ISAS - INTERNATIONAL SCHOOL FOR ADVANCED STUDIES

Studies on the intrabody trap technology

Thesis submitted for the degree of
Magister Philosophiae

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This thesis is dedicated to JD

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1 INTRODUCTION

1.1 The intracellular antibody technology

In the last decade, the advancements in antibody engineering allowed the development of the intracellular antibody technology. The possibility to screen virtually infinite repertoires of natural antibodies via techniques such as the phage display, along with the advancements of molecular biology in general, gave this technique all the tools needed for its development. Today the ectopic expression of engineered antibodies is an established method to interfere with the function of specific intracellular targets (Cattaneo and Neuberger, 1987, Carlson 1988 and, for a review, Cattaneo and Biocca, 1997), achieving what is called the phenotypic knock out of the selected targets. The advent of monoclonal antibodies (Koehler, et al., 1975), along with the phage display (McCafferty et al., 1990; Winter et al., 1994) allow to isolate not only the antibody molecules, but also the rearranged genes that encode for the antibody proteins. Therefore the development of recombinant DNA technology justify the ambitions to apply this approach not only to research fields but also for therapeutical contexts (Chen et al., 1994, Rabbitts, 1998 , Marasco et al., 1993) .

1.1.1 Ectopic antibody expression

The principle of ectopic antibody expression is to allow in vivo immunoglobulin expression in the same place and time where the antigen is present. In fact antibodies, when treated as recombinant proteins, can be expressed by mammalian cells as secreted proteins, but also as intracellular proteins and, when needed, targeted to different cell compartments. Several papers have demonstrated that antibodies can be correctly produced, folded and secreted by a whole spectra of different eukaryotic cells (Cattaneo

and Neuberger 1987, Capsoni et al., 2000, Cattaneo et al., 1999), thus following the normal production pathway in non lymphoid cells, but it is now clear that the binding specificity of antibodies may be conserved also when they are expressed in cytoplasm as well as other cellular compartments (Biocca et al 1990). Altogether these papers contribute to define the antibody ectopic expression as an available knock out method alternative and complementary to gene knock out, antisense RNA, and others.

It is possible to express different artificially rearranged forms of antibodies (Fig.1.1), however one of the most common forms is the so called single chain fragment (ScFv), were the variable regions are expressed covalently joined by a short linker. The ScFv form is the type of construct used for the work described on this thesis.

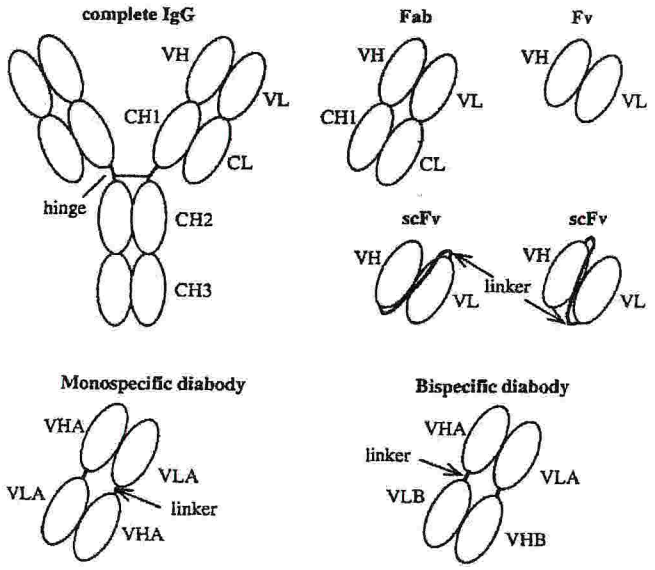

Fig.1.1

A complete antibody comprises two heavy chains and two light chains, with the two antigen binding regions made up of a heavy and light chain variable region. The VH-CH1 /VL-CL molecule is termed Fab and is usually monomeric in its binding ability. Monomeric binding units can also be created by expressing the variable regions, either alone (Fv) or covalently joined by a linker (ScFv - single chain Fv).

1.2 Selecting the appropriate ScFv

To perform a phenotypic knock out experiment it is necessary to select the appropriate ScFv gene: this antibody has to be sufficiently specific and selective, it has to be able to block the specific function of the chosen antigen, and it has to be functional and stable in the particular compartment where the binding is intended to occur.

To obtain a ScFv that binds with good affinity, avidity and specificity *in vitro* is possible by using monoclonal antibodies technology and/or phage display. However only a fraction of all the antibodies that show these properties will be able to interfere with the antigens functions *in vitro*, it is therefore necessary to screen a reasonable number of specific antibodies in order to find the ones, if any, useful for knocking out their target. In addition, ScFvs selected by their functional properties *in vitro* do not always function well also when expressed intracellularly due to problems that we now start to understand (see below) (Biocca et al. 1995).

1.2.1 Intracellular expression of ScFvs

At the present day some of the signals used by cells to target the synthesizing proteins have been identified, and it is now possible to utilize this knowledge to target the expression of recombinant proteins to particular cell compartments (Biocca et al., 1990, Biocca and Cattaneo, 1995).

The normal pattern of expression of antibodies in lymphocytes follows the secretory pathway, were their folding is assisted and controlled by a whole pool of accessory proteins (Gething and Sambrook 1992), nevertheless is usually possible to express antibodies in the secretory pathway of other cell types, even if the efficiency of the process may vary unpredictably for different ScFv fragments (Biocca and Cattaneo 1995). Anyway, antibodies that are intracellularly targeted to compartments such as endoplasmic reticulum, Golgi apparatus or the cell surface, usually retain the same binding properties showed in *in vitro* assays.

A completely different situation is found when antibodies are expressed in cytoplasm and/or nucleus. Although it is technically simple to target a ScFv to the cytoplasm, by omitting any targeting signal, creating the so called leaderless ScFv fragments, major

folding problems may arise due to the particular physico-chemical environment present in the cell cytoplasm. Normally the folding of antibodies is stabilized by several inter- and intrachain disulfide bonds, in the case of ScFvs a single conserved intradomain disulfide bond is present within each variable region. The formation of this intrachain bond is normally catalyzed by specific proteins of the endoplasmic reticulum, however the reducing environment of cytoplasm (Hwang et al., 1992) is able to fully prevent its spontaneous formation. It is thought that this bond is important for the correct folding and the stability of ScFvs (Martineau et al., 1998); generally, in its absence, many (but not all) antibodies fail to fold properly, or fold less efficiently, and loose their ability to recognize their specific antigen. However some natural antibodies lack the conserved cysteines, due to somatic mutations, and nevertheless fold properly (Rudikoff and Pumphrey, 1986). Some antibodies are still reactive even when these disulfide bonds are artificially cleaved (Proba et al., 1997, Proba et al., 1998). It is thought that a single disulfide bond contributes about 4-5 Kcal/mol to the overall stability of ScFvs, and it has been suggested that in some particular cases the stabilizing contributions of the other parts of the molecule may be sufficient to maintain the correct folding. The lack of understanding of the rules that underline the folding of macromolecules *in vivo*, makes it impossible to predict how a ScFv of known primary structure will behave in the cytoplasm, therefore no one can state if a particular antibody will be able to recognize its antigen also intracellularly without having previously tested it.

This means that to prepare an intracellular immunization experiment, one should be ready to test a whole set of ScFvs directed against the selected antigen, find out those able to recognize the antigen in cytoplasm, and verify if one of them is able to display a knocking out activity, with no guaranties that a suitable antibody will be found. To solve this strong limitation it is possible to use the molecular evolution approach (Proba et al., 1998), and very recently an alternative method was developed in our laboratory, based on the two hybrid system (Visintin et al., 1999).

1.3 Selecting intracellular antibodies with the two hybrid system

The two hybrid system is a powerful technique to screen for protein-protein interactions *in vivo*, used successfully in many different contexts, from functional genomics analysis to cancer research and others. Normally it is used to fish out, from a library of interest, the clones that are able to bind a given protein. The basic principle, explained in Fig.1.2, was first described by Fields and Song (1989) and Chien et al. (1991), based on the properties of transcription regulating factors, and DNA binding proteins, such as GAL4 (Keegan et al., 1986), LexA (Golemis and Brent, 1992) and VP16 (Vojtek et al., 1993).

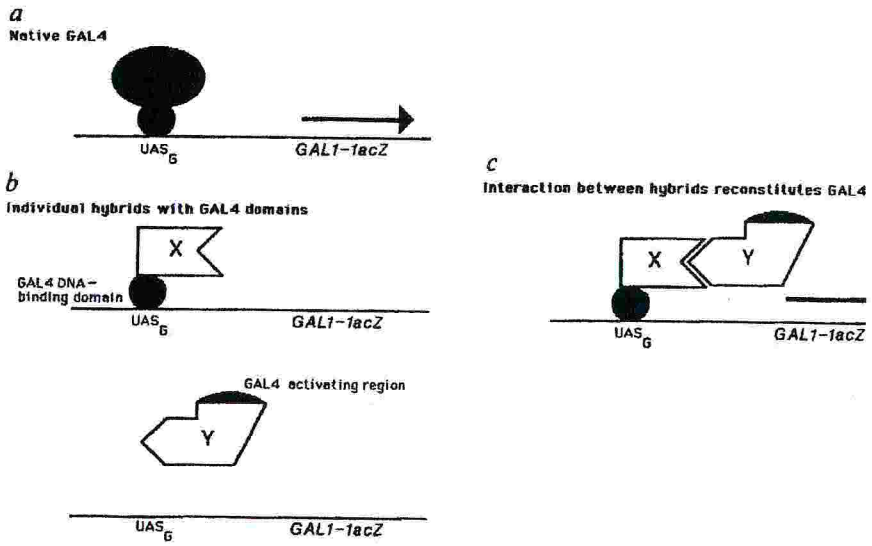

Fig.1.2
Model of transcriptional activation by reconstitution of GAL4 activity. a) The native GAL4 protein contains both DNA-binding and activating regions and induces GAL1-LacZ transcription. b) Hybrids containing either the DNA binding domain or activating region are unable of inducing transcription. c) A protein-protein interaction between proteins X and Y brings the GAL4 domains into close proximity and results in transcriptional activity.

The system used for our work is different from the original one, described in Fig. 1.2, in that the DNA binding domain of GAL4 was replaced by its LexA counterpart, while its

transactivation part was substituted with VP16 acidic activation domain, and the addition of the reporter gene HIS3 (see Chap. 2.6 for details).

In our case, the reason to use this system is simple: since it is designed to select genes that encode for proteins able to bind with a protein of interest, it may be as well suitable to isolate ScFvs able to recognize their protein antigen in an intracellular context. For our work the first model experiments were performed using already characterized ScFv-antigen couples (Visintin et al. 1999), such as the ScFv F8 and the p41 coat protein of the AMCV, ScFv against the HIV-1 integrase and others, and it was indeed possible to demonstrate that the system can discriminate couples able to react into the cytoplasm from others that do not.

The main interest of the technique however is represented by its presumable ability to screen for libraries of ScFv in order to fish out only those able to recognize its antigen in the cytoplasm and nucleus. To proof this, a model selection was performed against the microtubule-associated protein Tau, well known for its proposed role in the Alzheimer's disease (AD) etiology (Alonso et al., 1996). In particular, the deletion fragment Tau 151-421 was utilized which was shown to induce apoptosis in Cos cells (Fasulo et al., 2000).

Since the available protocols for yeast transformation provide an efficiency up to 10^6 $^{colonies}/_{\mu g\ DNA}$ it was not possible to select directly from a complete antibody library (diversity of 10^{11} clones), for this reason two cycles of phage display selections were performed, by panning on solid phase bound antigen (Visintin Ph.D. thesis).

The resulting enriched library was subcloned in the pray vector pVP16 and used for the two hybrid selection. The rescue of 100 positive clones permitted to identify five different ScFv, three of them, named ScFv2, ScFv14 and ScFv52 were true positives, as proven by individual re-testing in vivo and in vitro. They were subcloned both into a prokaryotic and an eukaryotic expression vector, all of them resulted reactive by ELISA and different biological assays.

These experiments proved the validity of the approach, in principle. However, having rescued only three different antibodies may not be sufficient, if the final intention is to identify ScFvs able to block the function of a particular protein. In order to have a good probability to find out at least one suitable ScFv it is necessary to have a larger panel of ScFvs against the antigen of interest. For this reason we decided that some adjustments on the main frame of the system were to be developed.

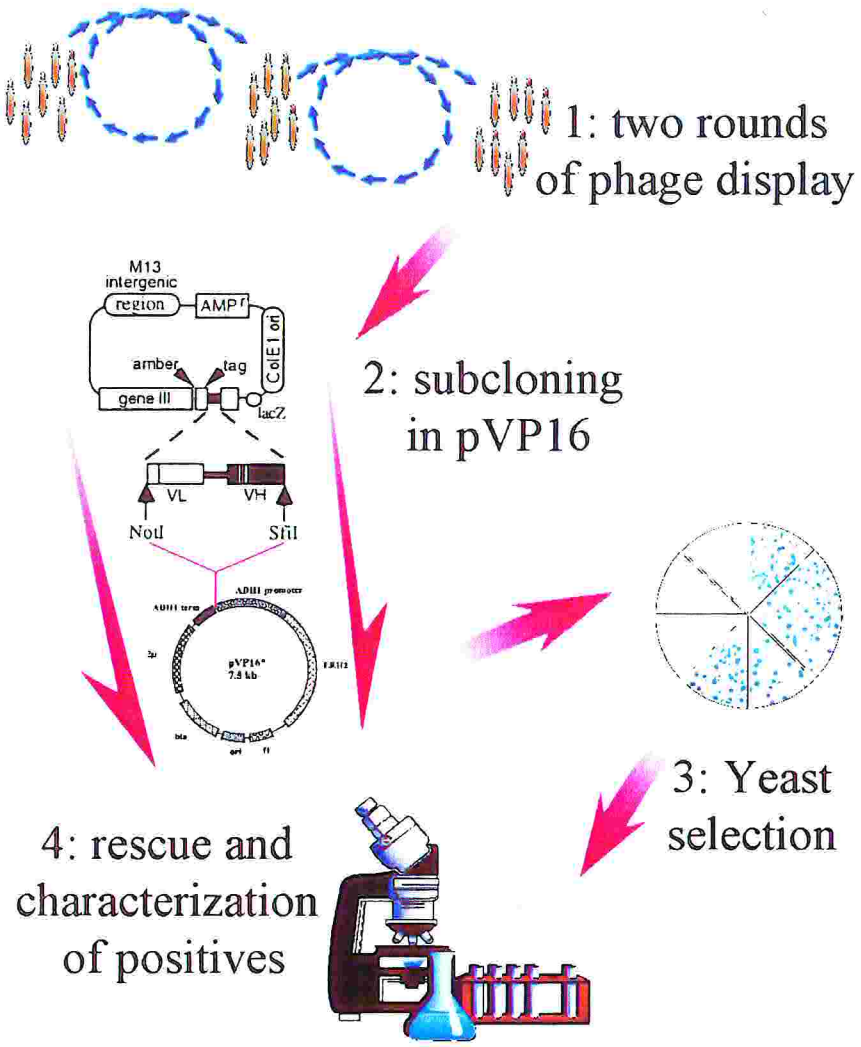

Fig. 1.3 The main steps of the ScFv selection
The general scheme of the system developed in our laboratory is summarized.

The main steps of the two-hybrid selection for intracellular antibodies technology, as developed in our laboratory, are summarized in Fig. 1.3: the picture is a schematic representation of the actual work that had been successfully done at the time when my contribution started.

1.4 The aim of the work

The problem of selecting antibodies suitable for cytoplasmic or nuclear expression seemed to be solvable with the two hybrid approach, still this system was to be refined and developed, in order to maximize the outcome, both in terms of numbers and of efficiency. Some work was also necessary to validate the applicability of the results already achieved. In this context my contribution was planned to be widespread, from following the path of the established results to exploring new directions to improve the technique.

My work consisted on trying to find the answer to three questions:

1. Is it possible to use a different source of ScFv genes for the two hybrid selection? In particular is it possible to start from a library obtained from hyper-immunized mice?
2. Is it possible to obtain more different ScFvs if starting the yeast selection after only one phage panning round?
3. The use of ScFv for the phenotypic knock out in mammalian cells would be facilitated by the availability of stable mammalian cell lines expressing them. Is it true that ScFv fragments isolated with the yeast trap system can be used to make stable cell lines?

The general representation of my work is illustrated in Fig. 1.4, were my contributions are added in color to the black and white scheme of the work already done.

1.4.1 The experimental strategy

To develop the system, two approaches were undertaken, the first was studied in order to achieve different results contemporary. We planned to perform another pilot selection utilizing the green fluorescent protein (GFP) as the bait antigen. The idea was to identify new intracellular antibodies both starting from a phage display selection and with a library derived from hyper-immunized mice. This strategy should assure many benefits: first to experiment the possibility of using a different type of input library; second to compare the results obtained from the two different starting points; third having the possibility to assay easily if some of the found antibodies would be able to interfere with the antigen biological activity, thanks to its directly measurable fluorescence; and, last but not least, develop

some intracellular antibodies directed against a reporter protein used more and more in the lab works.

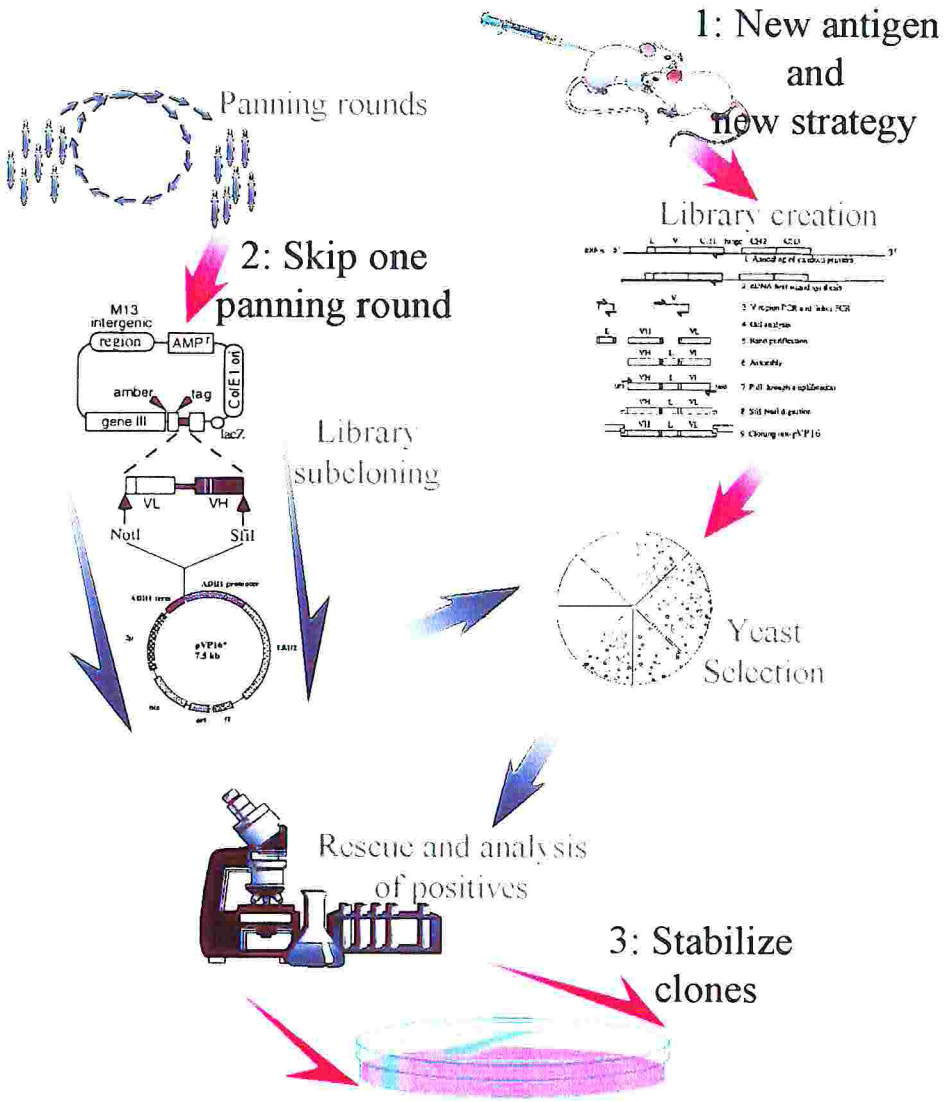

Fig. 1.4, My foreseen contributions

1) To verify the possibility to start from a library derived from hyper-immunized mice, a model selection against a new antigen was planned, 2) A new yeast selection against Tau 151-421 was also planned, starting from the first phage panning round. 3) Finally we wanted to formally prove that it was possible to stabily express the selected genes in mammalian cell lines.

The second line of work was to explore the possibility to maximize the output diversity of the yeast selection, simply by maximizing the numbers of different input clones. On the first experiment the library used in yeast was obtained with two rounds of phage display

panning, to reduce the size of the library to a predicted value, while retaining a sufficiently high diversity against the target antigen.

It is well known that after each selection cycle the diversity of a phage library decreases and a few clones tend to dominate. For the subsequent yeast trap many different and reactive clones are needed, in other words it would be inappropriate to have only a few very strong binders, as one would expect after three or four cycles. On the other hand, one would like to have the highest possible percentage of reactive antibodies. The choice of selecting form a second round has proven to be correct: correct because indeed allowed to find relevant and truly positive clones, but it had to be improved because these clones were only a few.

To try to maximize the output one possible approach is to perform the yeast selection starting from a larger library, in particular the chosen option was to start from the library rescued after only one round of phage display.

Finally, I wanted produce some stabilized clones for the already characterized ScFv in mammalian cells C6, in order to demonstrate the possibility of doing so, and to facilitate the ScFvs *in vivo* characterization necessary for eventual knock out experiments.

2 METHODS

2.1 Chemicals and molecular biology reagents.

Enzymes for modification of DNA were obtained from Boehringer Mannheim, Gibco BRL, New England Biolabs, Pharamcia, Promega or Stratagene.

Plasmid preparations were made with Qiagen mini- or midi- plasmid kits or Talent mini-prep plasmid kit. All other routine molecular biology reagents were from Sigma, Merk, Calbiochem or Carlo Erba.

2.2 Molecular biology methods

Standard methods for molecular biology were used as described in Sambrook et al. (1989).

2.2.1 Bacterial strains and plasmids

- *Escherichia coli* **DH5α** supE44 ΔlacU169 (Φ80lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi1 relA1.
- *Escherichia coli* **TOP10** mrcAΔ(mrr-hsdRMS-mcrBC) Φ80lacZΔM15 DlacX74 deoR recA1 araD139 Δ(ara-leu)7697 galU galK rpsL endA1 nupG (Invitrogen).

The map of the pQE-31 plasmid is reported in Fig. 2.1, in Fig. 2.2 there is pEGFP-N1.

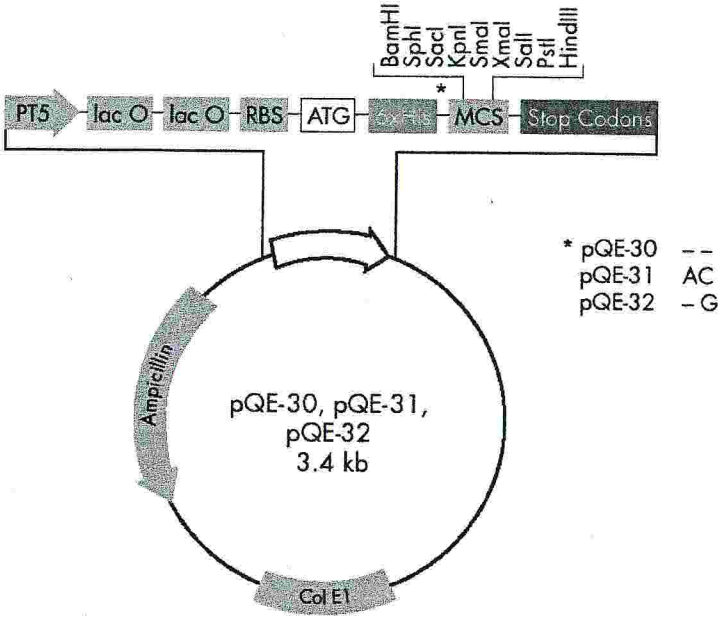

Fig. 2.1 pQE-31

This plasmid was provided by Dott. K. Ainger with the EGFP gene already inserted, it was used for the protein purification.

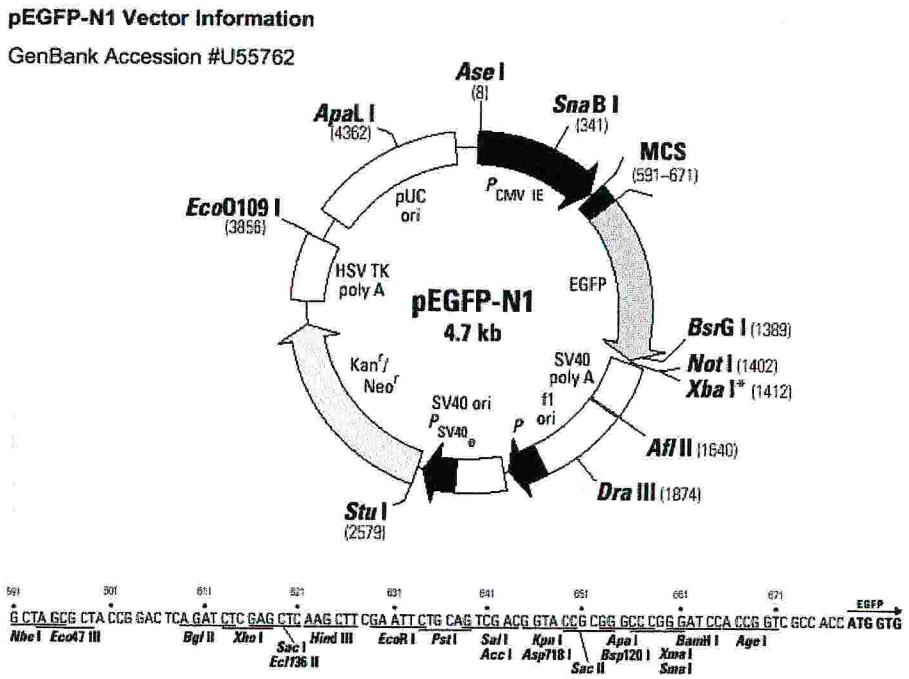

Fig. 2.2 PEGFP-N1

Restriction Map and Multiple Cloning Site (MCS) of pEGFP-N1 Vector. (Unique restriction sites are in bold) The NotI site follows the EGFP stop codon. The XbaI site (*) is methylated in the DNA provided by CLONTECH

In Fig.2.3 there is the map of the pScFv-expressCyto (Persic et al., 1997) used for the stabilized clones in C6 cells.

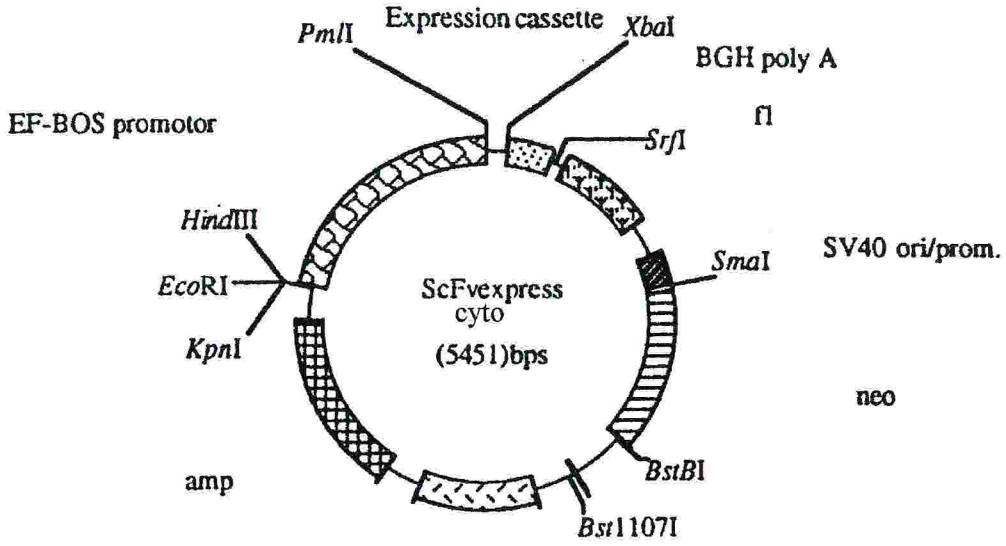

Fig. 2.3 pScFv-expressCyto

The yeast vectors pBTM116 and pVP16 are represented respectively in Fig. 2.4 and Fig. 2.5.

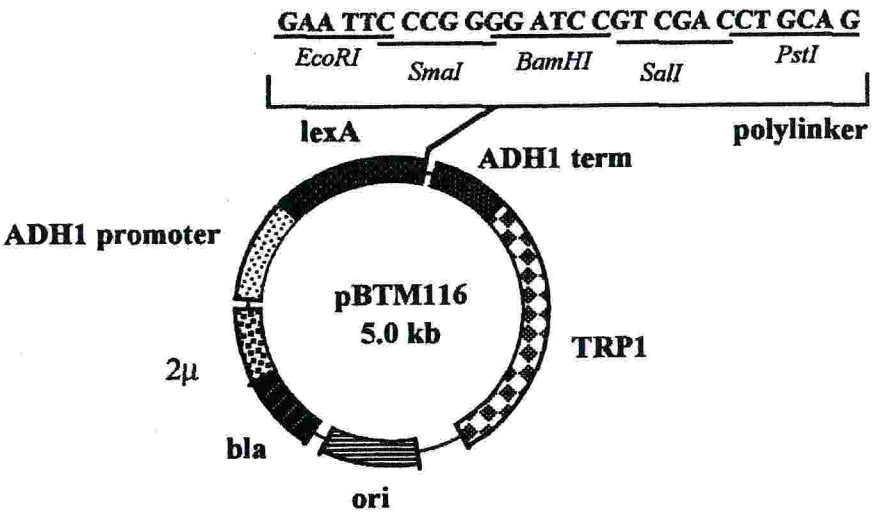

Fig. 2.4 pBTM116

The polylinker permits to clone the bait gene down stream the LexA DNA binding domain, in my case the EGFP constructs were cloned between the EcoRI and BamHI restriction sites.

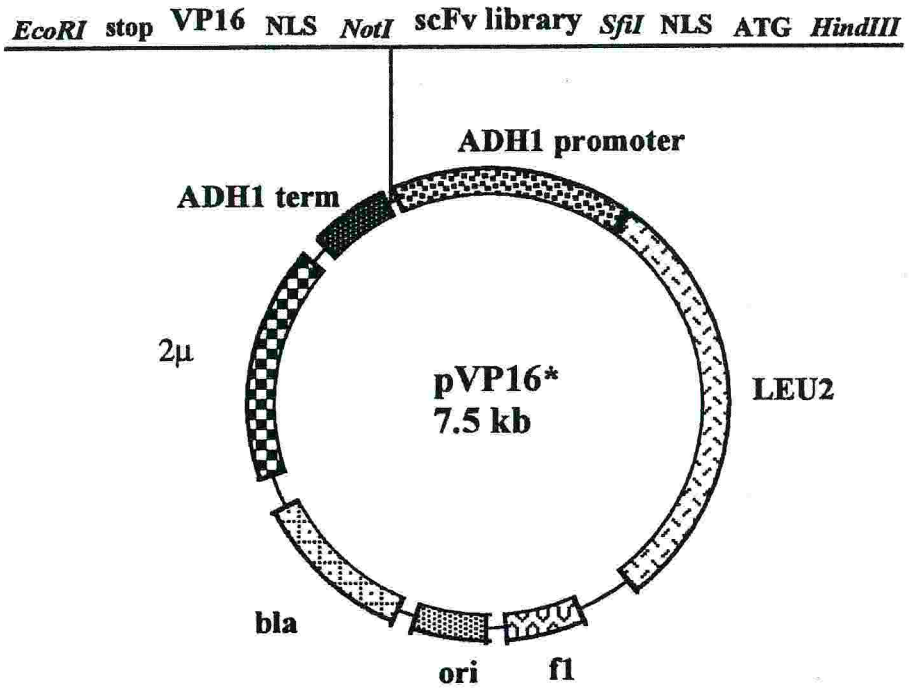

Fig. 2.5 pVP16
The ScFvs library was cloned between SfiI and NotI restriction sites.

2.2.2 Polymerase chain reaction (PCR)

Different enzymes were used for different purposes: to produce DNA for cloning Vent and Pfu polymerases were used, for other reasons (analytic or fingerprinting) the RedTaq polymerase (SIGMA) was used.

In the following the different PCR executed for this work are described.

ScFv amplification for fingerprint analysis:

To screen individual colonies for fingerprint analysis the ScFv genes were amplified by PCR with RedTaq polymerase. The template is provided directly from the bacteria. For N colonies the PCR mix was prepared on ice:

-(N+1) X 1µl primer For* (10pmol/ µl)

-(N+1) X 1µl primer Back* (10pmol/ µl)

-(N+1) X 2µl RedTaq buffer

-(N+1) X 2µl dNTPs (2mM)

-(N+1) X 0.6µl RedTaq

-(N+1) X 13.4µl H_2O

* All the used primers are described at the end of this chapter.

20µl were dispensed for each sample, the selected colonies were picked with a sterile loop or toothpick, and diluted in 100µl of water, the same toothpick was then immersed in a single reaction tube.

After overlaying the tubes with mineral oil the following cycles were performed:

94°C 5'

94°C 1'⎫
52°C 1'⎬ 30 cycles
72°C 1'⎭

72°C 5'

4°C to ∞.

The RedTaq buffer already contains glycerol, therefore to check the result on gel it is not necessary to use any sample buffer.

EGFP amplification for cloning:

to insert the appropriate restriction sites to the EGFP gene the following PCRs were performed.

- PCR mix:

2.5µl primer For* (10pmol/ µl)

2.5µl primer Back* (10pmol/ µl)

5µl Vent buffer

5µl dNTPs (2mM)

20µl H_2O

-7µl were taken apart for the negative control. The DNA template was provided by touching with a 200µl tip the surface of a mini-prep solution of pEGFP-N1, the same tip was then immersed in the PCR mix.

To perform a hot start reaction the polymerase mix was prepared.

- Vent mix:

0.5µl Vent pol.

14.5µl H_2O

After overlaying the tubes with mineral oil the following cycles were performed:

94°C 5' during this step 3µl of the Vent mix were added to the negative control, 12µl to the main reaction.

94°C 1'
70°C 1' } 26 cycles
72°C 1'

72°C 5'

4°C to ∞.

This reaction was performed for the complete EGFP bait, for the ΔEGFP bait with Vent polymerase and with Pfu polymerase, in this case the last elongation step had to be increased to 10'.

EGFP amplification to screen transformed colonies:

. For N colonies the PCR mix was prepared on ice:

-(N+1) X 1µl primer For (10pmol/ µl)

-(N+1) X 1µl primer Back (10pmol/ µl)

-(N+1) X 2µl RedTaq buffer

-(N+1) X 2µl dNTPs (2mM)

-(N+1) X 0.6µl RedTaq

-(N+1) X 13.4µl H_2O

20µl were dispensed for each sample, the selected colonies were picked with a sterile loop or toothpick, and diluted in 100µl of water, the same toothpick was then immersed in a single reaction tube.

After overlaying the tubes with mineral oil the following cycles were performed:

94°C 5'

94°C 1'
52 or 70°C 1'
72°C 1'
} 30 cycles

72°C 5'

4°C to ∞.

Both the pBTM116 and EGFP primers were used, but it was impossible to use one pBTM116 primer paired with one of the EGFP because of the high difference in the annealing temperature.

PCR primers:

- pVP16 PCR and sequencing primers

VP16 sense:

5'GAG TTT GAG CAG ATG TTT A3'

Primes 39 bases upstream the BamHI site onto pVP16.

VP16 antisense:

5'TGT AAA ACG ACG GCC AGT 3'

Primes 54 bases downstream the BamHI site onto pVP16.

The sequencing primers have the same sequence, but present the fluorophore IRD800 attached at the 5' termini.

- pBTM116 PCR and sequencing primers

BTM116 sense:

5'CAG AGC TTC ACC ATT GAA3'

Primes 49 bases upstream the BamHI site onto pBTM116.

BTM116 antisense:

5'GAA ATT CGC CCG GAA TT3'

Primes 19 bases downstream the BamHI site onto pBTM116.

The sequencing primers have the same sequence, but present the fluorophore IRD800 attached at the 5' termini.

- EGFP PCR primers

BaitEGFP sense:

5'CCG GTC **GAA TTC** ATG GTG AGC AAG GGC GAG GAG3'

The EcoRI restriction site is in bold.

BaitEGFP antisense:

5'TGA TCT **GGA TCC** GCG GCC GCT TTA CTT GTA CAG3'

The BamHI restriction site is in bold.

2.2.3 Sequence analysis

All the sequence analysis were performed with the automated LI-COR DNA sequencer 4000L and the Sequitherm EXCEL II Long-Read DNA Sequencing Kit-LC, with 2pmoles of IRD-800 modified oligonucleotide primer, to allow laser detection at 800nm. In general 1 ml of template DNA was necessary for each reaction, while the reactions were executed following the standard protocol provided with the sequencing kit.

2.2.4 Cloning: ligations and transformations

To maximize the overall efficiency all the plasmids used for cloning were treated for 20' with alkaline phosphatase (C.I.P from Promega) and the transformations were executed by electroporation. All the standard reactions were executed as described in Sambrook et al. 1989, ligations were carried over night at 16°C.

2.2.5 Fingerprint of ScFvs

The fingerprint reactions were executed by M. Visintin following the reported protocol.
When fingerprinting N clones, make a restriction enzyme mix containing:
-(N+1) X 17.8 µl water
-(N+1) X 2 µl restriction enzyme buffer (New England Biolabs buffer 2)
-(N+1) X 0.2 µl BstNI
-(N+1) X 0.2 µl BSA (10 µg/ µl)

-Add 20 ml of the mix to each well of a 96 well plate.
-Add l0 ml of PCR mix from different clones to each well.
-Cover the wells with parafilm and put to incubate at 60^0C for 3 hours.
-Add 5 ml 6X gel loading buffer to each well and load on 2% Metaphor/Nusieve TBE gel.
-Run 60V 60-120'.

2.3 Transfection of C6 cells

To produce stabilized C6 clones able to express the ScFvs in the cytoplasm, the following protocol was used.
Solutions:
1)TE: 1mM Tris (pH7.5) 0.1mM EDTA
2)2.5M $CaCl_2$
3)2X HeBS (pH 7.1): 280mM NaCl, 0.5mM Na_2HPO_4, 1mM NaH_2PO_4, 50mM Hepes
4)G418 in medium (50mg/ml) without serum, add 1N NaOH to neutral pH; store in aliquots at -20C for few months. Use 1mg/ml final.

Procedure:
1)Seed 10^6 cells per 100mm Petri dish one day before in 7ml medium (Once good HeBS is found, it can be kept at RT) (X is the number of plates).

2)Dissolve DNA at 40μg/ml in TE (solution 1) in a volume of X/2 (0.5) ml. Use Falcon 2059 or 2063 tubes.

3)Add 0.X/2m1 (0.05) ml of 2.5M $CaCI_2$ (250mM final), dropwise, mixing between drops DNA may precipitate.

4)Add DNA/$CaCl_2$ solution, dropwise to equal volume of sterile 2xHeBS (X+X/2 ml 0.55m1). Best done by running DNAICaCI2 down pipette. Precipitate should be floccular or filamentous and usually forms immediately. Should be left to mature over 45mm.

5)After 45mm, gently mixing by pipetting, add 1ml to each plate (to 7ml of medium). Rock then gently.

6)After one day pour off the medium and start selection with G418. (DMEM+10% FCS; never overgrow the cells).

Perform the first selection with 1mg/ml G418 final. Change medium every three days.

2.4 Immunofluorescence

This method was used to detect antibody fragments within cells.

1. Start with $5x10^4$ cells on polylysine-coated glass coverslips placed at the bottom of a petri dish and transfected according to the previous protocol.
2. Wash three times with PBS.
3. Fix the cells with 3,7 % paraformaldheide in PBS for 10' at room temperature (1 ml).
4. Wash once with PBS.
5. Permeabilize the cells with Tris-HCl 0.1 M pH 7.6/0.2% Triton X100 (1ml) for 4' at room temperature.
6. Wash three times with PBS.
7. Incubate with 3% BSA in PBS for 10' (1ml).
8. Wash once with PBS
9. Add appropriate volume of primary antibody 1:30 9E10 (Chan et al. 1987) in PBS 3%BSA (Incubate at RT for at least 1h. Incubations should be done in a moist

chamber (an airtight box with a piece of damp tissue paper at the bottom) to prevent drying out of the cells.

10 Wash coverslips once with PBS, thrice with PBS/0.05% tween-20, thrice with PBS

11 Add α-mouse Ig-Fict (DAKO) 1:2000 in PBS 3%BSA Incubate at RT for at least 1h.

12 Wash as before

13 Seal with nail polish and wait until it dries up.

2.5 Protein treatment

2.5.1 EGFP expression

For the expression of EGFP the main guidelines were followed from Baird et al. 1999. One bacterial colony was inoculated in 50ml of LB-100μg/ml ampicillin, and left ON at 37°C. From this culture, 4ml were transferred into 400ml of pre-heated LB-100μg/ml and left to grow to an OD_{600} of 0.6 at which time the expression was induced with 1mM isopropyl β-D-thiogalactoside. Bacteria were allowed to express the recombinant protein for 6 hours at room temperature with shaking, and then at 4°C over night standing still.

In the end it was possible to clearly see the green fluorescence in room light.

2.5.2 Preparation of cleared lysate

The protocol followed was modified from the QIA*expressionist* handbook (1997) Preparation of cleared lysates under native conditions

Materials: cell pellet, lysis buffer (50mM NaH_2PO_4, pH 8.0; 300mM NaCl), lysozyme.

1. Thaw the cell pellet for 15' on ice and resuspend cells in 10ml lysis buffer.
2. Add lysozyme to 1 mg/ml and incubate on ice for 3'.

3. Sonicate on ice. Use six 10'' bursts at 200-300W with a 1' cooling period between each burst.
4. (Optional) If the lysate is very viscous, add RNAse A (10pg/ml) and DNAse I (5pg/ml) and incubate on ice for 10-15' (skipped).
5. Centrifuge lysate at 10000 x g for 20-30' at 4°C to pellet the cellular debris. Save supernatant. A certain proportion of the cellular protein, including the 6xHis-tagged protein, may remain insoluble and will be located in the pellet. For more complete recovery of the tagged protein, this material must be solubilized using denaturing conditions as described in Protocol 9 on page 64 before purification under denaturing conditions.*
6. Dialyze at 4°C over night with PBS.

* The recovered pellet appeared to be intense green, the personal impression was that the sonication protocol was not strong enough to lysate completely all the cells. In addition it seemed important to preserve the native folding of EGFP, therefore the pellet was simply resuspended in other 10ml of lysis buffer and the protocol followed once again.

2.5.3 EGFP-His-tag affinity chromatography

Materials: dialyzed cleared lysate, NTA-Ni matrix, Washing buffer 1 (WB1) (50mM NaH_2PO_4, pH 8.0; 250mM NaCl), Washing buffer 2 (WB2) (50mM NaH_2PO_4, pH 8.0; 500mM NaCl), Elution buffer 1 (EB1) (WB2, 15mM imidazole), Elution buffer 2 (EB2) (WB2, 100mM imidazole).

1. Wash 1ml of NTA-Ni matrix in a 15ml falcon with 10ml of PBS, centrifuge at 800rpm 5' and discard supernatant. Repeat twice.
2. Add to the dialyzed NaCl to 300mM. Transfer in a 50ml falcon the lysate and add the washed matrix. Leave at 4°C for 2h with gentle shaking.
3. Spin down the resin as before, keep supernatant and resuspend matrix in 40ml of WB1.
4. Spin down the resin once again, keep supernatant and transfer matrix in the chromatography column, use WB1 to ensure good column packing.

5. Wash with at least 30 ml of WB1, proceed when the OD of the flow through is stabilized.
6. Wash as before with WB2.
7. Elute aspecific binders with at least 15ml of EL1, proceed only when the OD of the flow through is stabilized.
8. Elute bound protein with 10ml of EL2, collect fractions, follow elution with the aid of the chart recorder.
9. Dialyze at 4°C over night with PBS.

All the steps could be easily followed thanks to the intense fluorescence of EGFP, only the visibly green eluted fractions were kept and dialyzed. Two separate columns were used, one with the first cleared lysate, the second with the lysate obtained from the resuspension of the first debris pellet.

2.5.4 Evaluation of the protein concentration

The protein concentration of the two eluted and dialyzed solutions was estimated with a standard Lowry assay, as well as with an SDS-PAGE.

To produce the calibration curves for the Lowry assay the following BSA dilutions in PBS were used: 2μg/μl, 1.5μg/μl, 1μg/μl, 0.5μg/μl, 0.2μg/μl. as well as pure PBS. To calculate the best fit only the values between 0 and 1μg/μl were kept, because higher values seemed to fit badly and the measured value for the EGFP samples were much lower.

An SDS-PAGE was made to confirm the Lowry results, and to verify that the protein purity was good.

2.5.5 EGFP coupling with BSA

The coupling reaction was setup following the Harlow and Lane suggestions (1988) for coupling of synthetic peptides, since in our case the coupling involves a whole protein some changes were done.

For single-step coupling:

1. Prepare an equimolar solution EGFP and BSA in PBS (0.5 mg of EGFP were used and the volume adjusted to 2ml). Add a stir bar to the solution and place on a magnetic stirrer in the fume hood.
2. Prepare a solution of 0.2% glutaraldehyde in PBS. Slowly, add an equal volume of glutaraldehyde to the peptide/protein solution with constant agitation. One milliliter of a 0.2% solution is approximately 20 $_1$ttmoles.
3. Incubate at room temperature for 1 hr.
4. Add glycine from a 1 M stock in PBS (check pH and readjust to 7.2, if necessary) to a final concentration of 200mM. Incubate with stirring for 1 hr.
5. Separate the peptide-protein conjugate from glutaraldehyde and glycine by dialysis against PBS.
6. Wash and concentrate the dialyzed solution with a Centricon column.

The final concentration was around 0.5mg/ml, suitable for intraperitoneal immunization for mice.

2.5.6 Western blot (WB)

To verify the expression of the bait construct crude yeast extracts were made in the following way:

1. Inoculate cells in 5ml of appropriate medium, leave growing at 30°C-230RPM for approximately 18h
2. When the OD_{600} reaches 1.2 transfer enough cells to OD_{600}=0.15 in 5 ml of pre-warmed media.
3. Grow at 30°C to OD_{600}=0.5-0.7
4. Pellet 1.5ml of cells in a tabletop centrifuge at maximum speed for 1'.
5. Discard supernatant and resuspend pellet in 50ml of 2X Laemmli sample buffer.*
6. Vortex and place immediately on dry ice.
7. Boil samples for 5', spin like before. The supernatant is ready to be loaded on the gel.

*10ml of 2X Laemmli buffer are made with

- 6ml of SDS 10%
- 2ml glycerol

- 1ml β-mercaptoethanol
- 30µl TRIS-HCl 1M pH 6.8
- 2mg bromophenol-blue
- H_2O enough to 10ml

The gel was at 8% acrilamide, the primary antibody α-LexA from Invitrogen diluted 1:5000, the secondary α-rabbit Ig-HRP (DAKO) at 1:2000.

The C6 cells western blot was done with whole cell lysates prepared as follows: cells were grown on 90mm petri dishes.

1. Place plates on ice, wash three times with PBS
2. Cover cells with 1ml of RIPA-SDS buffer-Complete EDTA free (from Roche)
3. Scrap cells from plate, collect buffer and leave 45' at 4° with gentle shaking
4. Pellet cells at maximum speed 4°C for 3-5'.
5. Transfer supernatant to a new tube.
6. Add 50ml of 2X sample buffer to the pellet, resuspend it by careful sonication while keeping on ice.

Only supernatants were loaded on the gel (12% acrilamide), primary antibody is 9E10 1:50, secondary is α-mouse Ig-HRP (DAKO) at 1:2000.

RIPA buffer:

20mM Tris-HCl pH 7.8

150mM NaCl

1mM EGTA

0.1% SDS

1% Triton-X100

0.5% Sodium-deoxycholate.

2.6 Yeast methods

2.6.1 Yeast strain and media

The yeast strain used is *Saccharomyces cervisiae* L40, its genotype is: Mata his3Δ200 trp1-901 leu2-3,112 ade2 LYS2::(lexAop)$_4$-HIS3 URA3::(lexAop)$_8$-LacZ GAL4.

The expression of the HIS3 and LacZ genes are respectively driven by minimal HIS3 and GAL1 promoters, fused to multimerized LexA binding sites. The expression of HIS3 enables yeast to grow in the absence of histidine, while the expression of LacZ can be detected with the color assay using 5-bromo-4chloro-3indolyl-β-D-galactoside (X-gal).

YC medium contains the following components per liter: 1.2g yeast nitrogen base, without amino acids and ammonium sulfate (Difco), 5 g ammonium sulfate, 10g succinic acid; 6 g NaOH, 0.75g amino acid mix lacking tryptophan (W), histidine (H), uracil (U), leucine (L), and lysine (K); 2% glucose and, as required, 20g agar (Difco), 0.1g W, 0.05g H, 0.1g U, 0.1g L, 0.1g K. Amino Acid Mix lacking WHULK is composed of the following: 1 g each of adenine sulfate, arginine, cysteine, threonine; and, 0.5 g each of aspartic acid, isoleucine, methionine, phenylalanine, proline, serine, and tyrosine.

The different selective media as YC-WHULK or YC-WL are composed in the same way, but without the amminoacids indicated in the name.

YPAD medium contains 10g yeast extract, 20g bacto-peptone, 0.1g adenine, 2% glucose, and 20g agar, as required, per liter. YFA medium is YPAD without glucose.

2.6.2 LiAc transformation (small scale)

This protocol is a modification of methods published by Clontech laboratories (Gietz et al. 1992, Hill et al. 1991, Schiestl and Gietz 1989).

Materials: 10X LiAc buffer (1M LiAc, pH *7.5* adjusted with dilute glacial acetic acid filter-sterilized), 50% (w/v) filter-sterilized PEG 4000 (store the solution in a tightly sealed glass bottle to avoid evaporation), 10X TE buffer (100mM Tris, 10mM EDTA, pH7.5, filter-sterilized), 100% DMSO, 10mg/ml denatured herring testes carrier DNA (Clontech).

Procedure:

Day 1: Inoculate few colonies of L40 in 50 ml of YPD and incubate for 16-18 hr with shaking at *250* rpm at 30^0C to place the culture at mid log phase the next day ($0D_{600}$> 1.5).

Note: use only glass flasks carefully washed with ultrapure, pyrogen-free water and sterilized by autoclaving 15 mm at 121^0C.

Day 2:

1. Dilute the overnight culture to OD_{600} 0.2-0.3 in 300 ml of YPD pre-warmed to 30°C.Grow at 30°C for 3 hours with shaking (230 rpm)
2. Pellet the cells by centrifugation (1000 X g for 5') at room temperature, discard the supernatant and resuspend the pellet in 50ml of H_20.
3. Centrifuge the cells again as in 2., decant the supernatant.
4. Resuspend the pellet in 1.5 ml of freshly prepared 1X TE/LiAc (10mM TE, 0.1M LiAc).
5. For each transformation prepare in a tube a mixture of: 0.1µg bait construct, 0.1µg pVP16 vector construct (if you need to test specific antigen-antibody partners) 0.1µg denatured herring testes carrier DNA. Add 100 µl of the resuspended cells to each transformation tube.
6. Add 0.6 ml of a sterile PEG/LiAc solution (0.1M LiAc, 10mM TE, PEG 4000 40%) to the tube and vortex to mix.
7. Incubate 30 minutes at 30^0C with shaking (230 rpm).
8. Add 70 µl of DMSO, mix gently by inversion and heat shock for 15 mm in a 42°C water bath.
9. Chill cells on ice.
10. Pellet cells by centrifugation (20'' at maximum speed)
11. Remove supernatant and resuspend cells in 0.5ml of TE. Spread 100 µl for single transformation or 250 µl for co-transformations on appropriate selective media plates.

2.6.3 LiAc transformation (maxi scale)

This protocol is a modification of methods published by Clontech laboratories (Gietz et al., 1992, Hill et al., 1991, Schiestl and Gietz, 1989).

1. Grow a 5ml overnight of the L40 yeast strain containing the pBTM116 bait plasmid in YC-WU.
2. Inoculate 100ml of YC-WU with an aliquot of the overnight culture. The goal is to find a dilution that places the 100ml culture at mid log phase the next day ($0D_{600}$ not greater than 4).
3. Dilute the culture from (2) to $0D_{600}$ of 0.3 in 1 liter of YPAD, pre-warmed to 30°G. Grow at 30°C for 3 hours.
4. Pellet cells by centrifugation at room temperature (1500 g, 5').
5. Wash pellet in 500ml of 1X TE (10mM Tris, 1mM EDTA, pH 7.5): resuspend pellet in TE, then centrifuge. Resuspend pellet in 20ml 1X LiAc, 0.5X TE (made from 10X filter sterile stocks; 10X LiAc: 1M LiAc, pH 7.5, adjusted with diluted glacial acetic acid; 10X TE: 100mM Tris, 10mM EDTA, pH 7.5). Transfer to a sterile 1-liter glass flask or beaker.
6. Add a mixture of 500µg library plasmid DNA and 1.0ml of 10mg/ml denatured salmon sperm DNA. The library DNA can be prepared with Qiagen columns or by cesium chloride gradients.
7. Add 140ml of 1X LiAc, 40% PEG 3350, 1X TB. Mix and incubate 30 minutes at 30°C, with gentle shaking.
8. Add 17.6ml DMSO. Swirl to mix. Heat shock in a 42°C water bath for 6 minutes, with occasional mixing. Immediately dilute with 400ml YPA and rapidly cool to room temperature in a water bath.
9. Pellet cells at room temperature. Wash with 500ml YPA. Resuspend pellet in 1.0l pre-warmed YPAD. Incubate at 30°C for one hour, with gentle shaking.
10. Pellet cells from 1 ml. Resuspend in 1 ml YC-UWL. Plate 10 and 1µl aliquots (10^{-5} and 10^{-6} of total) on YC-UWL to measure the primary transformation efficiency. This protocol should yield 10 to 100 million transformants.
11. Pellet cells from the remaining 99ml. Wash pellet in 500ml YC-UWL. Resupend in 1l of pre-warmed YC-UWL. Incubate, with shaking, at 30°C for 16 hours.
12. Pellet cells. Wash twice with 1l YC-WHULK. Resuspend final pellet in 10ml YC-WHULK.
13. Plate 10^{-5} and 10^{-6} of total on YC-UWL plates to compare to the number of primary transformants from (10). This allows a calculation of the number of doublings and

the number of His positive colonies that should be screened to roughly cover the number of primary transformants.

14. Plate aliquots on YC-WHULK plates and incubate at 30°C for 2 to 3 days. Because His positive colonies grow poorly if the density on the plates is too high, the optimal plating volume is dependent on the recovery time in YC-UWL and on the efficiency of the transformation. With a 16 hour recovery, plate 10 plates of 5µl, 10µl, 25 µl, and 50 µl. Save the remainder of the 10ml at 4°C as a backup in the event the dilutions are inappropriate; the yeast are stable for at least one week when stored in this fashion.
15. Transfer His positive colonies on two YC-WHULK plates: a master plate and a duplicate plate for β-galactosidase filter assay; arrange the colonies in a grid like pattern.

2.6.4 β-galactosidase filter assay

The following protocol is a modification of the method described by Breeden and Nasmyth (1985)

1. Transfer yeast colonies or patches to a dry nitrocellulose filter circle (Schleicher and Schuell BA85 or BA-S85).
2. Lift filter and place colony side up on a pre-cooled aluminum boat floating upon a sea of liquid nitrogen. After 20 seconds, immerse boat and filter for 5 seconds. Remove filter from boat (the filters are very fragile) and place at room temperature, colony side up, until thawed.
3. In the lid of a petri dish, place 3 ml of Z buffer (60mM Na_2HPO_4, 40mM NaH_2PO_4, 10mM KCl, 1mM $MgSO_4$, pH 7.0) containing 30µl of 50µg/µl X-gal in N,N-dimethyl-formamide. Lay a Whatman filter circle #1 in the Z buffer, followed by the nitrocellulose filter, colony side up, avoiding air bubbles at each step. Cover with the bottom of the petri dish. Incubate at 30°C 45' to 5h. Monitor the color development over time.

2.6.5 Plasmid segregation

To recover the selected genes it is necessary to segregate the bait against the pray plasmid in the following way.

1. Inoculate 5ml of YC-L with each selected clones, grow two days at 30°C.
2. Plate on YC-L enough cells in order to achieve a density of 100-200 cells per plate (approximately 100µl of a 1:1000 dilution of the two days cultures). Incubate plates two days at 30°C.
3. Replica plate the YC-L plates from (2) first to YC-WL and then to YC-L plates. Incubate two days at 30°C. Identify colonies that require W for growth, i.e. colonies that grow on YC-L but not on C-WL, these colonies have segregated the bait plasmid.

2.7 Oligo sets for library cloning

The general cloning strategy is based on the PCR assembly approach and it is discussed and described in Chap. 4.7. The oligo and linker sequences are based on the ICGEB-EMBO-SISSA course on "Selecting from phage display libraries" (1996).

The linker 220 sequence is:

```
 S   G   G   S   T   S   G   S   G   K   P   G   S   G   E
TCC GGA GGG TCG ACC AGC GGT TCT GGG AAA CCA GGT TCC GGT GAA
AGG CCT CCC AGC TGG TCG CCA AGA CCC TTT GGT CCA AGG CCA CTT

 G   S   S   G   S
GGC TCG AGC GGT ACC
CCG AGC TCG CCA TGG
```

In the following there is the list of all the primers needed for the library construction.

VHback:	Name	deg.	µl
TCC GGT GAA GGC TCG AGC GAK GTR MAG CTT CAG GAG TC	HB1	8	4
TCC GGT GAA GGC TCG AGC GAG GTB GAG GTB CAG CAG TC	HB2	9	4
TCC GGT GAA GGC TCG AGC CAG GTG CAG CTG AAG SAS TC	HB3	4	3
TCC GGT GAA GGC TCG AGC GAG GTC CAR CTG CAA CAR TC	HB4	4	4
TCC GGT GAA GGC TCG AGC CAG GTY GAG GTB CAG CAR TC	HB5	12	7

TCC GGT GAA GGC TCG AGC CAG GTY CAR CTG CAG CAG TC	HB6	4	2
TCC GGT GAA GGC TCG AGC CAG GTC CAC GTG AAG CAG	HB7	1	1
TCC GGT GAA GGC TCG AGC GAG GTG AAS STG GTG GAA T	HB8	4	2
TCC GGT GAA GGC TCG AGC GAV GTG AWG YTG GTG GAG TC	HB9	12	5
TCC GGT GAA GGC TCG AGC GAG GTG CAG SKG GTG GAG TC	HB10	4	2
TCC GGT GAA GGC TCG AGC GAK GTG CAM CTG GTG GAG TC	HB11	4	2
TCC GGT GAA GGC TCG AGC GAG GTG AAG CTG ATG GAR TC	HB12	2	2
TCC GGT GAA GGC TCG AGC GAG GTG CAR CTT GTT GAG TC	HB13	2	1
TCC GGT GAA GGC TCG AGC GAR GTR AAG CTT CTC GAG TC	HB14	4	2
TCC GGT GAA GGC TCG AGC GAA GTG AAR STT GAG GAG TC	HB15	4	2
TCC GGT GAA GGC TCG AGC CAG GTT ACT CTR AAA GWG TST G	HB16TG	8	5
TCC GGT GAA GGC TCG AGC CAG GTC CAA CTV CAG.CAR	HB17	6	3.5
TCC GGT GAA GGC TCG AGC GAT GTG AAC TTG GAA GTG TC	HB18	1	0.7
TCC GGT GAA GGC TCG AGC GAG GTG AAG GTC ATC GAG TC	HB19	1	0.7

A mixture of the above primers in the ratios given comprises the VHback mix. The underlined sequence is the overlap region with the linker DNA.

VHfor:	Name	deg.	µl
TGA GGA GAC GGT GAC CGT GGT	HF1	1	1
TGA GGA GAC TGT GAG AGT GGT	HF2	1	1
TGC AGA GAC AGT GAC CAG GGT	HF3	1	1
TGA GGA GAC GGT GAC TGA GGT	HF4	1	1

A mixture of the above primers in the ratios given comprises the VHfor mix

VLback:	Name	deg.	µl
GA YAT CCA GCT GAC TCA GCC	LB1	2	1
GA YAT TGT TCT CWC CCA GTC	LB2	4	2
GA YAT TKT GMT VAC TCA GTC	LB3	8	8
GA YAT TGT GYT RAC ACA GTC	LB4	8	3.5
GA YAT TGT RAT GAC MCA GTC	LB5	8	4
GA YAT TMA GAT RAM CCA GTC	LB6	16	7
GA YAT TCA GAT GAY DCA GTC	LB7	12	6
GA YAT YCA GAT GAC ACA GA	LB8	4	1.5
GA YAT TGT TCT CAW CCA GTC	LB9	4	2
GA YAT TGW GCT SAC CCA ATC	LB10	8	3.5
GA YAT TST RAT GAC CCA RTC	LB11	16	8
GA YRT TKT GAT GAC CCA RAC	LB12	16	3
GA YAT TGT GAT GAC BCA GKC	LB13	12	6
GA YAT TGT GAT AAC YCA GGA	LB14	4	2
GA YAT TGT GAT GAC CCA GWT	LB15	4	2
GA YAT TGT GAT GAC ACA ACC	LB16	2	1
GA CAG GCT GTT GTG ACT CAG GAA TC	LBL	1	1

A mixture of the above primers in the ratios given comprises the VLback mix.

VLfor:	Name	deg.	µl

GGT ACC GCT CGA GCC TTC ACC GGA CGT TTK ATT TCC AGC TTG G	LF1/2	2	2
GGT ACC GCT CGA GCC TTC ACC GGA CGT TTT ATT TCC AAC TTT G	LF4	1	1
GGT ACC GCT CGA GCC TTC ACC GGA CGT TTC AGC TCC AGC TTG G	LF5	1	1
GGT ACC GCT CGA GCC TTC ACC GGA CCT AGG ACA GTC AGT TTG G	LFL	1	0.25

A mixture of the above primers in the ratios given comprises the VLfor mix. The underlined sequence is the overlap region with the linker DNA.

PullFor:	Name	deg.	µl
TCA CCT GAT **AGC GGC CGC** TGA GGA GAC GGT GAC CGT GGT	HF1	1	1
TCA CCT GAT **AGC GGC CGC** TGA GGA GAC TGT GAG AGT GGT	HF2	1	1
TCA CCT GAT **AGC GGC CGC** TGC AGA GAC AGT GAC CAG GGT	HF3	1	1
TCA CCT GAT **AGC GGC CGC** TGA GGA GAC GGT GAC TGA GGT	HF4	1	1

A mixture of the above primers in the ratios given comprises the pullFor mix. The underlined sequence is the overlap part with the 3' region of VH DNA. In bold there is the NotI restriction site.

PullBack:	Name	deg.	µl
AA TGG ACT ATG **GCC CAG CCG GCC** GAY ATC CAG CTG ACT CAG CC	LB1	2	1
AA TGG ACT ATG **GCC CAG CCG GCC** GAY ATT GTT CTC WCC CAG TC	LB2	4	2
AA TGG ACT ATG **GCC CAG CCG GCC** GAY ATT KTG MTV ACT CAG TC	LB3	8	8
AA TGG ACT ATG **GCC CAG CCG GCC** GAY ATT GTG YTR ACA CAG TC	LB4	8	3.5
AA TGG ACT ATG **GCC CAG CCG GCC** GAY ATT GTR ATG ACM CAG TC	LB5	8	4
AA TGG ACT ATG **GCC CAG CCG GCC** GAY ATT MAG ATR AMC CAG TC	LB6	16	7
AA TGG ACT ATG **GCC CAG CCG GCC** GAY ATT CAG ATG AYD CAG TC	LB7	12	6
AA TGG ACT ATG **GCC CAG CCG GCC** GAY ATY CAG ATG ACA CAG A	LB8	4	1.5
AA TGG ACT ATG **GCC CAG CCG GCC** GAY ATT GTT CTC AWC CAG TC	LB9	4	2
AA TGG ACT ATG **GCC CAG CCG GCC** GAY ATT GWG CTS ACC CAA TC	LB10	8	3.5
AA TGG ACT ATG **GCC CAG CCG GCC** GAY ATT STR ATG ACC CAR TC	LB11	16	8
AA TGG ACT ATG **GCC CAG CCG GCC** GAY RTT KTG ATG ACC CAR AC	LB12	16	3
AA TGG ACT ATG **GCC CAG CCG GCC** GAY ATT GTG ATG ACB CAG KC	LB13	12	6
AA TGG ACT ATG **GCC CAG CCG GCC** GAY ATT GTG ATA ACY CAG GA	LB14	4	2
AA TGG ACT ATG **GCC CAG CCG GCC** GAY ATT GTG ATG ACC CAG WT	LB15	4	2
AA TGG ACT ATG **GCC CAG CCG GCC** GAY ATT GTG ATG ACA CAA CC	LB16	2	1
AA TGG ACT ATG **GCC CAG CCG GCC** GAC AGG CTG TTG TGA CTC AGG AAT C	LBL	1	1

A mixture of the above primers in the ratios given comprises the pullBack mix. The underlined sequence is the overlap part with the 5' region of VL DNA. In bold there is the SfiI restriction site.

3 RESULTS

3.1 Construction of an EGFP bait

Every two hybrid experiment starts with the construction and characterization of the "bait" fusion protein, i.e. the selected antigen in this work. The gene used was the enhanced green fluorescence protein (EGFP) from the pEGFP-N1 plasmid distributed commercially by Clontech. To insert the appropriate restriction sites, necessary for the sub-cloning into pBTM116, a pair of PCR primers was designed, containing the restriction sites for BamHI and EcoRI in the correct frame. The PCR was performed with Vent DNA polymerase (see Chap2.2.2). The DNA obtained was gel purified, digested and ligated to the prepared plasmid. The DNA from ten clones was purified, and tested for the insertion of the EGFP gene by PCR and fingerprinting. All of them resulted positive, therefore three of them were sequenced using the BTM116 and EGFP primers *sense* and *antisense* (see Chap.2.2.2). The sequences showed no mutations from the published sequence of EGFP, therefore the assays in yeast were performed.

To verify the ability to activate the transcription of the reporter genes the following transfections were performed (see Chap. 2.6.2):

- BTM116-EGFP alone, plated onto YC-W and YC-WHUK plates.
- BTM116-EGFP cotransformed with VP16, plated onto YC-WL and YC-WHULK plates.

A bait should not be able to grow in absence of histidine (His) when expressed alone, or in presence of native VP16. Unfortunately this is not always the case, even the DNA binding site of LexA, when expressed alone, is able to promote a low expression of the reporter genes, permitting the cells to survive in the absence of His, and resulting mild positive also to the β-gal assay. In the case of LexA-EGFP the situation was similar, the transfected clones were able to survive on YC-WHUK and to result positive for β-gal

activity. In the presence of VP16 the false positive clones were also present, even if they showed a decreased ability to grow on selective media (Fig.3.1).

Even if the activation in presence of VP16 was very low, we decided that this bait was not suitable for a library screening. To decrease the aspecific activation two methods are found in literature, the first consists in simply adding 3-aminotriazole (3-AT) to the selective plates; the second is to try different deletion constructs of the bait, it is said that in some cases even the deletion of a few amminoacids resulted in the disappearance of the aspecific activity.

Both the solutions offer different advantages and disadvantages: even in the presence 3-AT the numbers of false positives seem to remain higher than when using an ideal bait, and anyway its concentration must be carefully adjusted to minimum levels; the deletion method on the contrary does not give any guarantee, in the sense that every construct may or may not activate on its own in an unpredictable way.

We knew from literature data (Siegel and Isacoff, 1997), and from our own experience that the deletion of the last C-terminal eight amminoacids of EGFP is possible without disrupting its folding and its fluorescence activity; thus we decided to try this deletion to construct a new bait protein (LexA-ΔEGFP). The rationale being that if by chance this bait would behave properly no other setup steps would be needed.

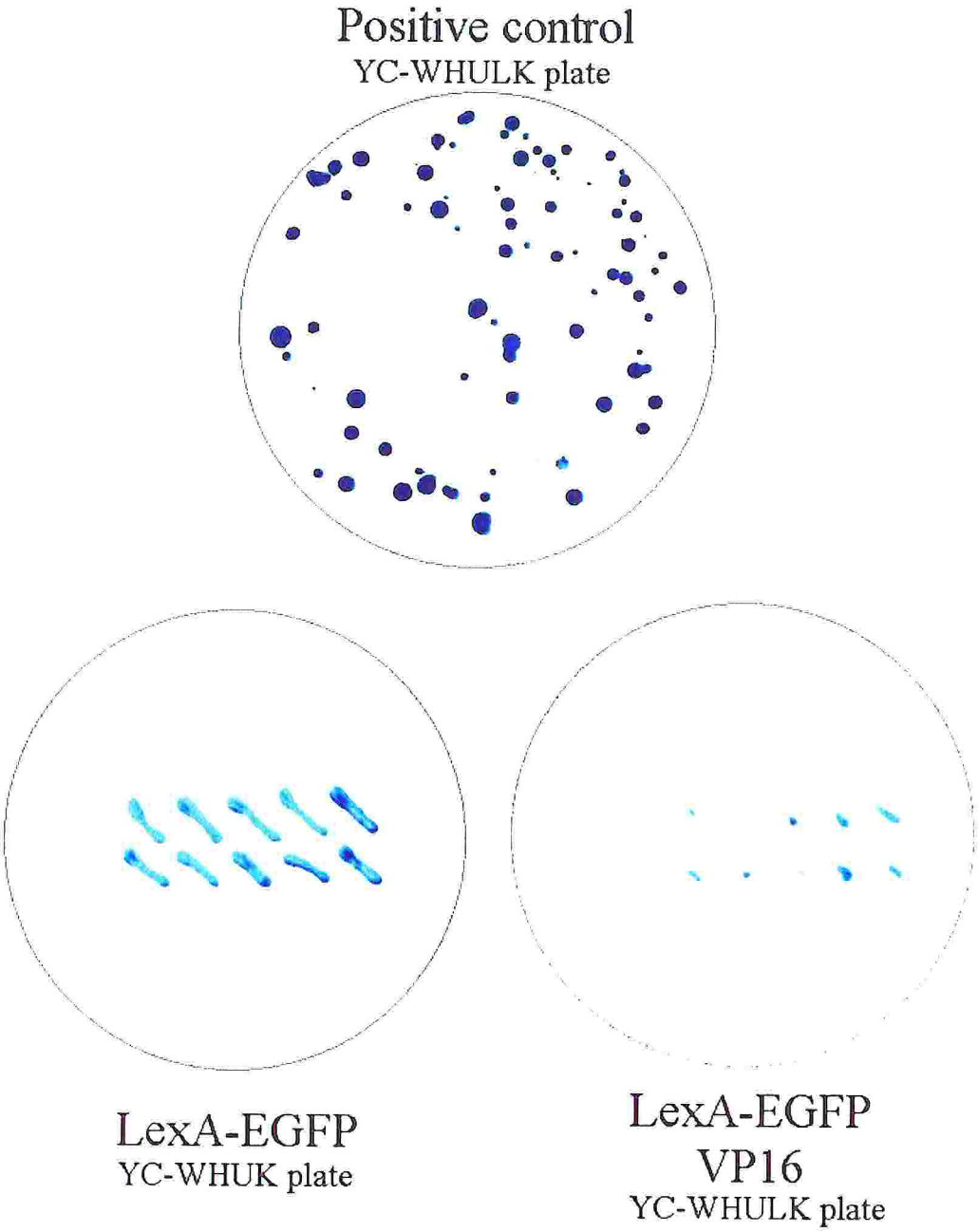

Fig. 3.1 β-galactosidase filter assay of LexA-EGFP.
The first filter was obtained with pVP16-ScFv F8 and the pBTM11-p41 (a coat protein of the AMCV) used as positive control. In the second the β-gal activity of the bait alone was assayed, in the third the bait cotransformed with VP16. Note that the color saturation of the picture was set at maximum levels, in order to optimize the visibility of all the colonies.

3.1.1 The LexA-ΔEGFP bait

A new antisense primer was studied in order to allow the cloning into pBTM116 of the selected portion of EGFP in frame with LexA (see Chap. 2.2.2). Again the PCR reaction was performed with Vent polymerase, the same steps for the cloning followed as before, and the DNA from ten positive clones sequenced. All these clones presented a one base deletion in the first part of the EGFP gene, resulting in a frame shift of nearly all the gene. To minimize the probability to introduce mutations during the PCR, a new reaction was performed with Pfu polymerase utilizing an adapted cycling protocol (see Chap. 2.2.2). This time no mutations were detected with the sequence analysis of five positive clones. One of them was chosen for the activity assays on yeast (Fig. 3.2); to verify that the fusion protein was expressed a Western blot with α-LexA antibody was performed (Fig 3.3).

Fig 3.2 β-gal assay for LexA-ΔEGFP.
The cells were grown on YC-W and YC-WL because no colonies could grow in absence of His. The picture is once more at the highest possible levels of color saturation, to allow the appearance of the uncolored colonies. The same positive control (not shown) as before was used.

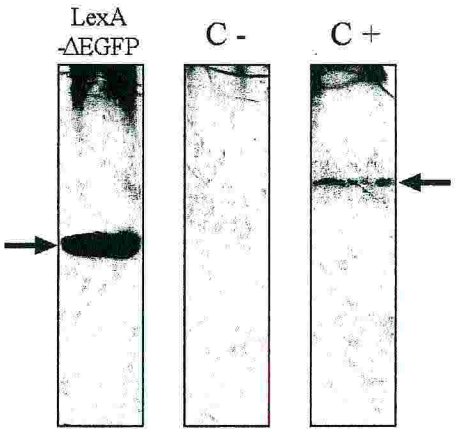

Fig.3.3 Western blot with α-LexA primary antibody.
In the first line crude extract of L40 transformed with pBTM116-LexA-ΔEGFP, the second is L40 untransfected, the third with pBTM116-Lamin

Luckily no clones were able to survive on selective media. Also the β-gal assay gave clearly negative results, both with LexA-ΔEGFP and LexA-ΔEGFP with VP16 (Fig. 3.2). This deletion construct of LexA-ΔEGFP was considered suitable for the foreseen selections.

3.2 Expression and purification of EGFP

Before performing the selection in yeast, two things were needed: to produce a ScFv library from hyper-immunized mice, and to perform at least one panning round of a phage display ScFv library, thus a consistent amount of purified EGFP was needed.

A common system for protein expression and purification in *E. coli* is based on the His tag affinity purification, the gene of EGFP inserted into the pQE31, an expression vector commercialized by Qiagen was kindly provided by Dot. K. Ainger. This vector permits the IPTG regulated expression of the inserted gene fused with a short His tag. The protocol for the expression followed the lines of a method previously developed to optimize GFP expression and folding in *E. coli* (Baird et al., 1999), the purification procedure is described in Chap. 2.5.

BSA µg	0	0	1	1	2.5	2.5	5	5
	0.077	0.078	0.108	0.110	0.168	0.166	0.242	0.284
EGFP 5µl	0.110	0.123						

Tab. 3.1 Lowry assay.
The absorbance values for the BSA standards, and for the EGFP purified from the first column are reported.

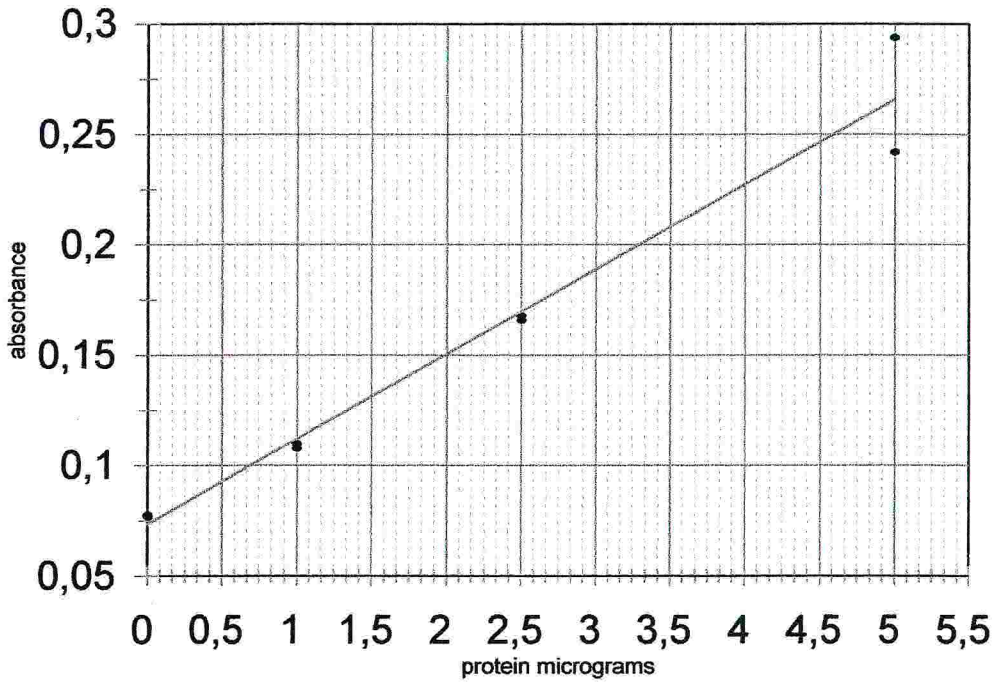

Fig. 3.4 best fit of Lowry assay.
The values of the BSA standards are plotted, the best fit was calculated with QuattroPro8. The calculated equation is Y (Abs)=0,07362 + X (µg) x0,03847. The mean absorbance of the EGFP from the first column is 0.1165, this means the concentration of the initial sample is 0,222 µg/µl. The elution volume was of ~3.2 ml for a total of ~0.7mg of purified protein. Similar calculations permitted to evaluate the amount of protein from the second column as ~1mg.

Two column purifications were performed (see Chap. 2.5.3), the first from the lysate, the second after a subsequent resuspension and sonication of the pellet. The final yield of the purification was estimated via SDS-PAGE as well as a Lowry assay (Tab. 3.1, Fig. 3.4); the first purification gave 0.7 mg of purified protein, the second 1 mg, for a total of 1.7mg.

To immunize the mice two approaches were chosen: since the GFP structure is known to be very compact, it may be useful to conjugate it to a carrier protein, anyway the conjugation may as well mask some antigens and create some new irrelevant, for this reason I decided to immunize some mice with native EGFP, and some with EGFP coupled with BSA.

Of the total amount of purified protein, 0.7mg were destined for the phage display, the remaining 1mg was divided in two parts of 0.5mg, one was coupled with BSA as described in Chap. 2.5.5. A total of four mice are at present time in the immunization process, two with free EGFP, two with the conjugated form.

3.3 The yeast screen of the phage derived library

To verify if it was possible to increase the number of different ScFv obtainable with the two hybrid screening, a new selection was performed starting from the library rescued after one cycle of phage panning.

With the previous selection it was possible to identify 3 different ScFvs able to bind the 151-421 Tau fragment, the input library for the yeast experiments was obtained after two cycles of phage display. We decided to try to perform a new two hybrid experiment, the only difference being to start form the library rescued from the first cycle of panning. The work described in this section was done in collaboration with M. Visintin.

The genes of the ScFvs recovered from phagmids were cloned into pVP16 with the restriction enzymes SfiI and NotI.

This library was utilized for a maxi scale transformation in yeast as described in Chap. 2.6.3. The efficiency of the PCR was estimated performing a series of bacterial colony PCR amplifications and a digestion of the ScFvs amplified genes with BstNI. The

fingerprint analysis permitted to estimate a diversity around 90%, while the yeast transformed library size was about 2.2 x 10^6. Of all these clones 10^4 survived in absence of His, among them, 90% were positive also to the β-gal assay.

To recover the different ScFv genes it was necessary to segregate the bait plasmid pBTM116 against the pray pVP16 as described in Chap. 2.6.5. After the segregation it is possible to purify the plasmid DNA with the standard Qiagen mini-prep kit, following a modified protocol (Rose et al., 1990). Fifty different colonies were picked for the segregation, the pray plasmids purified and re-transformed in *E coli*. The usual fingerprint analysis were performed, 11 different patterns were identified, among them 3 corresponded to the fingerprints of ScFv 2, 14 and 52 obtained with the previous experiments. Six of the other ScFv were tested once again in the two hybrid (Fig.3.5), four of them resulted real positives (Tab. 3.2); to control the true specificity of the identified ScFv they were tested against the irrelevant bait LexA-lamin (Hollenberg et al., 1995). To discriminate true positives from aspecific binders, the assays summarized in Tab 3.2 were performed:

Bait	Prey	YC medium	HIS3 phenotype	LacZ phenotype
/	VP16-ScFv	-L	/	White
/	VP16-ScFv	-WHUK	No growth	/
pBTM116-Lamin	VP16-ScFv	-WL	/	White
pBTM116-Lamin	VP16-ScFv	-WHULK	No growth	/
LexA-Tau151-422	VP16-ScFv	-WHULK	Growth	Blue

Tab. 3.2 Complete two hybrid assay of selected ScFv.
Only the ScFv that show exactly the characteristics listed above were considered truly positive. These ScFv were called A2, A3, A6 and C12.

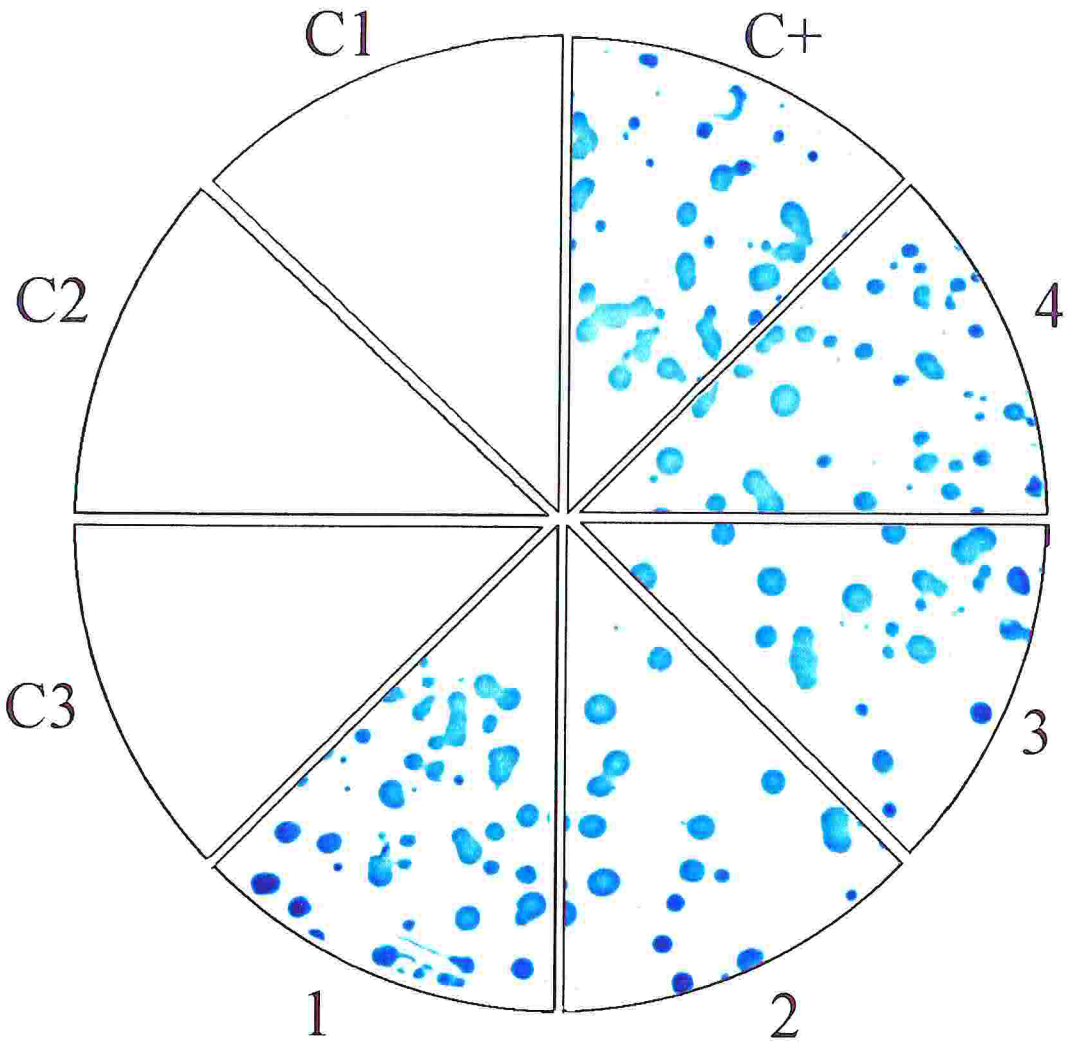

Fig. 3.5 β-gal assay of the selected ScFv.
Panel 1 to 4: the selected ScFv tested again with LexA-Tau151-422; 1) ScFvA2, 2) ScFvA3, 3) ScFvA6, 4) ScFvC12, C+) pVP16-ScFvF8 and pBTM11-p41. C1), C2) and C3) are respectively A2, A3 and A6 cotransformed with pBTM116-Lamin.

The experiment was therefore considered successful, these results seemed to indicate that in this way it is possible to isolate at least two/three times more different ScFvs than with the second panning cycle. More experiments are certainly needed to characterize these new ScFvs, anyway, the results of the previous selection allow a good confidence in saying that these antibodies should represent specific intracellular binders.

3.4 Stable transfection of yeast selected ScFv

One important point that needed to be formally proven to confirm the validity of the two hybrid selection for intracellular antibodies was the possibility to stabilize mammalian cells able to express the selected ScFvs. This step may seem naive, but since the final purpose is to express functional antibodies in the cytoplasm of eukaryotic cells, it was necessary to proof the possibility to do it.

The ScFvs used were ScFv 2, 14, 52 and R4 (Martineau et al., 1998) as a control. They were subcloned into the pScFv-expressCyto vector (Persic et al. 1997), again with the aid of the restriction enzymes SfiI and NotI. The transfection was performed onto C6 cells as described in Chap.2.3, and the selection was carried on in Dulbecco's MEM with 1mg/ml of G418. For each transfection four clones were analyzed by Western blot (Fig. 3.6), the positive ones were controlled also by immunofluorescence (Fig3.7).

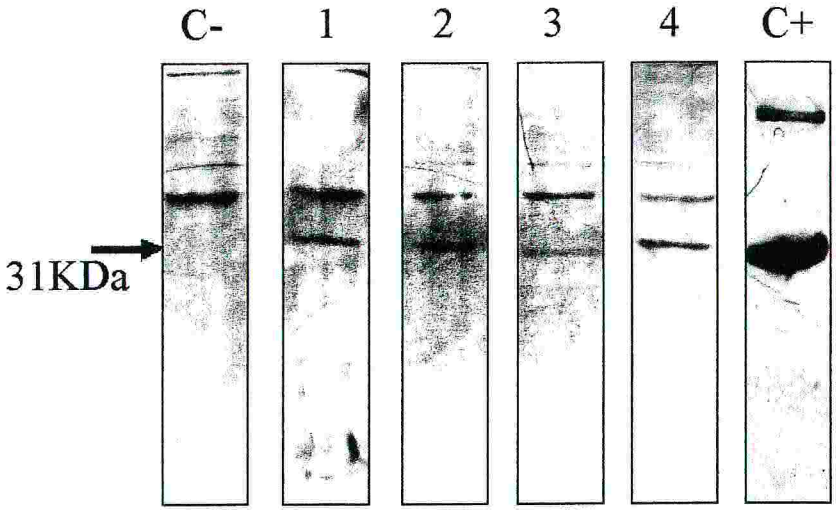

Fig. 3.6 Western blot of stabilized clones.
The primary antibody is monoclonal 9E10, lines C-, 1, 2, 3, 4, crude cell extract of respectively, C6 untransfected, ScFvR4-clone 1, ScFvR4 clone 5, ScFv2 clone 1, ScFv14 clone 3; C+ is the ScFv α-TrkA purified from *E. coli*.

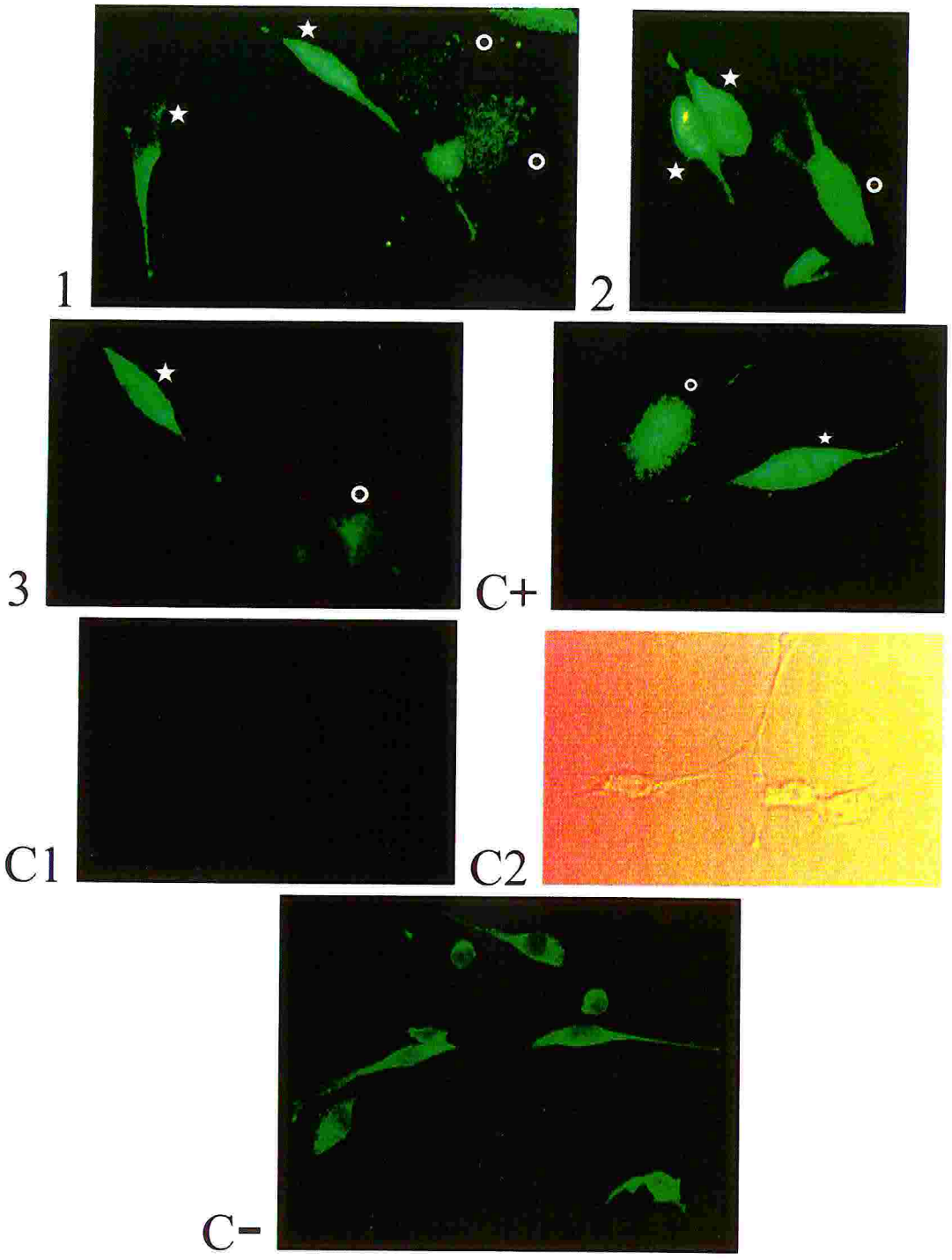

Fig. 3.7 Immunofluorescence of C6 stabilized clones.
The primary antibody used is 9E10. 1) ScFvR4-clone 1. 2) ScFv2-clone 1. 3) ScFv14-clone 3. C+) positive control with α-TrkA. C1) ScFv14-clone 3 with an irrelevant primary antibody. C2) as C1 under visible light. C-) Untransfected C6 cells. All the preparations were made with a mixture of equal amounts of untransfected C6 cells and of cells of the stabilized clones, in order to provide an internal positive control in each preparation. This expedient was necessary to overcome the problem of the high background signal of 9E10. The positive cells are marked with a star, the negative with a circle.

Some major problems were encountered in the immunofluorescence experiments, the high background signal typical of monoclonal 9E10 combined with the low expression levels of the ScFvs forced us to setup an unconventional procedure. In order to distinguish between positive and negative cells it was necessary to compare the fluorescence levels in a completely unbiased way. The only way to do it seemed to have positive and negative cells present in the same frame, in order to appreciate the difference on a single picture, eliminating the intrinsic variability present between different slides and photographs. Thus we decided to mix positive and negative cells in the same preparation. In this way it was possible to discriminate in the same snapshot between positive and negative cells. To validate this observations it was necessary to pay particular attention to the controls, all the clones were tested with an irrelevant primary antibody (α-NGF), to verify that the high background was indeed due to the 9E10 antibody, as well as that the differences seen on the fluorescence were not due to intrinsic differences of the cell populations. The crucial point however was to verify the substantial homogeneity of the negative control of untransfected cells. My observations never contradicted this important premise, unfortunately it is impossible to show pictures of a significant number of cells in this theses, however the image chosen for the negative control contains a number of cells clearly equally fluorescent.

We have therefore demonstrated that stabilized mammalian cell clones can express the ScFv, opening the way to the intracellular immunization experiments, or even to the construction of transgenic animals, once function-neutralizing ScFv fragments would be identified.

4 DISCUSSION

4.1 The EGFP project

Although the current results are clearly preliminary, the work already done is rather promising, and sets the ground for an improvement and refinement of the intrabody trap technology. The work followed three directions: i) exploiting the two-hybrid intrabody trap to isolate intrabodies from the spleen of immunized mice, ii) increasing the diversity of the input antibody pool by reducing the number of pre-selection panning cycles to just one and, finally, iii) producing stable transfected cell clones with the selected anti-Tau scFv fragments, to be used in functional studies to assess the ability of the selected ScFvs to achieve an effective knock-out of the protein activity.

For the hyperimmune spleen approach, the Green Fluorescent Protein (GFP) was chosen as a model antigen, since we argued that isolating a panel of validated anti GFP intrabodies may turn out very useful. Particularly intriguing is the possibility of isolating scFv fragments able to modulate the fluorescence emitted by GFP. Having found a good EGFP bait construct, together with the setup of a reliable protocol for EGFP purification, represents a strong starting point for the foreseen experiments: soon it will be possible to verify if the immunization of the mice has been effective, and if this will not be the case, more protein can be easily purified, and different coupling procedures can be utilized for more immunizations. In the meanwhile the phage display selection cycles can be performed, followed by the yeast selection.

The choice of EGFP has the advantage that its activity can be easily measured via instruments such as a fluorimeter, and maybe also a fluorescence-activated cell sorter or a confocal microscope. We are planning to setup a system to verify the direct fluorescence of yeast cells with a fluorimeter; this would certainly require more work to prepare reliable protocols and controls, but at the present time all the necessary tools (expression plasmids, purified protein and others) are available. The phage display based approach should by itself provide an acceptable number of intracellular antibodies for the fluorescence tests.

The particular structure of the GFP may be the cause of some problems, towards the planned goal, since the protein is extremely compact and the fluorophore is hidden deep inside its structure. This could result in a limited immunogenic strength, as well as in a fluorescence activity particularly insensible to external factors, such as binding antibodies. To overcome the first possible problem, the conjugation to BSA should result appropriate, and the coupling conditions may be adjusted after the analysis of the first immunized sera. The second problem may not arise at all, due to the particular folding properties of EGFP. In fact, this protein is well known for the slowness of its post-translational folding and maturation, as well as for the low temperature dependence of the process. In the experimental conditions of the yeast two-hybrid, the antigen and the antibodies are expressed simultaneously: we think that there is the possibility that the interaction between antigen and antibody may occur during the EGFP folding process, thus inhibiting or slowing down the formation of mature and fluorescent protein. This should allow demonstrating the validity of the procedure to isolate biologically active intracellular antibodies from immune libraries. Moreover, fluorescence-modulating antibodies may have interesting applications in various cell biology experiments.

The part of the project involving the immunized mice is significant by itself, independently from the biological activity of the binding antibodies that may be found. The experiments will be considered successful if they will lead to the isolation of new intracellular binders, because this approach would result in another way to isolate novel intracellular antibodies, using the yeast two hybrid as part of the selection procedure. The success of this work will depend on a crucial step in the procedure: cloning the library of antibody variable regions from the immune lymphocytes, in the format of ScFvs, into the yeast two-hybrid vector. The following section outlines my plans on the construction of this library.

4.2 The proposed approach for the ScFvs library construction

When planning to create a ScFvs library there are two general approaches that can be chosen, to separately clone the VH and VL genes, or to clone them together after a PCR based assembly of the two.

In both cases the first step is the separate PCR amplification of the two variable regions; in the first procedure the subsequent step is to clone separately the VH and VL amplified genes in the chosen vector, one after the other. This means that it is necessary to have a vector already able to accept the variable genes in the correct way, it has to present two pairs of different restriction sites, chosen between the low frequency cutters, separated with the linker encoding DNA. It is obvious that the ideal situation would be to have a yeast two hybrid vector able to accept in such a way the two pools of genes, otherwise one would be forced to use an intermediate shuttle vector. To this aim, it would be necessary to modify VP16 by adding a linker DNA flanked with two suitable restriction enzymes. These modified vectors are being prepared, but are not validated yet.

The advantages of choosing this approach are the following: by performing two separate cloning steps it is possible to carefully evaluate the result of each of them, thus, there would be the possibility to maximize the diversity of both the VH and the VL genes. The disadvantages are mostly practical: it is necessary to modify the acceptor plasmid, and also to perform two separate library cloning steps, this means a good deal of work, with all its uncertainties. Moreover, this also implies the use of four different restriction enzymes that, even if rare cutters, would inevitably cut within a number of interesting variable region genes. It is my opinion that this approach is the best option when the aim is to clone a library as large as possible: it permits to monitor the efficiency of the process after each crucial step, thus allowing all the needed feedback.

The other method is based on the PCR assembly, prior to the cloning step in the acceptor vector (Fig. 4.1): after the separate amplification, the two sets of variable regions are mixed together and linked in the desired way with a second PCR, at this point it is possible to perform a last PCR to add the appropriate restriction sites at both ends of the assembled fragments. The cloning is therefore executed in only one step, while the only way to monitor how the various reactions work is to check the DNA band position and size on

agarose gels after each step. This will not enable to verify if there is a significant loss in the diversity of the amplified genes, but only if the reactions worked in the overall expected way. For this reason the disadvantage of this approach is that, if for some reason the diversity of the library would result too small, it would be difficult to identify the exact origin of the loss. In any case a certain number of genes may be lost anyway as a result of the many PCRs performed, which will introduce amplification biases. On the other hand, the amount of DNA prepared in this way should be more than enough to facilitate the final cloning. This approach seems therefore suitable when the aim is not to clone a huge library, but to maximize the chances to succeed in the cloning of a fairly diverse number of clones.

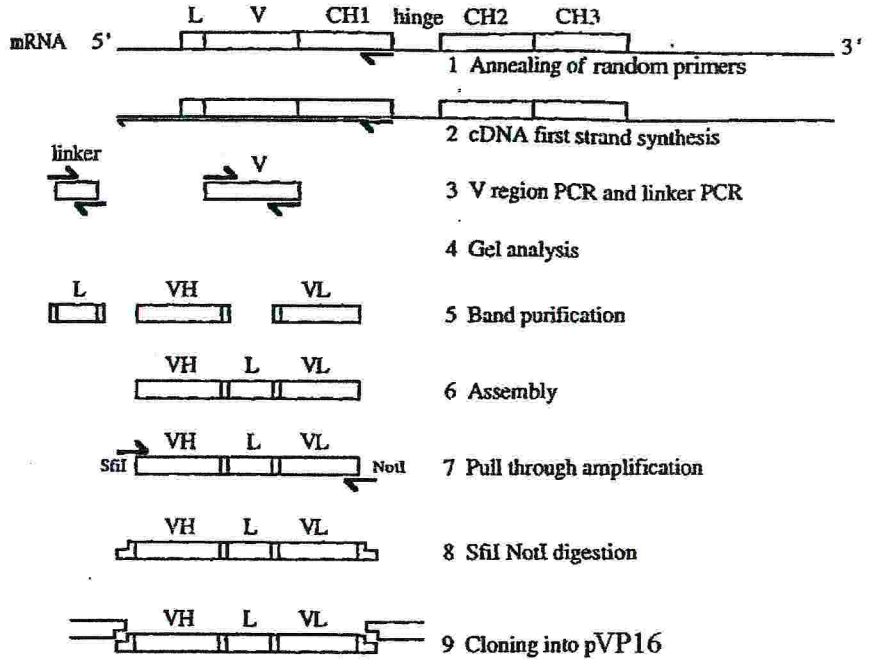

Fig. 4.1 Proposed method for the library construction.
Steps 1-2 involve random primers, as suggested by McCafferty (1996). For step 3 the primer sets for the variable regions are referred as VHback, VHfor, VLback and VLfor, in Chap. 2.7. Steps 6 and 7 may be performed simultaneously by using the pullFor and pullBack mix in the assembly reaction instead of VLBack and VHFor (see Chap. 2.7).

In our case the limiting factor is represented by the size of the library that can be transformed into yeast, that is around 10^{6-7}, since the efficiency of transformation in yeast is limited to 10^{5-6} $^{colonies}/_{\mu g\ DNA}$ it would be technically prohibitive to attempt transforming representative pools of larger libraries. Therefore some observations are necessary, because this may appear in contrast with the size of the ideal library that can be obtained from hyper-immunized mice.

From a murine spleen it is possible to obtain approximately 5x 10^7 different lymphocytes, but cloning the rearranged Ig genes implies the combinatorial reshuffling of the VL and VH genes, with the loss of the original VH/VL pairings, creating a theoretical diversity so high that is almost impossible (or impractical) to handle, with the ordinary technologies. While in general the reshuffling is a positive effect of the cloning because it increases greatly the diversity of the final library, in our case it is not, if we consider the limited number of clones transformable in yeast. The reshuffling implies that among the 2.5x 10^{15} possible combinations only something in the order of 5x 10^6 (the 10% of the initial repertoire) can represent the original antibodies specific for the antigen of interest. This means that it is nearly impossible to fish-out the original antibodies with the two hybrid system; however, the promiscuity of the VH/VL pairing with respect of the antigen specificity of the assembled antibody comes in help. Ever since the first phage display antibody libraries were produced, the promiscuity of the Vh-Vl pairing appeared in all its relevance (Clackson et al., 1991), showing that in many cases, but not in all cases, a single variable region, either VH or VL, is sufficient to determine the antibody specificity, leaving little relevance to the other variable partner. These findings, along with our own experiences, suggest that the percentage of antigen-reactive antibodies in a library derived from a hyperimmune spleen may even reach 0.1%. Incidentally, this number is very near to the estimated specificity of a phage library after one panning round selection on antigen, the kind of input library that seems to give the best results for the yeast trap (see below).

For all these reasons, my choice would fall on the PCR assembly based approach. Rather than trying to produce the largest library possible, I would choose the most reliable method, the goal being to gain 10^{7-8} diverse clones. In this thesis I propose a set of primers based on the ICGEB-EMBO-SISSA course on "Selecting from phage display libraries" (1996), summarized in Chap. 2.7. They represent a degenerated set of oligonucleotides designed to anneal to the largest possible number of known murine variable regions. Suitable sequences are added, when needed, to their 5' end, to allow their assembly and

cloning. The proposed linker (for a review: Bird and Walker, 1991) is the 220 as described in the already cited course. The PCR based approach protocols can be found in McCafferty et al. 1996.

4.3 The selection for new anti Tau ScFvs

Previous work in our lab (Visintin et al 1999; Visintin 2000 Ph.D. thesis) had shown that intracellular binders can be isolated from a large phage library pre-selected with two panning cycles on antigen (a fragment of human Tau). However, growing a phage library after each panning step leads to a reduction in diversity, creating strong biases and selecting rapidly dominating phage antibody clones, due to selection of phage on the basis of "non relevant" unwanted reasons. I therefore performed an experiment to verify if the number of panning cycles, required to enrich the input library and to reduce its size, could be limited to just one. The results were very encouraging, since the intrabody trap selection after just one panning cycle against the Tau fragment gave excellent results: having found four novel intracellular ScFv binders by screening just fifty ELISA- positive clones is maybe the best result one could ideally expect.

A very important point is that we were able to select again three of the four clones that were found with the previous selection. This means that the dimension of the first cycle library does not exceed the screening power of the yeast two hybrid, in other words it is possible to screen directly the library obtained with just one round of phage panning, if the starting library is of good quality, and the panning protocol is found to be reliable.

For further work it is of course necessary to characterize these new antibody genes, in order to confirm their presumed specificity. However, having seen the previous results, it seems extremely likely that at least most of them will result to be real intracellular binders.

Altogether these results allow to conclude that the two hybrid selection is indeed a powerful technique to select intracellular antibodies, and we are looking forward for new selections performed against other antigens of particular interest for ongoing projects of our laboratory.

Since my work was focused more on developing the system than in the utilization of the results, it seems appropriate to point out the possible improvements that my experience suggests. The output of the screening in yeast may have been underestimated: in the first

selection performed by M. Visintin, hundred yeast colonies were screened, but only a few carried different variable region genes, maybe between all the other positive clones there could be some more interesting ScFvs, but it is unlikely that more than one ore two have been left apart. The screening from the first phage display round was limited to only 50 yeast colonies, still 11 different genes were present: this means that we can be almost sure that a significant number of ScFv is still to be found among the selected clones. We screened only fifty genes for a very practical reason, the segregation and DNA purification procedure is very expensive in terms of material, work and time, and in the end represents a limiting factor. To overcome this real bottleneck, two different strategies could be investigated separately, on one hand it would be very useful to be able to perform a PCR amplification of the ScFvs DNA directly from yeast, this would enable the experimenter to verify by fingerprint analysis which clones are different from one another before the segregation process. On the other hand, the segregation would be much easier if performed in bacteria, this was not possible because the used plasmids, pBTM116 and pVP16, both carry the resistance only to ampicillin, however it is possible to modify one of the two, in order to change the resistance gene, thus allowing to perform the segregation in *E. coli*.

4.4 The C6 cells stabilized clones

The last part of my work can be considered as a side branch, however, as previously stated, it was important to demonstrate the possibility to express, via stabile transfections, the ScFvs identified and identified by the intrabody trap technology. The availability of stable clones is an essential prerequisite to perform functional knockout studies with these ScFv fragments. These experiments will provide the final validation of the anti Tau ScFvs isolated by the intrabody trap.

4.5 Conclusions

The work here presented was programmed as a medium range project, still the results achieved so far are to be considered in the more general context of the work necessary to support a newly developing technique.

With the selection of the phage display first round library, the approach of the yeast selection has passed over a significant bottleneck. The technique has demonstrated its reliability and its potential in a incontrovertible way: we know now that we can reasonably expect to use the intrabody trap to find out a reasonable number of intracellular antibodies against the chosen antigen, starting from a naive ScFv library, via the described two step procedure. Even antibodies that are rare in the input library will be accessed and isolated with the procedure as outlined in this work.

In addition, significant steps were taken towards opening a new way to utilize the technique, by starting from a library derived from hyperimmune spleen: the EGFP experiments have reached their crucial point, and soon we hope to be able to complete also this new path.

Having confirmed that the system works robustly, from this point on, it is possible to work with the aim of using it systematically for research and application purposes.

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