Program schedule of practical course

Mutagenesis of the nuclear localization signal of the nuclear rabies virus P3 protein and cloning in a mammalian expression vector

28 november – 9 december 2005

Danielle BLONDEL
UMR INRA-CNRS. Laboratoire de Virologie Moléculaire et structurale
Gif sur Yvette FRANCE

Day 1 : **Monday 28 November**
I. Introduction
1- Replication of rabies virus
2- Rabies phosphoprotein P
3- Rabies P3 protein
4- Presentation of the practical course
PCR method to introduce mutation

II. Practical course

Week 1

Day 1 : Monday 28 November
1. 1 Amplifications PCR 1 and PCR2 to obtain both P3 et P3 mutated (P3m); mutations will be introduced by 2 steps of PCR using sest of oligonucleotides.

Day 2 : Tuesday 29 November
2. 1. PCR3 amplification of the full-lenght gene for P3 an,d P3m
2. 2. Purification on agarose gel of PCR products (P3 and P3 m)

Day 3 : Wenesday 30 November
3. 1. Digestion of P3 and P3m and of the cloning vector by restriction enzymes.
3. 2. Purification of the vector on agarose gel
3. 3. Purification of P3 and P3m inserts

Day 4 : Thursday 1 December
4 .1. Ligation of the inserts (P3 and P3m) in the digested vector
4. 2. Transformation of bacteria

Day 5 : Friday 2 December
5. 1. Growth of colonies

Week 2

Day 6 : Monday 5 December
6 . 1. Screening of positive colonies by minipréparation
6. 2. Amplification of one positive clone for each construct

Day 7 : Tuesday 6 December
7. Purification of plasmids

Day 8 : Wenesday 7 December
8. Transfection of cells

Day 9 : Thursday 8 December

Day 10 *:* Friday 9 december
10. Fixation of cells and analysis of localization of P3 and P3m in cells by immunofluorescence

I. Introduction

1. Replication of rabies virus

Rabies virus belongs to the Lyssavirus genus of the rhabdovividae family which includes also the Vesiculovirus genus whose VSV is the prototype. Rhabdovirus particles are envelopped virus and have a typical bullet shaped. The genome consists of negative sense single stranded RNA (about 12kb) encoding 5 viral proteins. It is encapsidated by the nucleocapsid N to form the nucleocapsid that is associated with the RNA polymerase L and its cofactor, the phosphoprotein P. The nucleocapsid is condensed by the matrix protein M into a tightly coiled structure and is surrounded by the viral membrane that contains a unique glycoprotein (G).

The virus cycle is cytoplasmic. The virus enters the cells via the endocytic pathway and this step is followed by the fusion between the viral and the endosomal membrane that allows the release of the nucleocapsid inside the cells. In the cells, the nucleocapsid (N-RNA) serves as a template for transcription and replication (Figure 1). There is a single site for transcription initiation at the 3'end of genomic RNA. After initiation, the polymerase complex (L and P) transcribes a copy of the leader RNA and then subsequently the individual mRNA of the viral genes from the 3' to 5'(N-P-M-G-L). There is 30% attenuation of transcription at each gene boundary leading to a gradient of transcripts from 3' to 5'. The replication process yields nucleocapsids containing full-length antisense genome RNA, which in turn serves as a template for the synthesis of sense genome RNA (Figure 1). The N protein favors replication process by its ability to bind to the nascent leader RNA and to the intergenic junctions, resulting in the synthesis of full lenght antigenomic sense RNA. The neo-synthesized genome either serves as a template for secondary transcription or are compacted by M proteins to allow budding of the virion from the cellular membrane.

2. Rabies virus P protein : a multifunctional protein at the interface between the virus and its host.

The rabies virus P protein plays a central role in the network of protein-protein interactions by providing a bridge between the polymerase L, N-RNA template and cellular factors. P is a non-catalytic cofactor and a regulatory protein that plays a role in viral transcription and replication : it stabilises the RNA polymerase L to the N-RNA template and binds to the soluble N preventing N aggregation and keeping N in a suitable form for specific encapsidation of viral RNA. The P protein contains two N protein-binding domains: one

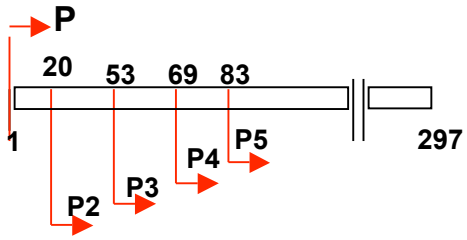
domain located in the amino-terminal 177 residues binds to N and the other in the carboxy-terminal region binds to N-RNA (Figure 2). The C-terminal domain folds as a single compact domain as shown from the recently solved crystal structure of the carboxy-part of P (Mavrakis et al. 2004. *J Mol Biol* 343: 819-831.

The major L-binding site resides within the first 19 residues of P. P protein has specific functions in the host cells. Diverse reports demonstrate the direct interaction of P with different cellular proteins. Interaction of P with the dynein light chain LC8 could mediate the transport of viral nucleocapsid in the nervous central system, but the importance of this interaction during natural infection merits further study (Raux et al. 2000; *J Virol* **74**, 10212-10216; Jacob et al. 2000; *J Virol* **74)**. Interestingly, rabies P protein interacts directly with two proteins playing an important role in IFN-induced antiviral response, STAT1 the critical component of IFN signaling and PML, the organizer of the nuclear bodies (Blondel et al.2002 *Oncogene*, **21,** 7957-7970, Vidy et al 2005, *J Virol* **79** in press).

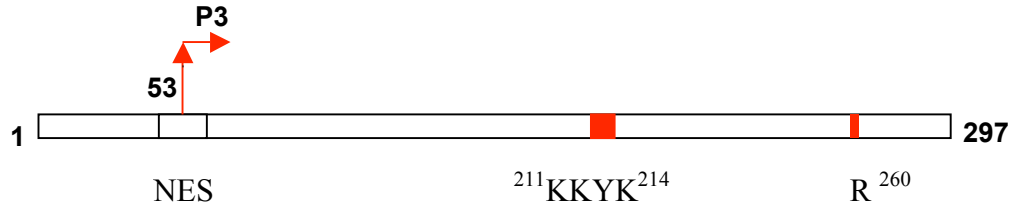
In addition, P protein impairs IRF-3 phosphorylation leading to the inhibition of IFN production. This multifunctionality of P is probably linked to the high polymorphism of expression of the protein. It is phosphorylated by two kinases: the RVPK (Rabies virus protein kinase), and the protein kinase C (Gupta et al. 2000; *J Virol* **74**, 91-98). Both kinases phosphorylate specific sites on the P protein, leading to the formation of different phosphorylated forms of the P protein with different motilities in SDS-PAGE.

3. Rabies virus P3 protein

Rabies P gene encodes P and additional shorter P products (P2, P3, P4 and P5) whose translation is initiated from downstream and in frame AUG codons by a leaky scanning mechanism (Chenik et al. 1995, J.Virol. **69,** 707-712). These small versions of P have different intracellular distribution. The nuclear localization of P3-P5 are due to the presence of a nuclear localization signal (NLS) located in the C-terminal part of the protein whereas the cytoplasmic distribution of P-P2 is the result of a nuclear export signal located in the N-terminal part of the protein (Pasdeloup et al. 2005, Virology **334**, 284-293).



The P3 protein contains a nuclear localization signal NLS and is nuclear. P3 mutated in the NLS is cytoplasmic.



4. Presentation of the practical course

The aim of this practical course is the construction of a plasmid encoding P3 in fusion with GFP (P3-GFP) and P3-GFP mutated in the NLS in the mammalian expression vector expressing GFP: pEGFPN1.

The NLS ^{211}KKY**K**^{214}-**R**^{260}is located in the carboxy-terminal part of the P protein (the numbers indicate the amino-acid) and is conformational.

Briefly, first the NLS of P3 gene will be mutated by substitution of a residue Lysine in Alanine (K ^{214}-->A) introduced by PCR and then both P3 and P3(K ^{214}-->A) will be cloned in fusion with the green fluorescent protein GFP in the vector pEGFP-N1. Second, recompbinant plasmids will be transfected in mammalian cells and the localization of both proteins will be analyzed by immunofluorescence.

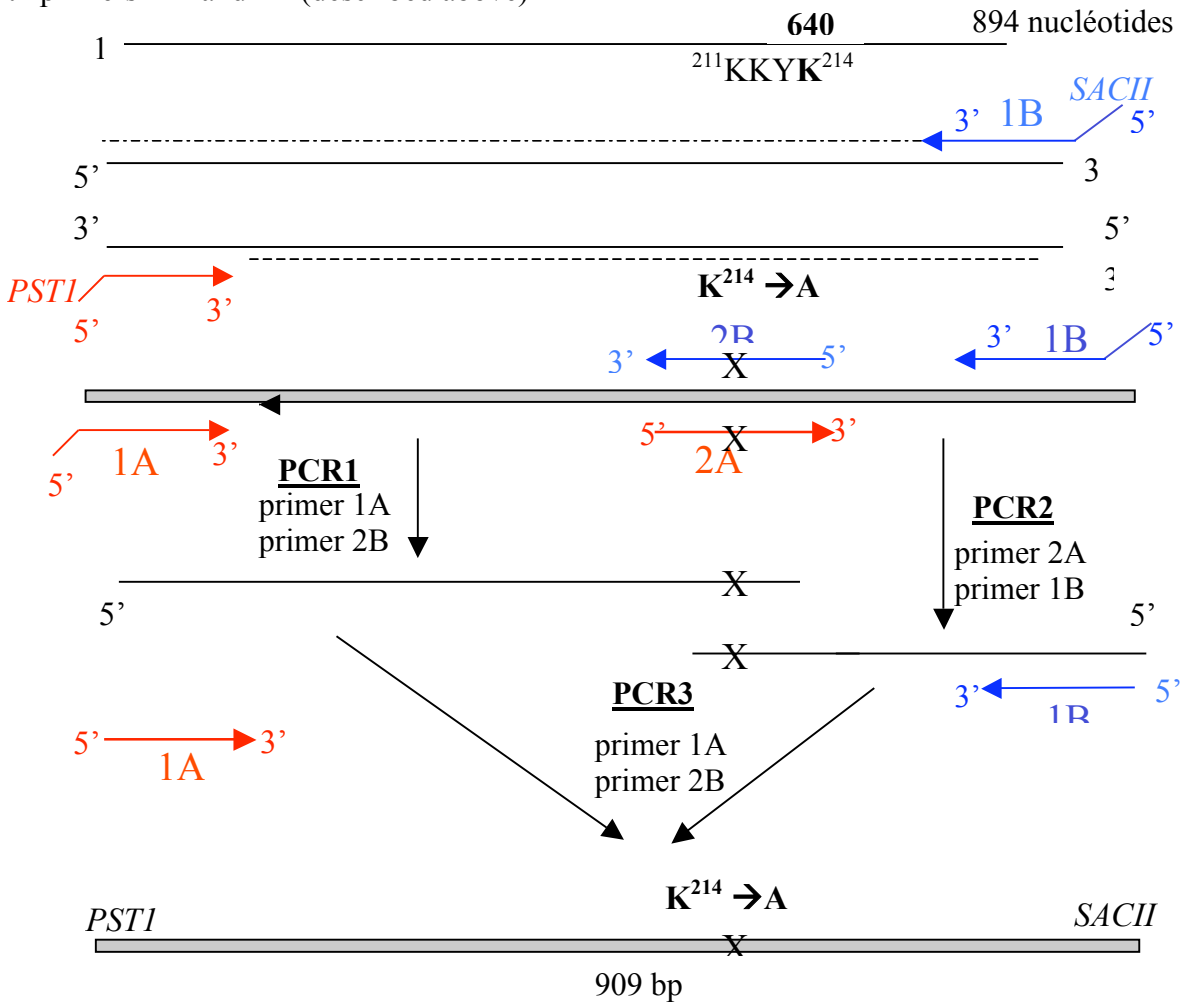
5. PCR amplification as a tool to introduce mutations

1. First made two PCR (PCR1 and PCR2) from the template containing rabies P gene with a set of primers *(by convention sens primer are termed A and antisens primer are termed B)*:

- PCR1
  - Primer 1A : sens primer corresponding to the 5' end of the mRNA of P, contains a PST1.restriction site
  - Primer 2B : antisens primer contains the mutation AAG→GCG (K ^{214}-->A)
- PCR2
  - Primer 2A : sens primer contains the mutation AAG→GCG (K ^{214}-->A)
  - Primer 1B : antisens primer complementary to the 3'end of the mRNA of P contains a SAC II restriction site .

Primers 2A and 2B are complementary

2. Second, made PCR3 by using as template an aliquot of PCR1 product and of PCR2 product with primers 1A and 1B (described above)



WEEK 1

Day 1 : Monday 28 November

1. PCR1and PCR2 amplifications and analysis of the PCR fragments by agarose gel 1% in TBE 0,5 X buffer

Materials for PCR:

- Plasmide p-P-GFP as template
- primers 1A, 1B, 2A, 2B (50 picomoles /ul)
- Taq polymerase (Expand high fidelity PCR from Roche) and its buffer 10X
- dNTPs 10mM of each
- water ultra pure
- Primers for:

  - *Construction of pP3-GFP in PEGFP N1*

  PCR with primers 1A and 1B
  Forward primer 1A : 5'GCCCTGCAGCTGAGGACATGAAGC 3' (PST1 site)
  Backward primer 1B : 5'GCCCCGCGGGCAGGATGTATA3' (SACII site)

  - *Construction of pP3($K^{214}A$) in pEGFP-N1*

  Forward primer 2A : 5'CCAAGAAGTAC**GCG**TTTCCCTCCCGA3'
  Backward primer 2B : 5'TCGGGAGGGAAA**CGC**GTACTTCTTGG 3'
  PCR1 : primers 1A and 2B; PCR2 : primers 2A and 1B
  PCR3 : primers 1A and 1B

General PCR protocol

- Set up 2 master mixes as follows :

  - *mix 1* (for one reaction)
    2 µl 10mM dNTPs
    1 µl upstream primer
    1 µl downstream primer
    45 µl Sterile water
    add the denatured template

  - *mix 2*
    10 µl buffer 10X
    1 µl enzyme Expand Hight fidelity Taq
    39 µl Sterile water

Template DNA (50-100ng)
1 µl in 9 µl sterile
denaturation 95°C, 2min

- Pipet together 50 µl mix 1 and 50 µl mix 2 and put the mixture in 2 PCR tubes. You have 2 tubes / reaction.

- Place the tubes in Thermocycler and perform PCR

Programme/

1.94°C, 5min
2. 94°C, 30s
3. 55°C, 30s } 10cycles
4. 72°C, 1min
5. 94°C, 30s
6. 55°C, 30s } 15 cycles
7. 72°C, 1min
8. 72°C, 4 min
9. Pause 20°C

1. 1. **Amplifications PCR 1 and PCR 2**

- Denature the template pP(1µl in 9µl water) 95°C 2min.
- Prepare 3 eppendorf tubes

• *mix 1* for PCR1	for PCR2
2 µl 10mM dNTPs	2 µl 10mM dNTPs
1 µl primer 1A	1µl primer 2A
1 µl primer 2B	1 µl primer 1B
35 µl Sterile water	35 µl Sterile water
add 100ng of pP as template	add 100ng of pP as template

- *mix 2* (you can prepare the mix for 2,5 reactions (PCR1 and PCR2)
  2,5 X 10 µl buffer 10X
  2,5 X 1 µl enzyme Expand Hight fidelity Taq
  2,5 X 39 µl Sterile water

- Pipet together 50 µl mix 1 and 50 µl mix 2 and put the mixture in 2 PCR tubes. You have 2 tubes / reaction
- Place the tubes in Thermocycler and perform PCR

1. 2. **Analysis of the PCR 1 and 2 fragments on agarose gel (1%) in TBE .**

<u>Materials for gel:</u>

- Agarose
- BET
- Buffer TBE 10X : 0, 9M Tris-HCl pH 7,5 to make TBE 0,5 X
  0, 9M Acide Borique
  20mM EDTA pH8
- DNA molecular weight (DNA of phage lambda digested by HindIII- EcoRI)
- Blue 6X

- Mix the tubes of the same PCR reaction
- Take 3 µl/100 µl PCR and add 1µl of blue and 2µl water
- Take 5 µl of marker
- Charge the marker, PCR1, PCR2, negative control
- Migration in TBE X 0,5 with constant voltage (2cm of migration)

We expect for PCR1 a band of 515 bp and for PCR2 a band of 272 bp.

Put the PCR reactions at – 20°C

Day 2 : Tuesday 29 November

2. 1. **PCR3 amplification of the full-lenght gene for both P3 and P3 K^{214}A and analysis on gel.**

- Prepare the mix 2 as previously described and the mix1 as follow:

2 µl 10mM dNTPs
1 µl primer 1A
1 µl primer 1B
44 µl Sterile water
1ul of PCR1 reaction
1ul of PCR2 reaction

- Prepare the mix 2 as previously described
- Analyze the PCR3 reactions P3 and P3 K^{214}A on an agarose gel.

We expect a band of 763 bp

2. 2. **Purification of the P3 and P3 K^{214}A fragments on agarose gel**

Materials:

- TAE X 20 buffer : 0,8M Tris-HCl pH 7,5; 1,3M Acetic Acid ; 20mM EDTA pH8
- kit NucleoSpin extract (Macherey-Nagel)
  buffers NT1, NT2, NT3 and NE

- Prepare the agarose gel 0,8% in TAE and charge
- Charge100 µl + 20µl blue.
- Excise the band from the gel with a scalpel and put it in an eppendorf tube.
- Transfer to an eppendorf tube and weight the gel slice
- Add 300µl NT1
- Incubate sample at 50°C (5 – 10min) until the gel is dissoved
- Place a NucleoSpin Extract colum into a 2ml tube and load the sample
- Centrifuge for 1min at 8000 g . Dicard flow-through and place the column into the tube
- Wash with 600µl buffer NT3
- Centrifuge for 1 min at 11000 g ; discard flow-through and place the column in the tube
- Add 200 µl NT3. centrifuge for 2min at 11 000 g to remove buffer NT3
- Place the column into a clean tube; add 50µl NE incubate for 1min
- Centrifuge for 1min at 11000 g.

Day 3 : Wenesday 30 November

3. 1. **Digestion of the P3 and P3 $K^{214}A$ fragments and of the plasmid pPEGF-N1**

- Prepare 3 tubes eppendorf
- Mix:

50µl of the purified fragment (**P3 and P3 $K^{214}A$)**	20µl plasmid pEGFP-N1 (1µg/µl)
10µl buffer 10X	10µl buffer 10X
4µl PST1 (10 U/µl)	4µl PST1 (10 U/µl)
4µl sacII (10 U/µl)	4µl sacII (10 U/µl)
32µl water (100µl final)	62µl water (100µl final)

- Digestion for at least 2hours at 37°C.

3. 2. **Purification of the digested P3 and P3($K^{214}A$)**

Material

- kit nucleospin extract (Macherey-Nagel) to remove enzyme.

- Add 400µl NT2 to 100µl reaction
- Place a NucleoSpin Extract colum into a 2ml tube and load the sample
- Centrifuge for 1min at 8000 g . Dicard flow-through and place the column into the tube
- Wash with 600µl buffer NT3
- Centrifuge for 1 min at 11000 g ; discard flow-through and place the column in the tube
- Add 200 µl NT3. centrifuge for 2min at 11 000 g to remove buffer NT3
- Place the column into a clean tube; add 50µl NE incubate for 1min
- Centrifuge for 1min at 11000 g.

3. 3. **Purification of the digested plasmid pEGFP-N1**

- Prepare a 0,8% agarose gel in TAE.
- Excise the band from the gel with a scalpel and put it in an eppendorf tube.
- Use the kit nucleospin extract as previously described
  keep the DNA at -20°C

Day 4 : Thursday 1 December

4. 1. **Ligation**

Materials:

- Ligase
- Buffer X 10

- Estimate the amounts of fragments and of plasmid by migration on agarose gel in TBE in parallel with a known amount of marker.

The ligation is performed with 100 ng of vector and 30 to 60 ng of insert

- Prepare 3 eppendorf tubes with:

	vector	insert	ligation buffer X 10	ligase	water (20µl final)
1	100ng	0	2 µl	1µl	
2	100ng	30 to 60	2 µl	1µl	
3	100ng	30 to 60	2 µl	1µl	

- Ligation for 1h at 25°C

4. 2. Transformation

Materials

- Competent DH5α bacteria
- Plates LB with kanamycine (30µg/ml)
- LB medium

- Prepare 1 tube/ ligation.
- Take 50 µl DH5α bacteria and add 3µl of the ligation
- Incubate the mixture for 30 min in ice then make a heat-choc at 42°C for 2min.
- Add 400 µl of LB and incubate at 37°C for 45 min.
- Spread 200µl of bacteria on LB kan plates and incubate overnight at 37°C.

Day 5 : Friday 2 December

5. 1. Growth of colonies

- Count colonies
- Pick 4 colonies in 3 ml of LB + kanamycin (30µg/ml)
- Incubate the tubes with shaking overnight at 37°C

WEEK-END

Saturday put the colonies at 4°C

WEEK 2

Day 6 : Monday 5 December

6. 1. Minipreparation

Materials

- Buffer 1 : Glucose 50 mM
  Tris pH8 25 mM
  EDTA 10 mM

- Buffer 2 : NaOH 0.2N 0.2 mL NaOH 10N
  SDS 1% 1 mL SDS 10%
  8.8 mL ddH_2O

- Buffer 3 : Acétate de K pH4.8, 3M (11,5ml acetic acid + 28,5ml water + 60ml 5M potassium acetate.

- Phenol-Chloroforme

- TE-RNase : 1 mL TE pH8 + RNase à 20 µg/mL.

-Transfer 1,5 ml of the culture in 3ml of LB containing kanamycine and centrifuge at 10 000 g for 1min.
- Remove supernatant and resuspend the cells by vortex in 100µl of ice-cold Buffer 1.
- Incubate for 5 min at RT
- Add 200 µl of buffer 2 and mix by inversion without vortex
- Incubate for 5 min in ice
- Add 150 µl of buffer 3
- Centrifuge at 10 000g for 5min
- Add an equal volume of phenol-chloroforme, mix par vortex 30sec
- Centrifuge at 10 000g for 5min
- Transfer the aqueous phase in a new tube
- Add 2,5 volumes of ethanol, mix and incubate for 5 min at RT.
- Centrifuge at 10 000g for 5min
- Remove the supernatant and rinse the pellet with 70% ethanol
- Dry the pellet and dissove the DNA in 20µl of TE with RNAase.

6. 2. Digestion of DNA of miniprep and identification of positive clones

- Prepare tubes with : 3 µl of DNA
  0,5 µl PST1
  0,5 µl SACII
  1µl Buffer X10
  5µl water

- Incubate for 1h at 37°C
- Migrate on 0, 8% agarose in TBE
  Identify the positive clones by analysis on gel

6. 3. Amplification of one positive clone for each construct

- Grow 100 µl of positive miniprep in 25 ml culture LB + Kanamycin
- Incubate the tubes with shaking overnight at 37°

Day 7 : Tuesday 6 December

7. Purification of plasmids pEGFP-P3 et PEGFP-P3($K^{214}A$)

- Nucleobond AX plasmid Kit PC 100 (Macherey-Nagel)

- Harvest the bacteria by centrifugation at 4500 g for 15 min at 4°C
- Discard the supernantant and add in the pellet
- Add 4ml buffer S1 + RNase A
- Add buffer S2. Do not vortex; mix by inversion and incubate for 2-3 min (max 5 min)
- Add 4 ml pre-cooled buffer S3. Mix by inversion
- Equilibrate the NucleoBond with 2,5 ml Buffer N2. Discard flow-through
- Filter the suspension after preweting the filter with drops of N2
- Load the cleared lysate on the nucleoBond column.
- Wash the column with 10ml buffer N3
- Discard the flow-through
- Elute the DNA with 5ml of buffer N5
- Precipitate with 3,5 ml Isopropanol
- Centrifuge at 15 000g for 30min at 4°C
- Discard the supernatant and wash the pellet by 2ml 70% ethanol.
- Centrifuge at15 000g for 10 min.
- Carefully remove ethanol and allow the pellet to dry at Room Temperature
- Add 30µl TE. Determine plasmid concentration by OD measure.

Day 8 : Wenesday 7 December

8. Transfection of BSR cells with CaPhosphate procedure

Materials

- Buffer HBSP : Hepes-buffered saline phosphate
  137mM NaCL
  5mM KCl
  0,7mM Na2HP04
  6mM dextrose
  21mM Hepes-NaOh ph7,1

- $CaCl_2$ 2M
- 15% EPT-glycerol

- BSR cells were grown in petri dish (8,7cm^2) 3cm diameter (80% confluency)

- Prepare 1 tube with DNA 2 to 5 µg / dish and add 125µl HBSP + 8,5 µl $CaCl_2$. Mix gently
- Incubate 20min at Room Temperature
- Remove medium from cells. Add DNA/ Ca phosphate coprecipitation on the cells
- Incubate 20 min at RT
- Add 1ml DMEM or MEM + 5% SVF
- Incubate for 4h at 37°C.
- Remove medium, add 1ml 15% EPT-glycerol ; incubate 3min at RT
- Remove glycerol and wash the cells with MEM or EPT
- Add MEM + 10% SVF
- Incubate the cells for 2 days at 37°C

Day 9 : Thursday 8 December
No experiment
Questions and Discussion.

Day 10 : Friday 9 December

Analysis of localization of proteins P3 and P3m in cells by immunofluorescence.

10.1. Fixation of cells

Materials:
- solution of 4% Paraformaldehyde (PFA) in EPT or PBS
- EPT or PBS

- Remove the supernatant. Wash the cells twice with PBS and add 1 ml of 4% PFA

10. 2. Observation of GFP fluorescence

<u>Discussion</u>