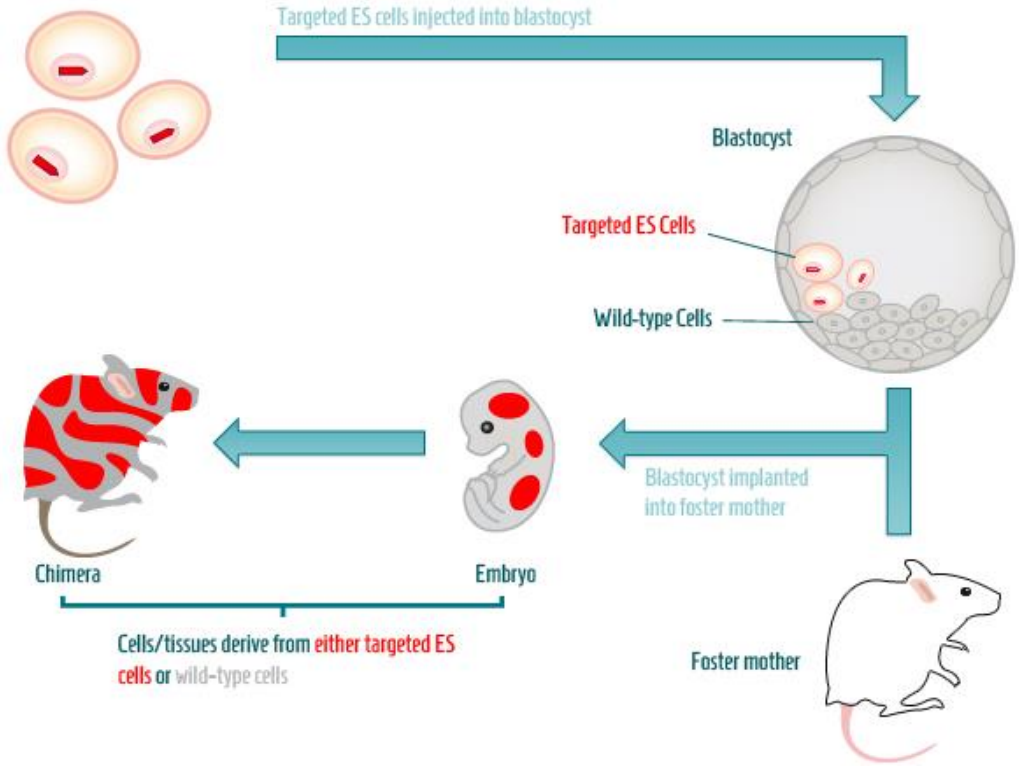


TRANSFERT DE GENES DANS DES CELLULES EN CULTURE

- **Comment modifier qualitativement ou quantitativement l'expression de nombreux gènes ?**

  ~~**Knock-Out !**~~

TRANSFERT DE GENES DANS DES CELLULES EN CULTURE

Carpenter and Sabatini. 2004. Nature Reviews Genetics, 5:11-22.

SYSTEMATIC GENOME-WIDE SCREENS OF GENE FUNCTION

Anne E. Carpenter and David M. Sabatini

By using genome information to create tools for perturbing gene function, it is now possible to undertake systematic genome-wide functional screens that examine the contribution of every gene to a biological process. The directed nature of these experiments contrasts with traditional methods, in which random mutations are induced and the resulting mutants are screened for various phenotypes. The first genome-wide functional screens in *Caenorhabditis elegans* and *Drosophila melanogaster* have recently been published, and screens in human cells will soon follow. These high-throughput techniques promise the rapid annotation of genomes with high-quality information about the biological function of each gene.

TRANSFERT DE GENES DANS DES CELLULES EN CULTURE

Kamath et al. 2003. Nature, 421: 231-237.

Systematic functional analysis of the *Caenorhabditis elegans* genome using RNAi

Ravi S. Kamath*†, Andrew G. Fraser*†§, Yan Dong*, Gino Poulin*, Richard Durbin‡, Monica Gotta*§, Alexander Kanapin‖, Nathalie Le Bot*, Sergio Moreno*¶, Marc Sohrmann‡§, David P. Welchman*, Peder Zipperlen* & Julie Ahringer*

* *Wellcome Trust/Cancer Research UK Institute and Department of Genetics, University of Cambridge, Tennis Court Road, Cambridge CB2 1QR, UK*
‡ *Wellcome Trust Sanger Institute, Hinxton, Cambridge CB10 1SA, UK*
‖ *EMBL-European Bioinformatics Institute, Wellcome Trust Genome Campus, Hinxton, Cambridge CB10 1SD, UK*
¶ *Centro de Investigacion del Cancer, CSIC / Univ. Salamanca, Campus Miguel de Unamuno, 37007 Salamanca, Spain*
† *These authors contributed equally to this work*

A principal challenge currently facing biologists is how to connect the complete DNA sequence of an organism to its development and behaviour. Large-scale targeted-deletions have been successful in defining gene functions in the single-celled yeast *Saccharomyces cerevisiae*, but comparable analyses have yet to be performed in an animal. Here we describe the use of RNA interference to inhibit the function of ~86% of the 19,427 predicted genes of *C. elegans*. We identified mutant phenotypes for 1,722 genes, about two-thirds of which were not previously associated with a phenotype. We find that genes of similar functions are clustered in distinct, multi-megabase regions of individual chromosomes; genes in these regions tend to share transcriptional profiles. Our resulting data set and reusable RNAi library of 16,757 bacterial clones will facilitate systematic analyses of the connections among gene sequence, chromosomal location and gene function in *C. elegans*.

TRANSFERT DE GENES DANS DES CELLULES EN CULTURE

Kamath et al. 2003. Nature, 421: 231-237.

Analysis of gene functions by RNAi

Loss-of-function RNAi phenotypes can be generated efficiently by feeding worms with bacteria expressing double-stranded RNA (dsRNA) that is homologous to a target gene[5–7]; we previously used this method to screen roughly 87% of predicted genes on chromosome I of *C. elegans* (ref. 2). To screen most of the predicted genes in *C. elegans* by RNAi, we constructed a library of bacterial strains, each capable of expressing dsRNA designed to correspond to a single gene. The library consists of 16,757 bacterial strains, which in total correspond to about 86% of the 19,427 current predicted genes in *C. elegans* with similar coverage across each chromosome (see Supplementary Tables 1 and 2). Using this library, we screened wild-type *C. elegans* hermaphrodites to identify genes for which RNAi reproducibly results in sterility, embryonic or larval lethality, slow post-embryonic growth, or a post-embryonic defect (Methods). Such phenotypes were obtained with 1,722 bacterial strains (10.3% of those analysed; Fig. 1a).

TRANSFERT DE GENES DANS DES CELLULES EN CULTURE

Boutros et al. 2004. Science, 303: 832-835.

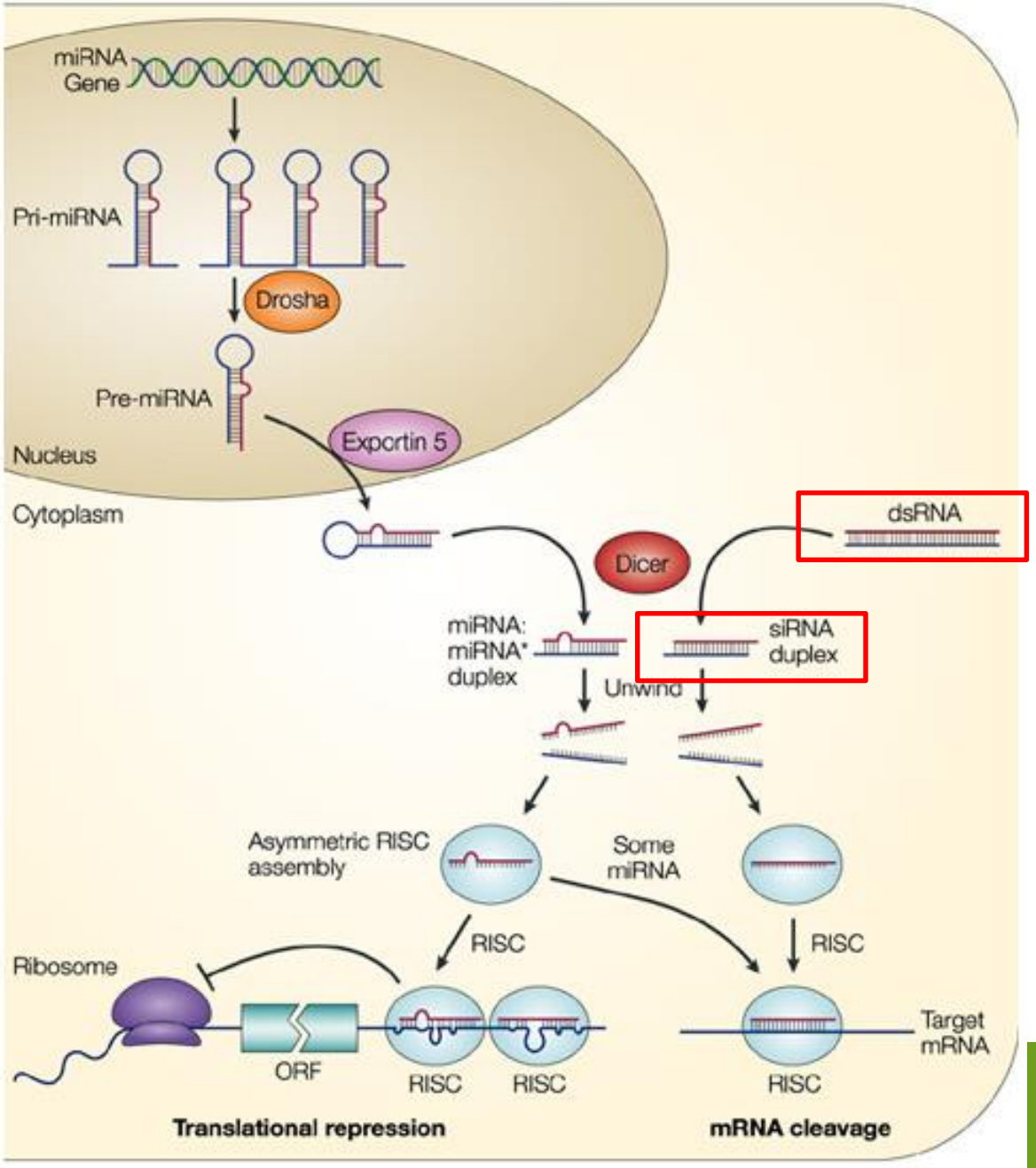
Genome-Wide RNAi Analysis of Growth and Viability in *Drosophila* Cells

Michael Boutros,[1*†] Amy A. Kiger,[1*] Susan Armknecht,[1,2] Kim Kerr,[1,2] Marc Hild,[3] Britta Koch,[3] Stefan A. Haas,[4] Heidelberg Fly Array Consortium,[3] Renato Paro,[3] Norbert Perrimon[1,2†‡]

A crucial aim upon completion of whole genome sequences is the functional analysis of all predicted genes. We have applied a high-throughput RNA-interference (RNAi) screen of 19,470 double-stranded (ds) RNAs in cultured cells to characterize the function of nearly all (91%) predicted *Drosophila* genes in cell growth and viability. We found 438 dsRNAs that identified essential genes, among which 80% lacked mutant alleles. A quantitative assay of cell number was applied to identify genes of known and uncharacterized functions. In particular, we demonstrate a role for the homolog of a mammalian acute myeloid leukemia gene (*AML1*) in cell survival. Such a systematic screen for cell phenotypes, such as cell viability, can thus be effective in characterizing functionally related genes on a genome-wide scale.

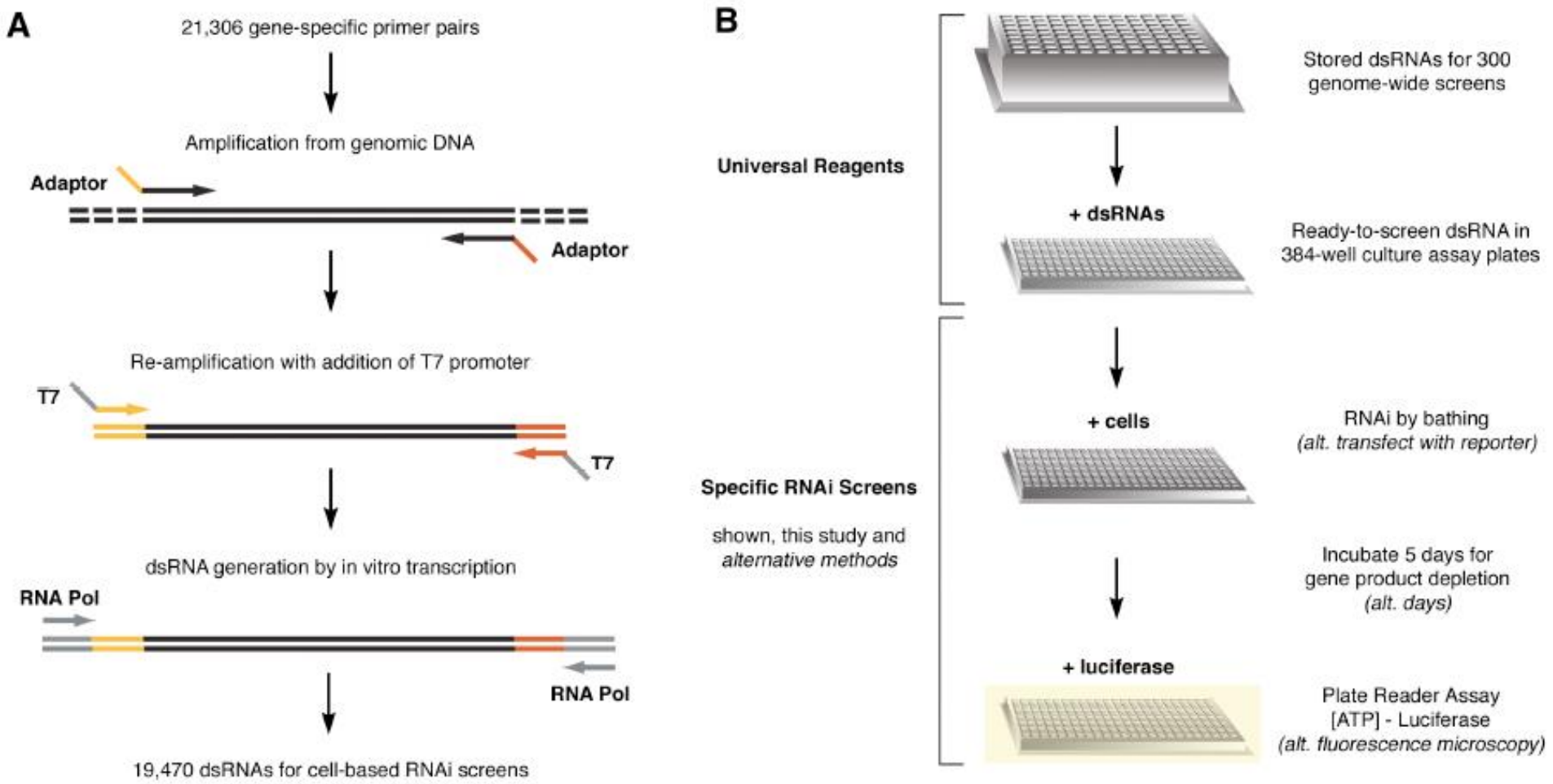
6 FEBRUARY 2004 VOL 303 SCIENCE www.sciencemag.org

RNA Interference, d'après He and Hannon. 2004. Nature Reviews Genetics, 5:522-531.

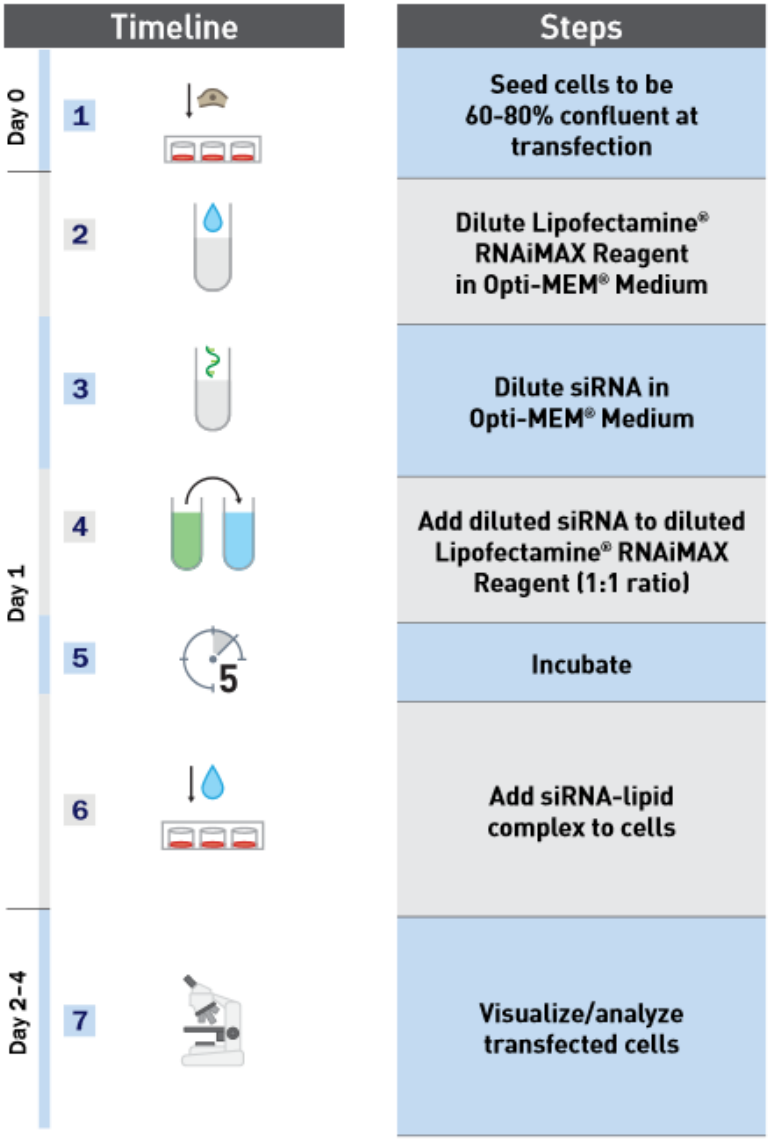


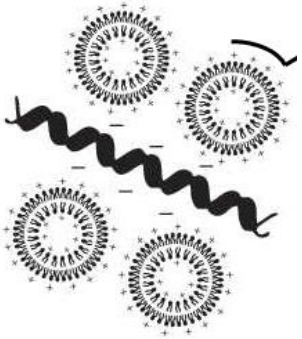
TRANSFERT DE GENES DANS DES CELLULES EN CULTURE

Boutros et al. 2004. Science, 303: 832-835.



TRANSFERT DE GENES DANS DES CELLULES EN CULTURE





TRANSFERT DE GENES DANS DES CELLULES EN CULTURE

Silva et al. 2005. Nature Genetics, 37:1281-1288.

Second-generation shRNA libraries covering the mouse and human genomes

Jose M Silva[1,4], Mamie Z Li[2,4], Ken Chang[1,4], Wei Ge[3], Michael C Golding[1], Richard J Rickles[2], Despina Siolas[1], Guang Hu[2], Patrick J Paddison[1], Michael R Schlabach[2], Nihar Sheth[1], Jeff Bradshaw[3], Julia Burchard[3], Amit Kulkarni[3], Guy Cavet[3], Ravi Sachidanandam[1], W Richard McCombie[1], Michele A Cleary[3], Stephen J Elledge[2] & Gregory J Hannon[1]

Loss-of-function phenotypes often hold the key to understanding the connections and biological functions of biochemical pathways. We and others previously constructed libraries of short hairpin RNAs that allow systematic analysis of RNA interference–induced phenotypes in mammalian cells. Here we report the construction and validation of second-generation short hairpin RNA expression libraries designed using an increased knowledge of RNA interference biochemistry. These constructs include silencing triggers designed to mimic a natural microRNA primary transcript, and each target sequence was selected on the basis of thermodynamic criteria for optimal small RNA performance. Biochemical and phenotypic assays indicate that the new libraries are substantially improved over first-generation reagents. We generated large-scale-arrayed, sequence-verified libraries comprising more than 140,000 second-generation short hairpin RNA expression plasmids, covering a substantial fraction of all predicted genes in the human and mouse genomes. These libraries are available to the scientific community.

RNA interference (RNAi) has been exploited in organisms ranging from plants to fungi to animals for deciphering gene function through suppression of gene expression. Particularly in systems where targeted genetic manipulation is difficult or time consuming, RNAi has transformed the way in which gene function can be approached on a single-gene or genome-wide level[1–5].

a siRNA

TRANSFERT DE GENES DANS DES CELLULES EN CULTURE

High-throughput RNAi screening in cultured cells: a user's guide

Christophe J. Echeverri and Norbert Perrimon‡*

Abstract | RNA interference has re-energized the field of functional genomics by enabling genome-scale loss-of-function screens in cultured cells. Looking back on the lessons that have been learned from the first wave of technology developments and applications in this exciting field, we provide both a user's guide for newcomers to the field and a detailed examination of some more complex issues, particularly concerning optimization and quality control, for more advanced users. From a discussion of cell lines, screening paradigms, reagent types and read-out methodologies, we explore in particular the complexities of designing optimal controls and normalization strategies for these challenging but extremely powerful studies.

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TRANSFERT DE GENES DANS DES CELLULES EN CULTURE

High-throughput RNAi screening in cultured cells: a user's guide

Table 3 | **siRNA libraries for use in mammalian cells**

Company	Species	Coverage	Reagent description
*Synthetic siRNA libraries**			
Ambion	Human, mouse and rat	Genome-wide	21 nt with 3′ overhangs; unmodified RNA
Dharmacon	Human, mouse and rat	Genome-wide	21 nt with 3′ overhangs; unmodified RNA
Qiagen	Human	Genome-wide	21 nt with 3′ overhangs; unmodified RNA
Invitrogen	Human	Kinase genes	25 nt with blunt ends; modified backbone
Vector-based shRNA libraries			
Open Biosystems	Human, mouse	Genome-wide	shRNAs with miR backbone in retroviral vectors, sold as bacterial stocks; second-generation design by the Hannon and Elledge laboratories
Open Biosystems, Sigma-Aldrich	Human, mouse	Genome-wide	shRNAs in lentiviral vectors; second-generation design by the RNAi Consortium

*Note that these and various other suppliers offer pre-designed small interfering RNAs (siRNAs) that target individual genes or small collections. shRNAs, short hairpin RNAs.

TRANSFERT DE GENES DANS DES CELLULES EN CULTURE

Sui et al. 2002. PNAS, 99:5515-5520

A DNA vector-based RNAi technology to suppress gene expression in mammalian cells

Guangchao Sui*, Christina Soohoo*, El Bachir Affar*, Frédérique Gay, Yujiang Shi, William C. Forrester, and Yang Shi†

Department of Pathology, Harvard Medical School, 200 Longwood Avenue, Boston, MA 02115

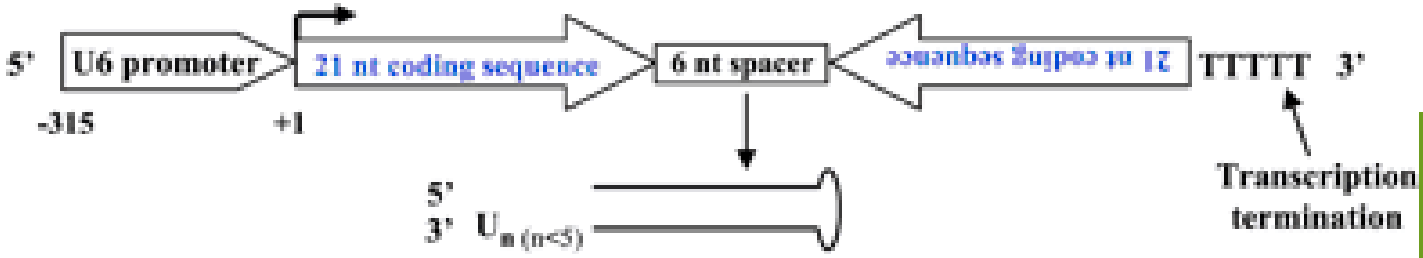
Communicated by Howard Green, Harvard Medical School, Boston, MA, February 28, 2002 (received for review February 19, 2002)

Double-stranded RNA-mediated interference (RNAi) has recently emerged as a powerful reverse genetic tool to silence gene expression in multiple organisms including plants, *Caenorhabditis elegans*, and *Drosophila*. The discovery that synthetic double-stranded, 21-nt small interfering RNA triggers gene-specific silencing in mammalian cells has further expanded the utility of RNAi into mammalian systems. Here we report a technology that allows synthesis of small interfering RNAs from DNA templates *in vivo* to efficiently inhibit endogenous gene expression. Significantly, we were able to use this approach to demonstrate, in multiple cell lines, robust inhibition of several endogenous genes of diverse functions. These findings highlight the general utility of this DNA vector-based RNAi technology in suppressing gene expression in mammalian cells.

encoded by the corresponding genes. Taken together, we have developed an RNAi approach that uses a DNA template to synthesize small RNA *in vivo*. This technique can be broadly used for analysis of gene functions and thus will significantly facilitate the use of the RNAi technology in cell culture and vertebrate animals.

Materials and Methods

Construction of Plasmids That Contain DNA Templates for the Synthesis of siRNAs Under the Control of the U6 Promoter. Plasmid pmU6 (−315/+1) (gift of S. Altman, Yale University, New Haven, CT) was used as a template for PCR isolation of the U6 promoter (−315 to +1) with an added *Apa*I cloning site at the transcriptional initiation site, which was cloned into Bluescript



TRANSFERT DE GENES DANS DES CELLULES EN CULTURE

Brummelkamp et al. 2002. Science, 296: 550-553

A System for Stable Expression of Short Interfering RNAs in Mammalian Cells

Thijn R. Brummelkamp,[1] René Bernards,[1,3] Reuven Agami[1,2,3]*

Mammalian genetic approaches to study gene function have been hampered by the lack of tools to generate stable loss-of-function phenotypes efficiently. We report here a new vector system, named pSUPER, which directs the synthesis of small interfering RNAs (siRNAs) in mammalian cells. We show that siRNA expression mediated by this vector causes efficient and specific down-regulation of gene expression, resulting in functional inactivation of the targeted genes. Stable expression of siRNAs using this vector mediates persistent suppression of gene expression, allowing the analysis of loss-of-function phenotypes that develop over longer periods of time. Therefore, the pSUPER vector constitutes a new and powerful system to analyze gene function in a variety of mammalian cell types.

TRANSFERT DE GENES DANS DES CELLULES EN CULTURE

Wang et al. 2003. PNAS, 100:5103-5106

Stable and controllable RNA interference: Investigating the physiological function of glutathionylated actin

Jun Wang*, Ephrem Tekle*, Hammou Oubrahim*, John J. Mieyal†, Earl R. Stadtman*, and P. Boon Chock*‡

*Laboratory of Biochemistry, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, MD 20892-8012; and †Department of Pharmacology, Case Western Reserve University, Cleveland, OH 44106

Contributed by Earl R. Stadtman, March 7, 2003

RNA interference is an effective method to silence specific gene expression. Its application to mammalian cells, however, has been hampered by various shortcomings. Recently, it was reported that introduction of 22-bp double-stranded RNAs (dsRNAs) would specifically suppress expression of endogenous and heterogeneous genes in various mammalian cell lines. However, using this method, we failed to knock out proteins of interest effectively. Here we report the development of a stable and controllable method for generating dsRNA intracellularly. Tetracycline-responsive transactivator-containing cells were transfected with a vector capable of tetracycline-induced bidirectionally overexpressing sense and antisense RNA to form dsRNA *in vivo*. With this method, glutaredoxin, monitored by Western blot, was knocked out by overexpressing 290-base sense and antisense RNA in NIH 3T3 cells controlled by tetracycline or doxycycline. By using these glutaredoxin knocked-out cells, we have demonstrated that actin deglutathionylation plays a key role in growth factor-mediated actin polymerization, translocalization, and reorganization near the cell periphery.

dsRNA inside cells as well as the half-life of the protein to be knocked out. Although introduction directly into cells of 22-bp dsRNA by transfection methods leads to specific gene silencing, this knockout is transient and usually is not sustained for the full period during the remaining gene product degradation inside the cells. Methods were therefore developed to transfect cells with vectors overexpressing dsRNA (22-mer) to sustain long-term gene silencing (20–22). The limit of 22-mer dsRNA interference is such that its efficacy depends on the selection of the most effective target site within the target mRNA to achieve complete gene silencing (23). The objective of this report is to establish an effective, specific, and controllable RNAi method by using full-length dsRNA generated intracellularly. This method was used to reveal the role of actin glutathionylation, regulated in part by glutaredoxin (GRx), in growth factor-induced actin polymerization, translocation, and reorganization near the cell periphery.

Materials and Methods

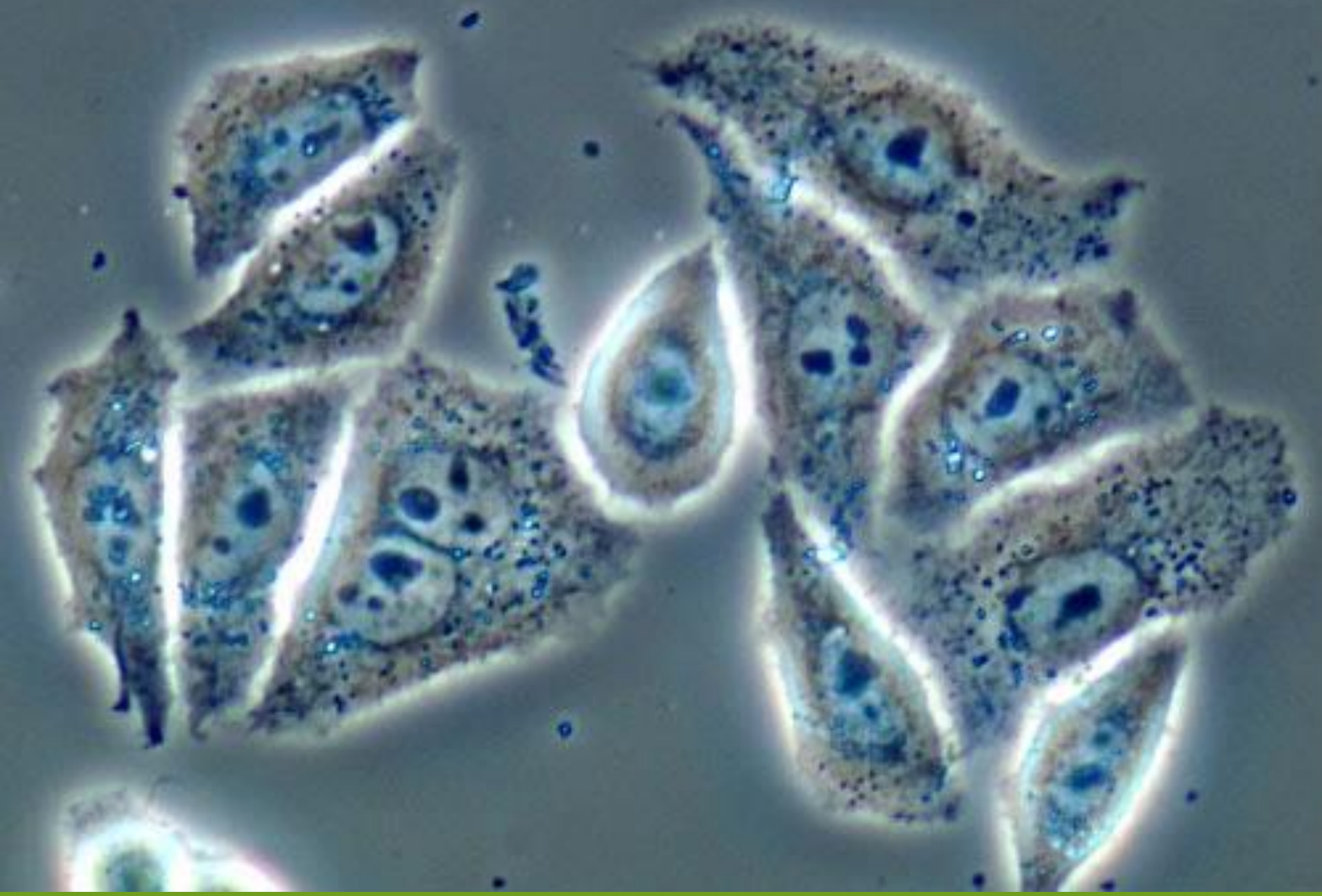
TRANSFERT DE GENES DANS DES CELLULES EN CULTURE

- **Pourquoi ?**
  - ✓ produire une protéine (à forte valeur ajoutée)
  - ✓ comprendre la fonction d'une protéine
  - ✓ comprendre la régulation de l'expression d'un gène
  - ✓ ...

- **Comment ?**
  - ✓ ajouter une (nouvelle) information génétique
  - ✓ modifier qualitativement ou quantitativement l'expression d'un gène

Peut maintenant se faire « simplement » au niveau du gène endogène (vs recombinaison homologue)

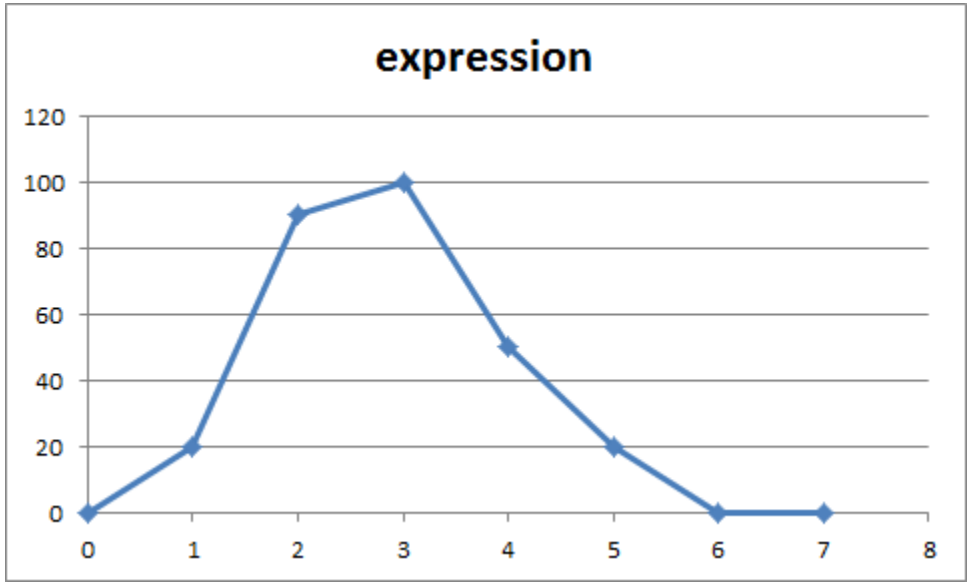

BAH MINCE ALORS !
J'ai comme l'impression qu'on peut tout transférer dans des cellules



TRANSFERT DE GENES DANS DES CELLULES EN CULTURE

le transgène s'exprime t'il de manière pérenne ?

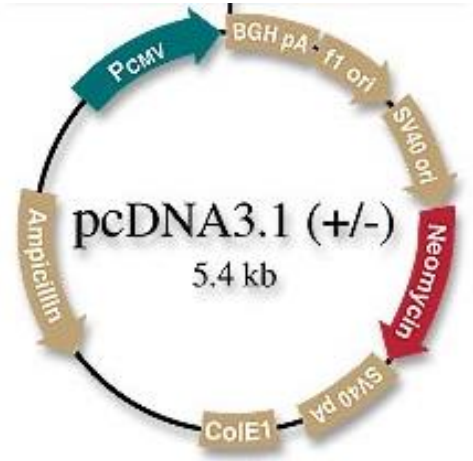
- **Expression transitoire :**
  directement à partir du plasmide, tant qu'il persiste dans la cellule
  « résistance » aux DNases

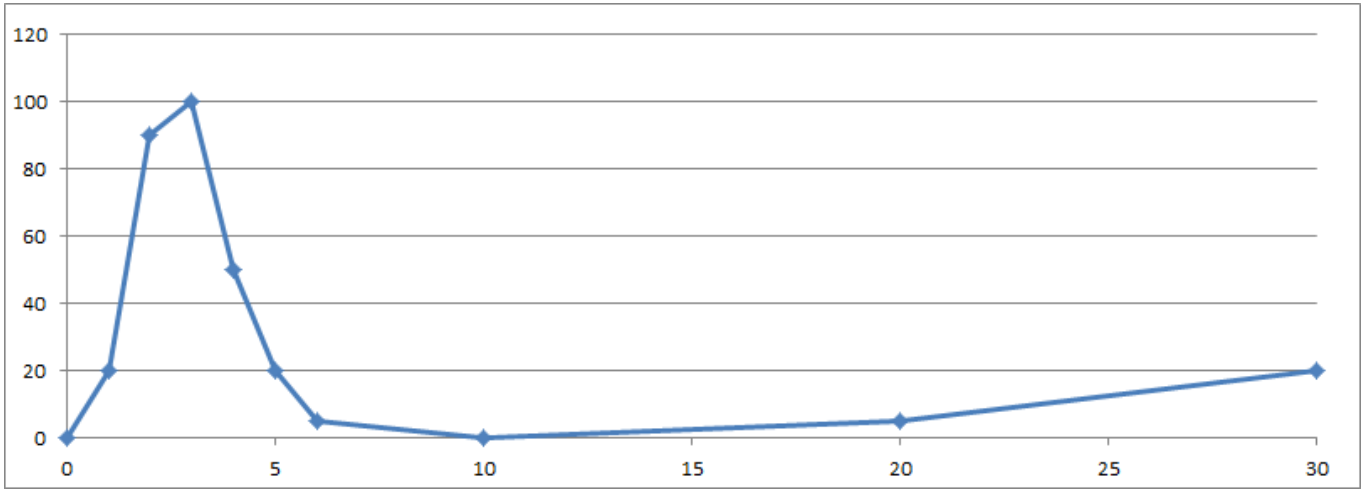


TRANSFERT DE GENES DANS DES CELLULES EN CULTURE

le transgène s'exprime t'il de manière pérenne ?

- **Expression stable :**
  maintien du transgène dans les cellules filles
  - ✓ origine de réplication propre au transgène (adénovirus)
  - ✓ **intégration dans le génome**
    - ⇨ **sélection des cellules filles ayant intégré le plasmide**





TRANSFERT DE GENES DANS DES CELLULES EN CULTURE

le transgène s'exprime t'il de manière pérenne ?

- **Expression transitoire :**

- **Expression stable :**

  maintien du transgène dans les cellules filles

  - ✓ origine de réplication propre au transgène (adénovirus)
  - ✓ **intégration dans le génome**

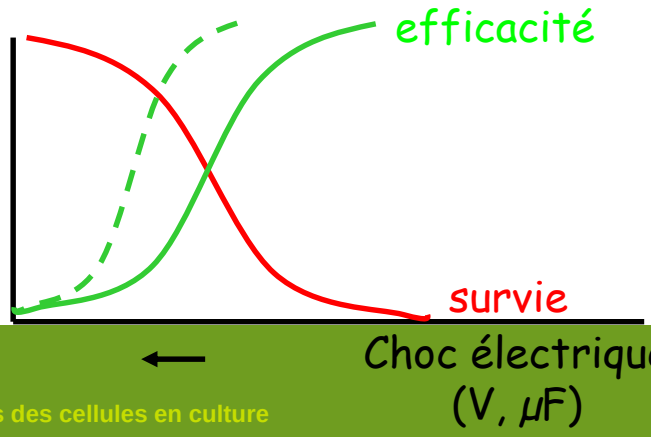
    facilitation de l 'intégration (ex : linéarisation, LTR, …)

TRANSFERT DE GENES DANS DES CELLULES EN CULTURE

- physique électroporation

Avantages :
facilité, « modicité de prix »
ADN nu (sans additifs)

Inconvénients :
ADN nu (non protégé des endonucléases)
relativement toxique (mb perméable)

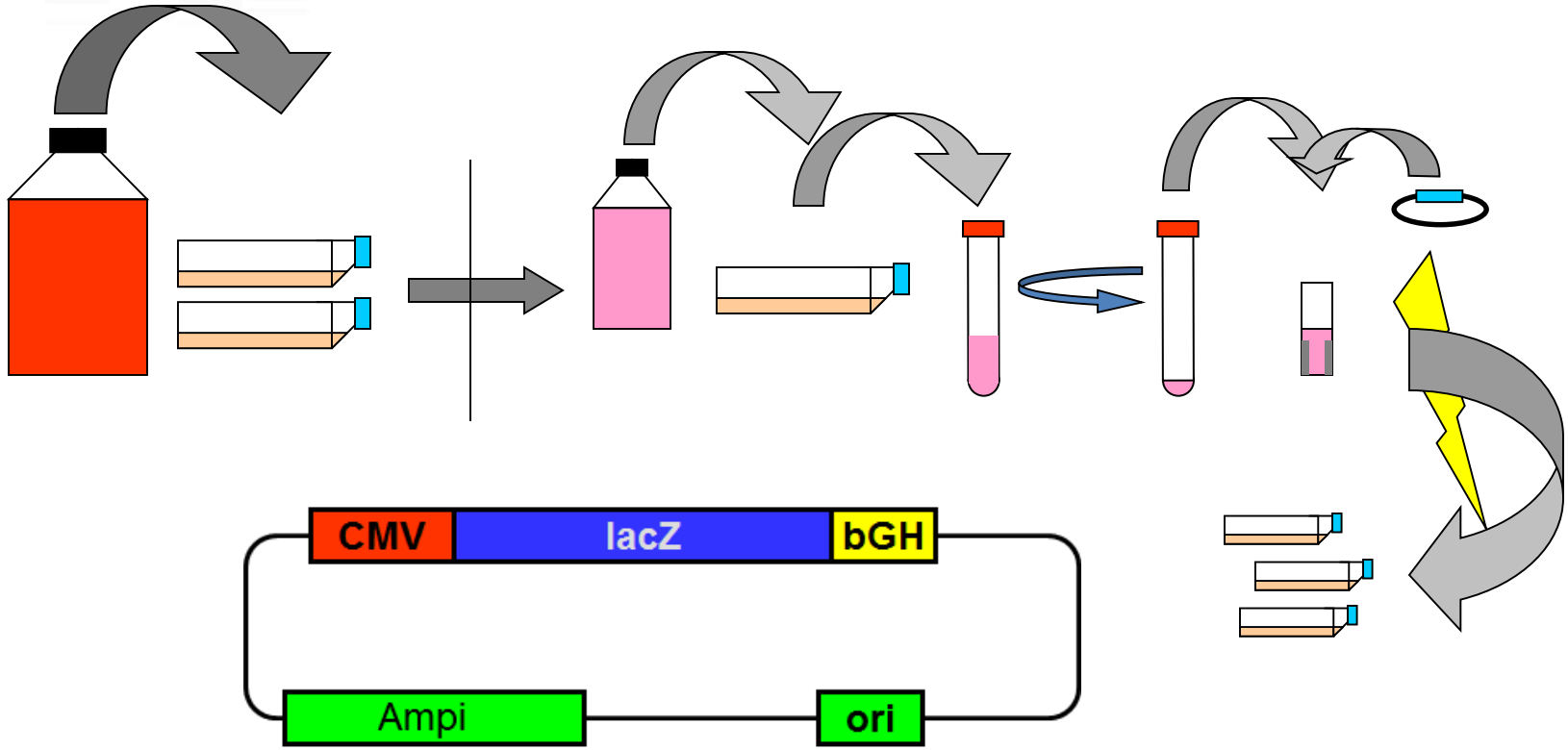


TRANSFERT DE GENES DANS DES CELLULES EN CULTURE

Baum C, Forster P, Hegewisch-Becker S, Harbers K. 1994. An optimized electroporation protocol applicable to a wide range of cell lines. Biotechniques 17(6):1058-62

A number of transfection methods for mammalian cells are available; however, many cell lines may appear resistant to efficient transfection, or at best, necessitate lengthy optimization procedures in recommended protocols. We describe here an **electroporation protocol that yields highly efficient gene transfer (20%-100% of surviving cells) in all 19 cell lines tested so far**, including lymphoid, myeloid, glial, fibroblast and COS cells. Unlike earlier electroporation protocols, **adaptation of this protocol to a cell line of interest only requires the optimization of a single parameter**, i.e., the voltage, and **can be performed within a day**. Furthermore, it is also **superior to transfection protocols for other methods** (calcium phosphate precipitation, lipofection, DEAE-dextran transfection and transferrinfection) **in terms of reproducibility, economy and average transfection efficiency** for a wide variety of cell lines.

TRANSFERT DE GENES DANS DES CELLULES EN CULTURE



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