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The neuroantibody approach: cloning and expression of anti NT-3 hybridoma variable regions for in vivo and in vitro studies.

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Index

Introduction

A recurrent observation in the development of the nervous system is the generation of neurones followed by their large scale elimination. This phenomenon, usually referred to as naturally occurring neuronal death, takes place at a particularly important time during the development of embryonic neurones -the period immediately following the arrival of their axons in the target fields (for a recent review see Barde 1989) . The extent of naturally occurring neuronal death varies considerably from region to region and can be dramatically increased by removing a prospective target early in development. In the absence of target, the number of neurones initially present is not changed, indicating that the target does not influence the number of neurones generated. Conversely the degree of naturally occurring neuronal death can be decreased by adding an extra target or by blocking neurotransmission Levi-Montalcini R., 1968; Laing and Prestige, 1978; Pittman and Oppenheim, 1978). These observations demonstrate that the neurones normally eliminated are not programmed to die, but can be rescued provided the conditions in which they grow are changed. In other words, there is a specialised intercellular communication that instructs selected neurones to survive while neighbouring neurones degenerate, termed "trophic interaction" (D. Purves, 1988). Polypeptide trophic factors are the best-studied mediators of trophic interactions (Levi-Montalcini R., 1968; Y.-A. Barde, 1989). In the generally accepted models, a neuron employs cell surface receptors on its axon to bind a trophic factor released by a neuron upon which it synapses (the target neuron). In some cases, the projection neuron retrogradely transports the factor from the binding sites in its target field back to its cell body (Yan, at al 1988), although the importance of retrograde transport for trophic factor action is still unclear. Binding of the trophic factor to its receptor subsequently activates second messenger cascades and induces gene transcription, the end result of which is the observed effect on neuronal survival, morphogenesis and differentiation.

In order to understand the specific nature of trophic interactions it is necessary to introduce perturbations in the developing nervous system. In

this light, a novel experimental strategy to specifically interfere with the function of trophic factors on the neuronal environment has been designed. This strategy is based on the ectopic expression of recombinant forms of monoclonal antibodies directed against specific trophic factors (Piccioli et al, 1991). Recently it has been shown that the immunoglobulins can be efficiently expressed as secreted (Cattaneo et al, 1987) or intracellular (Biocca et al, 1990) proteins by mammalian non lymphoid cells. In particular, the efficiency of antibody secretion by cells related to the nervous system was shown to be comparable to that of lymphoid cells. Thus, the local secretion of specific monoclonal antibodies by cells of the nervous system could be used in functional and developmental studies on the otherwise intact nervous system of transgenic mice. Before discussing more about our experimental approach, it is useful to describe the molecular characteristics of the neurotrophic factor family and of their receptors.

The Neurotrophin family

Nerve growth factor (for a review see Levi-Montalcini R., 1987) is the prototypical member of a family of neurotrophic factors, the neurotrophins, that now includes at least four other members: brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and most recently neurotrophin-4 (NT-4). The neurotrophins are highly homologous proteins, sharing roughly 50% amino acid sequence identities and display both overlapping and specific sets of neurotrophic activities on peripheral and central neurones. The distribution of the amino acid identities, among the neurotrophin sequences, reveals the occurrence of clusters of conserved residues. Various speculations can be offered to explain this remarkable structural conservation. One would be the necessity for the neurotrophins to fit within a particular common receptor structure ($p75^{NGFR}$) or within structurally related receptors- the members of Trk family. Another speculation for the structural relatedness of the neurotrophins is the possibility that some of the conserved residues might be involved in dimerization of the ligands. The dimeric nature of all the neurotrophins may play a key role in the mode of signal transduction, and ligand-induced receptor oligomerization is widely believed to initiate signal transduction, leading to autophosphorylation of the activated receptors (Ullrich et Schlessinger 1991) The recent elucidation of the crystal structure of NGF has localised in four loop regions most of the sequence variations among

the neurotrophins, and suggested that these regions may determine the different receptor specificities.

Whereas all neurotrophins support the survival of embryonic neural crest-derived sensory neurones (Thoenen and Barde 1980; Lindsay et al 1985; Hohn et al 1990, Ip et al 1992, Ibanez et al 1993), the survival of embryonic sympathetic neurones is only supported by NGF, while placode-derived sensory neurones are supported by BDNF and NT-3, but not by NGF (Thoenen and Barde 1980; Lindsay et al 1985; Hohns et al 1990). This specificity is believed to be achieved in part by the selective interaction between the different neurotrophins and high affinity tyrosine kinase receptors, belonging to the trk family, expressed on the surface of distinct neuronal populations. Thus, whereas NGF binds only to $p140^{trk}$ (TrkA) (Kaplan et al 1991, Klein et al 1991), BDNF and Nt-4 interact exclusively with $p145^{trkB}$ (TrkB) (Sopper et al 1991, Squinto et al 1991, Klein et al 1991, 1992, Ip et al 1992) while NT-3 interact with p 145^{trkc} (TrkC) and, to a lesser extent, also with TrkA and TrkB (Cordon-Cardo et al 1991; Lamballe et al 1991; Klein et al 1991, Squinto et al 1991). All the neurotrophins can also bind, with a similar affinity, to the so called low affinity nerve growth factors receptor p 75^{NGFR} (Ernfors et al 1990; Rodriguez-Tebar et al 1990; Hallbook et al 1991). Several functions have been suggested for the $p75^{NGFR}$. First, $p75^{NGFR}$ may function as a presentation receptor to concentrate NGF (Taniuchi et al 1986). Second, $p75^{NGFR}$ may be involved in the retrograde transport of NGF (Johnson et al 1987). Third, $p75^{NGFR}$ may aid Trks receptors in discriminating among neurotrophic factors (Rodriguez-Tebar et al 1992, Marta Benedetti et al 1993). The critical questions are why do two separate receptors exist for NGF in responsive cells, and what is the relevance of $p75^{NGFR}$ to neurotrophin function. A central dogma that has driven the neurotrophin field is that neuronal cell survival is dependent upon limiting amounts of target-derived growth factor. Hence, the number of surviving neurones is regulated by the supply of neurotrophic factors and the affinity of binding to their receptors. High affinity binding requires coexpression of both receptors, and recently it has been shown (Rabizadeh et al 1993) that the expression of $p75^{NGFR}$ induces neuronal death, if the receptor was constitutively unbound.

In situ hybridisation analysis of cells expressing messenger RNAs (m RNAs) for NGF, BDNF and NT-3 and their high-affinity receptors (TrkA, TrkB, and TrkC) revealed a complex temporal and spatial expression pattern for each of these mRNAs in the rat embryo. In many cases the temporal and spatial

pattern of expression fits with a target-derived mode of action. In general, the NGF, and in particular, the NT-3 genes are expressed in a wide variety of tissue, whereas BDNF is expressed more strongly in the CNS than in peripheral tissues. It is noteworthy that the hippocampal formation is the area of the brain where the expression of these three neurotrophin genes is highest (Hofes et al, 1990; Maisonpierre et al, 1990; Ernfors et al, 1990; Phillips et al, 1990). This finding has led to the suggestion that the neurotrophins might be involved in phenomena such as long-term potentiation and synaptic remodelling. Consistent with this idea, the electrical activity and excitatory neurotransmitters can indeed up-regulate expression of these genes. In particular, experimentally induced seizures rapidly increase the NGF mRNA levels (Gall et al, 1989) and administration of glutamate agonists drammatically up-regulates the levels of both NGF and BDNF mRNA (Zafra et al 1990) in the rat hippocampus. The hippocampal distribution for these neurotrophic factors is rather unique. NGF mRNA is found throughout the pyramidal cell layer and in the granule cells of dentate gyrus (Ernfors et al., 1990), BDNF mRNA levels are highest in CA2 and CA3 compared with CA1, and NT-3 mRNA is found in CA2, in medial CA1, and in the granule cells of the dentate gyrus (Ernfors et al, 1990; Phillips et al., 1990). Surprisingly, the neurotrophins BDNF and NT-3 are extensively coexpressed with their receptors, thus there is the potential for involvement of BDNF and NT-3 in autocrine self-stimulation, as well as in signalling from pyramidal cell to pyramidal cell and from pyramidal cell to dentate gyrus granule cell. Furthermore recent studies using dissociated and organotypic cultures of rat cerebellum demonstrate that the neurotrophins BDNF and NT-3 affect developing granule cells at distinct stages in differentiation. While early granule neurones in the external germinal layer responded to BDNF (as shown by an evident increase in cell number), more mature granule cells respond to NT-3. BDNF, but not NT-3, enhances survival of granule cells in cultures of embryonic cerebella. Thus, BDNF and NT-3 have distinct sequential functions that are likely to be critical in the development of the cerebellum. It was suggested that BDNF may promote the initial commitment, while NT-3 may direct the subsequent maturation of granule cells (Segal R. A., 1992).

Mechanisms of signal transduction and the Trk receptors

Binding of neurotrophins to their trk receptors is followed by a receptor dimerization. This is necessary for receptor activation and can expand the repertoire of receptor-ligand interactions and even the diversity of signals generated.

Neurotrophins are dimeric molecules, and the presence of six well conserved cystine residues allows them to adopt similar protomeric structures that contain a motif referred to as the cystine knot (Neil Q. Mc Donald et all, 1993). This structural motif and a conserved b strand structure is peculiar of many growth factors (TGFβ, PDGF etc..), and may be involved in the receptor activation by ligand-induced bridging of two receptors. After receptor dimerization there is an intermolecular tyrosine phosphorylation that triggers the interaction of the cytoplasmic portion of the receptor with intracellular proteins containing a particular domain termed Src homology domain (SH_2). This family of protein domains of approximately 100 amino acids is highly conserved and is a recognition motif for specific tyrosine phosphorylated peptide sequences. SH_2 domains are usually accompanied by another conserved domain of 50 amino acids, called SH_3 (Src homology). Mutagenesis studies of the SH_2 domain from the c-Abl kinase show that highly conserved sequences, such as FLVRES, are required for interaction with phosphotyrosines. It is evident that the short sequences flanking receptor phosphotyrosines are involved in the affinity and specificity of signalling molecules. While tyrosine phosphorilation acts as a switch to induce the binding of SH_2 domain bearing proteins, this motif is able to mediate the formation of heterodimeric protein complexes at or near the plasma membrane. These complexes are likely involved in the control of the activation of signal transduction pathways. The SH_3 domain, together with SH_2 domains may modulate interactions with the cytoskeleton and membrane and recently some results indicate that SH_3 domains may be responsible for the targeting of signalling molecules to specific subcellular localisation (Bar-Sagi D., 1993). Some signalling and transforming proteins contain SH_2 and SH_3 domains, unattached to any known catalytic elements, and may therefore serve as "adaptors" to link tyrosine kinases receptors to specific target proteins. Do SH_2 domains control the specificity of receptor signalling pathways? Autophosphorylation of growth factor receptors on

multiple sites generates specific binding sites for a variety of proteins containing intracellular SH_2 domains. Tyrosine autophosphorylation or interaction with tyrosine-phosphorylated regions modulates the functions of these proteins. According to this hypothesis the activity of a given receptor tyrosine kinase equals the sum of the activities of the signalling proteins that interact with its tyrosine-phosphorylated form. Elimination of tyrosine autophosphorylation sites should therefore prevent the activation of specific signalling pathways. Furthermore different cell types express, in addition to common target proteins required for general cellular functions, a cell type-specific set of SH_2-containing signalling proteins responsible for determining specialised cellular responses. These tissue-specific SH_2-containing proteins have a more specialised role in tyrosine kinase receptor-mediated signal transmission pathways. According to this view, ligand-induced stimulation of the NGF receptor will activate distinct signalling pathways in PC12 cells, as compared with fibroblasts, because these two cell types contain, in addition to common target proteins, a different repertoire of cell type-specific SH_2-containing protein. These yet to be discovered tissue-specific target proteins will interact with tyrosine-autophosphorylated NGF receptors and, upon activation, transmit signals leading, on one hand, to mitogenesis in fibroblasts and, on the other hand, to growth arrest and differentiation in PC12 cells. Indeed, Trk receptors are able to mediate many biological processes in the absence of the low affinity receptor, depending on the biological contexts in which they are expressed.

The Trks receptors share approximately 50% homology in their extracellular binding domains, the same degree of homology existing between their ligands NGF, BDNF and NT-3. The extracellular domain of the Trk tyrosine kinase is distinguished by a number of striking motifs, including three tandem leucine-rich repeats, maybe involved in amphipatic b structures, flanked by cysteine-rich motifs (Schneider and Schweiger, 1991) and two immunoglobulin like C-2 repeats. Recently, a truncated form of TrkB lacking the cytoplasmic domain, $p95^{TrkB}$ (Middlenas et al 1991) has been detected, containing all the leucine-rich and C-2IgG repeat structures of the full-length receptor, and derived from the same gene. The non catalytic $p95^{TrkB}$ molecule may act as a scavenger receptor to maintain high levels of neurotrophins, or participate in functions involved with cell adhesion or signalling. Alternative forms of rat TrkC with different functional capabilities have been described (Valenzuela, et al 1993). While some forms of TrkC lacking of the intracytoplasmic kinase domain resemble previously defined

truncated variants of TrkB, other forms of TrkC contain variable-sized aminoacid insertions within the tyrosine kinase domain (Lamballe et al 1993). These truncated forms of receptors may serve to recruit the ligand to regions where high concentration are required. Co-expression of truncated and full-length receptors in the same cell could lead to alternate mechanism of action. Furthermore, the existence of kinase domain variants may mean that these receptors respond differently to neurotrophins (Lamballe et al, 1993).

The specificity of action of different neurotrophins may therefore be explained by the differential in vivo pattern of growth factor expression, the level and localisation of receptor tyrosine kinase expression, and higher order structural features that permit multiple factors to bind to the same receptors. Another mechanism could involve $p75^{NGFR}$, which binds to all neurotrophic factors and may provide additional selectivity of binding (Rodriguez-Tebar et al 1990, 1992). Binding experiments with NGF, BDNF and NT-3 have demonstrated that each neurotrophic factor binds to $p75^{NGFR}$ with a low affinity, but the rates of association and dissociation vary markedly, with NGF having the fastest and BDNF the slowest off rate (Rodriguez-Tebar et al,1992).

Design of experimental strategy

In this work we focus on neurotrophin 3 (NT-3). This neurotrophin was chosen as it seems to display the greatest degree of promiscuity of binding and activation of the different Trk receptor family members when expressed in fibroblasts. This promiscuity could reflect an inherent redundancy among neurotrophic factors, the use of inappropriate concentrations of factors in these in vitro experiments, or the existence of a mechanism to ensure additive effects of neurotrophins. Thus, it is difficult to extrapolate in vivo neurotrophic action of NT-3 from these in vitro studies and from the in vivo distribution of NT-3 and the Trk receptors. NT-3 shows very limited effects upon TrkA-dependent differentiation in PC12 cells, but is more effective in stimulating TrkA in fibroblasts (Squinto et al 1991; Berkemer et al 1991; Cordon-Cardo et al 1991) strongly arguing that the cellular environment is important for neurotrophic action.

In order to elucidate and dissect the actions of NT-3 in the developing nervous system, we exploited the recently described neuroantibody strategy based on the ectopic expression of recombinant forms of a neutralising monoclonal antibody against NT-3. We are interested in studying the ways by which the developing nervous system compensates and develops in the presence of this interference. Our approach should allow the expression of inhibitory antibody molecules in the appropriate cellular compartment or extracellular space of any tissue of interest, and of the CNS in particular by the judicious use of suitable promoters and intracellular targeting tags. This strategy, among the other different methods used to inhibit the function of selected genes in mammalian cells, appears to be rather promising. Thus, it would be extremely advantageous to have an inducible system, in order to switch off the action of the particular neurotrophic factor, in a specific temporal window, by using an inhibitory recombinant antibody secreted in the extracellular space and thus competing with the neurotrophin receptor. Furthermore, by using an appropriate cell-type specific promoter, it may be possible to modulate either temporally or spatially the inhibitory effects on specific neuronal populations of interest.

On the other hand, gene disruption by homologous recombination ("knock out"), in its present form, leads to mutations produced in all the cells of the

organism from very early developmental stages. The procedure to generate mice with specific mutations by homologous recombination first involves the use of standard recombinant DNA technology to introduce the desired mutation into a cloned DNA sequence of a chosen locus. That mutation is then transferred via homologous recombination to the genome of a pluripotent, embryo-derived stem (ES) cell. By positive-negative selection procedure it is possible to enrich for ES cell containing targeted disruptions. Finally, microinjection of the mutant ES cell into mouse blastocysts is used to generate germ-line chimeras and interbreeding of heterozygous siblings yields animals homozygous for the desired mutation. However, if the approach of targeted disruption was applied to the disruption of the NT-3 gene or of its receptor, it would be very difficult to understand if some neuronal population is directly dependent on NT-3 or on some other neurotrophic factor induced by NT-3, given the existence of cascades of growth factor interactions. On the contrary, our approach, involving the use of inhibitory antibodies in transgenic animals, and in principle being not different to the creation of transgenic animals containing other genes, would allow a transcriptional control of the spatio-temporal pattern of expression of the transgenic antibody. Therefore the perturbing action can be mode more restricted both in space and in time, something that is not possible yet with the knock out approach in this present form.

Our starting point was a hybridoma cell line secreting a monoclonal antibody against NT-3 (provided within the context of a collaborative project by Y.-A. Barde, Max Planck Institute). The supernatant from this hybridoma, which contains a high titer of the corresponding monoclonal antibody (5-10µg/ml), is able to inhibit the action of NT-3 in biological assays performed on embryonic chick nodose ganglions. Furthermore, the specificity of this monoclonal antibody was tested in a survival assay using sensory neurones isolated either from chick nodose ganglions (NGs) or dorsal root ganglions (DRGs.) It was found that the monoclonal antibody completely inhibit the survival effects of NT-3 on these cells, while those of BDNF or NT-4 or NGF were not affected. The anti NT-3 monoclonal antibody (#12) was also shown to neutralise the action of NT-3 in vivo. Hybridoma cells secreting the anti NT-3 monoclonal antibody were also applied to the chorioallantois membrane of quails at the 3rd day of embryonic age (E3). Analysis of sections of peripheral ganglia after the period of normally occurring neuronal death

(E12) indicated that about 30% of the sensory neurones normally present in either the placode-derived ganglion nodosum, or the neural crest-derived dorsal root ganglion are eliminated by the antibody treatment (Gaese et al submitted). These data confirm the antibody specificity for in vivo studies, and persuaded us to clone its variable regions for its utilisation in transgenic mice model systems. The cloning of immunoglobulin coding sequences from the corresponding hybridoma cell lines does not only allow their stable expression, but also, if needed, their modification into forms suitable for their targeting to intracellular compartments where they will be able to interact with the corresponding antigen. Under normal conditions antibodies are secreted proteins, and harbour a hydrophobic leader sequence for secretion. For expression as secreted proteins in non-lymphoid cells no change to the leader is required.

Starting from a selected hybridoma cell line, all the steps required to obtain a monoclonal recombinant antibody can be summarised in the following points:

. cloning (and sequencing) of the hybridoma variable regions cDNA

. checking that the variable regions cloned produce functional immunoglobulin

. checking that the specificity of the immunoglobulin is correct

. cloning the appropriate targeting sequence in frame with the immunoglobulin variable regions in a vector which will provide the necessary transcriptional control elements

. choice of vectors for transgenic mice. This represents an important point: not all gene constructs work well in transgenic mice. The two most common problems are inappropriate expression patterns and failure to achieve adequate expression levels.

. production of transgenic mice by using embryonic stem cells (ES cells) engineered in vitro to secrete the cloned antibody under the control of suitable promoter or by microinjection of the plasmid DNA in fertilised eggs. A regionally localised pattern of antibody expression can be achieved alternatively by the transplant of engineered cells or by infection with retroviral or viral vectors. In this case a strong constitutive promoter should be utilised.

Results

Cloning of the variable regions of an anti NT-3 monoclonal antibody

The molecular structure of immunoglobulins is particularly advantageous for using the polymerase chain reaction (PCR) to amplify their encoding genes. Since the 5′ and 3′ ends of immunoglobulin V regions tend to be relatively conserved, mixtures of degenerate V region primers can be used to amplify V regions from hybridoma RNA or DNA (Orlandi, 1989). These V regions can be amplified directly from hybridoma genomic DNA or from immunoglobulin specific cDNA. If using cDNA, one has the advantage that the concentration of V region sequences is much higher than that in genomic DNA, but extra steps are required.

The synthesis of immunoglobulin specific cDNA (from hybridoma cells secreting anti NT-3 antibody) was carried out by using primer oligonucleotides corresponding to conserved sequences in the constant regions of the light and heavy chain genes. As a result of these reactions, we obtained two bands with different size, of about 360 base pairs (bp) for the heavy variable region and 320 bp for the light variable region.
Once VH and VL bands were obtained by PCR amplification of cDNA, two distinct strategies were followed in order to reconstitute antibody molecules or antibody domains and to express them.
The first one allowed the expression of soluble immunoglobulin fragments in bacteria (part I), the second one allowed, after sequencing, the expression of cloned V regions in mammalian cells (part II).

Part I: Expression of soluble immunoglobulin Fv fragment s (ScFv) in bacteria

Recently, it has been shown that functional antibody fragments (ScFv) can be produced in *E.coli* by secretion of the antibody domains into the periplasm where folding and the formation of disulphide bridges can occur.
The single chain Fv antibody fragment (ScFv) is made up of the heavy and light chain V regions connected by a flexible linker (fig. 2). In the pHEN1 phagemid vector (Hoogenboom et al 1991), the production of ScFv is driven

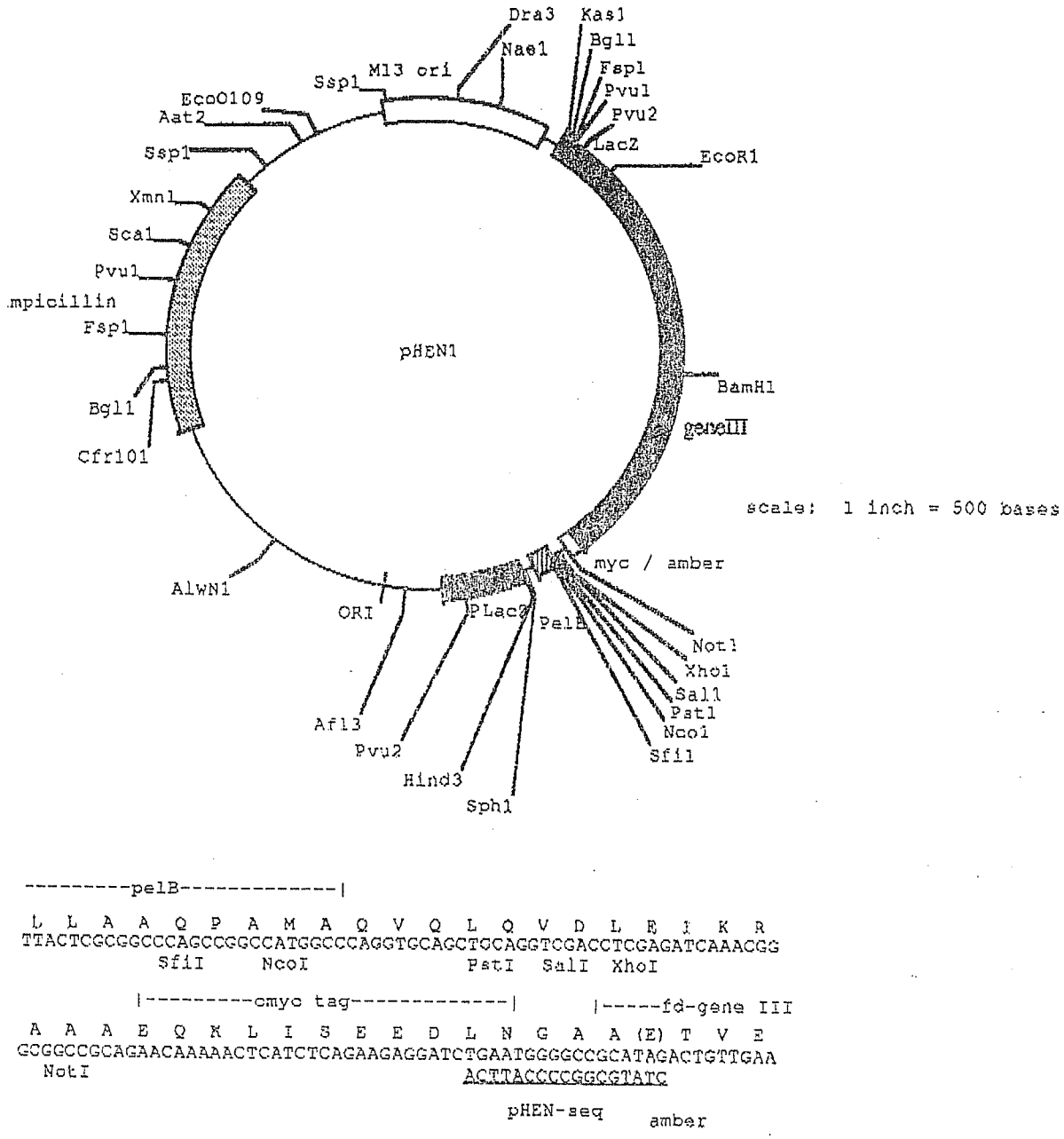


Fig. 1: map of pHEN1

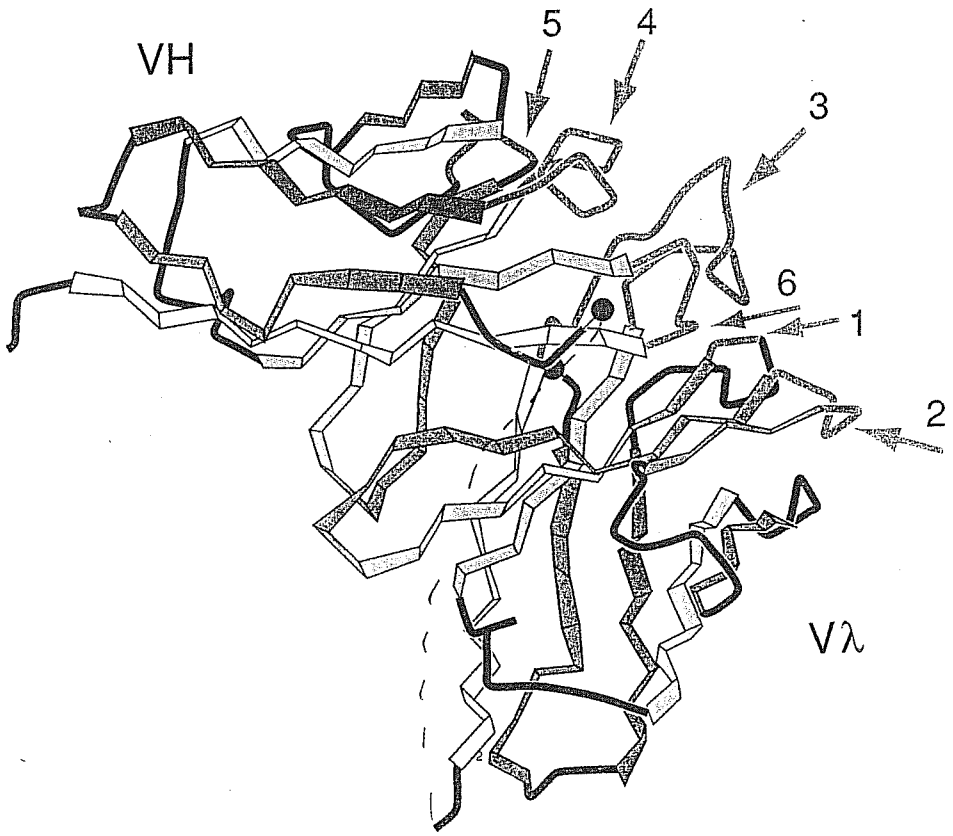


Fig. 2: Tridimensional rappresentation of single chain antibody fragment (ScFv). The model is taken from the solved X-Ray crystallographic structure of Fab and Fc domains of myeloma protein KOL, represented according to LesK A. and redrawn by an artist. (Nature, 1991, **349**, pag. 294)

by the lacZ promoter (induction by IPTG) while the export to the periplasm is directed by the *pelB* signal sequence (fig. 1). At the 3' end of the gene, a sequence encoding a portion of the c-myc protein is included, providing a C-terminal peptide on each soluble antibody fragment which can be recognised by the 9E10 antibody (Evan, 1985). This peptide tag facilitates detection in ELISA or Western blot and allows purification of large amounts of antibody fragments directly from bacterial culture medium by affinity chromatography with the 9E10 antibody.
The separately amplified VH and VL genes are linked in an assembly step (Method 2.2) to incorporate the linker DNA generated in (Method 2.1.1). The linker DNA has regions of homology with the 3' end of the amplified VH gene and the 5' end of the amplified VL gene, as well as a sequence encoding the $(Gly_4Ser)_3$ linker peptide which joins the two variable chains . In the same PCR reaction, the two outer flanking PCR primers provide restriction sites for cloning.

For cloning into pHEN1, the rare cutting enzymes SfiI and NotI sites are used. The cloning into pHEN1 of an assembled ScFv PCR fragment as a SfiI - NotI restriction fragment, is described in the methods section.
The following figure shows the different steps that we followed to obtain the anti Nt-3 ScFv construct (fig.3).

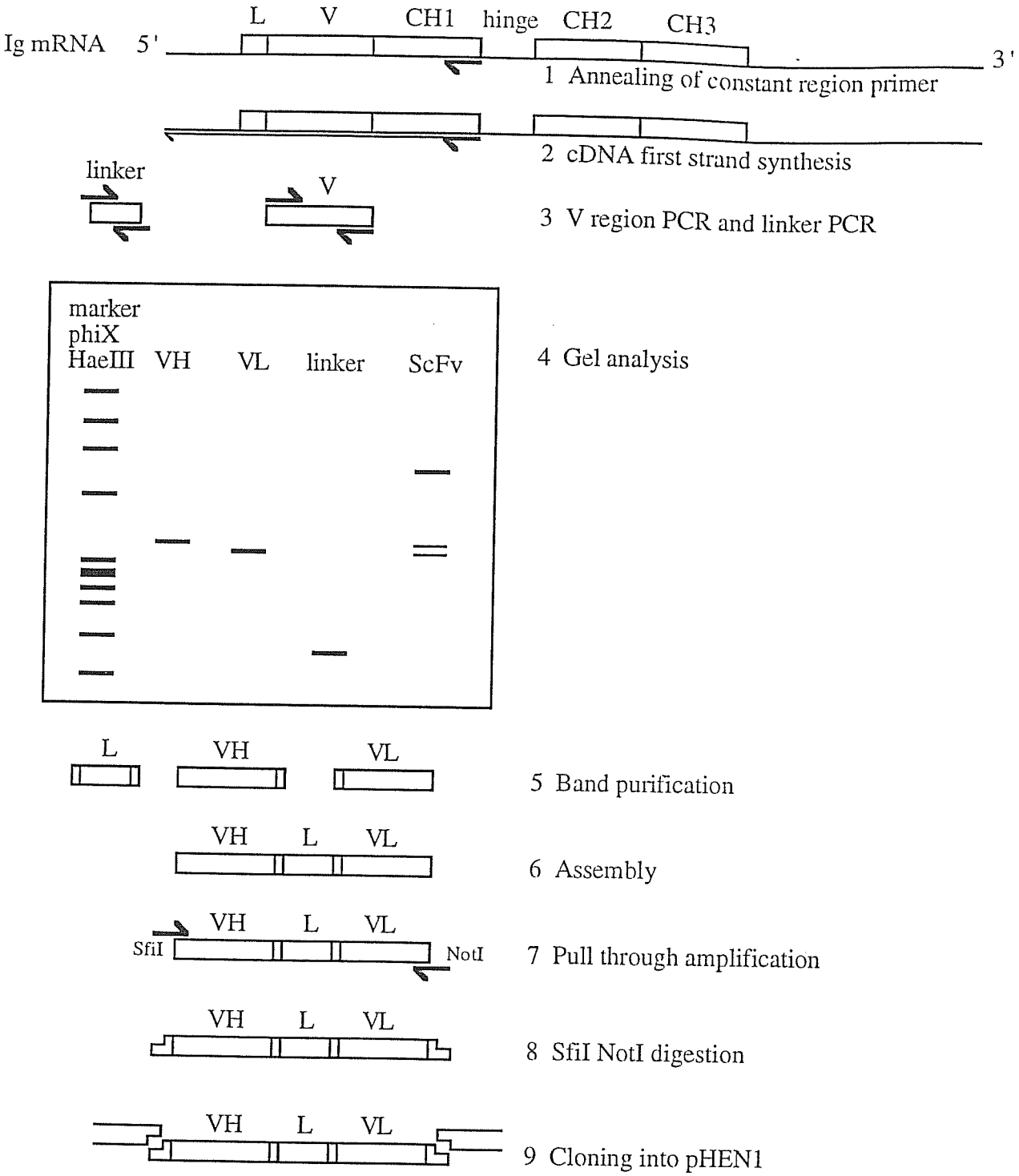


Fig. 3: ScFv assembly

Next, we tested if the ScFv construct in pHEN1 was indeed expressed after IPTG induction, and verified its localization (either in the periplasmic space or secreted in the bacterial culture medium). This was done by western blotting of the bacterial extracts., using the 9E10 mouse antibody (for details see methods)

The bacterial extracts were first analyzed in 12% SDS-denaturating gel and then transferred to a nitrocellulose filter
The figure below shows the results of western blotting:

1 2 3 4

Lane 1: anti NGF Scfv from bacterial periplasmic fraction, positive control; **lane 2:** non induced anti NT-3 ScFv from bacterial periplasmic fraction, negative control; **lane 3:** anti NT-3 ScFv from bacterial periplasmic fraction; **lane 4:** anti NT-3 ScFv from bacterial supernatant.
Usually, we put on gel 1/100 of the final volume from periplasmic extracts (see methods), while for bacterial supernatant, 400µl were TCA precipitated and then resuspended in the loading buffer.

The ScFv protein secreted by E.coli cells was used in futher studies to characterize its antigen binding properties.

Part II: Sequencing and expression of cloned V regions in mammalian cells

For sequencing of the amplified VH and VL regions and in order to test their specificity it is necessary to insert the PCR amplified bands into M13 based vectors, M13VKPCR and M13VHPCR (see fig.5a)
This was done by using standard techniques. These M13 vectors provide the immunoglobulin VH promoter, a eukaryotic leader sequence for secretion, the leader intron, and the first half of the intron downstream of the V region, and offer also the possibility of making single strand DNA for easier sequencing. The vectors contain V regions which need to be replaced with the newly amplified PCR fragment. As indicate in the Table I the primers used in the PCR reaction introduced restriction sites for six base cutters into extremities of the V-regions, thus allowing force cloning of the fragments. More than one clone has been sequenced (n=7) to ensure that no PCR errors have been introduced. It is also of the utmost importance to derive clones from at least two independent PCR reactions, as we did.
Nucleotide and deduced aminoacid sequences of anti NT-3 light (VK) and heavy (VH) variable regions are show in fig. 4
The sequence obtained was translated in all three frames. The immunoglobulins are very conserved proteins, also in their variable domains. Thus, they show a peculiar amino acidic "pattern", which is responsible for the overall canonical structural features of all immoglobulins. For this reason the presence of cystein residues in particular positions (Cys95, Cys68), and other specific residues, involved in the CDRs frameworks is diagnostic in order to verify the successful cloning of VH and VK regions.
A comparison of the deduced aminoacid sequences (for both VH and VK) with the Kabat -immunoglobulin data bank confirmed that the DNA sequences cloned by PCR did indeed correspond to immunoglobulin variable regions. The second point considered was to verify that the sequences of the variable regions obtained are different from all the other variable regions known, and in particular from that of other variable regions which have been cloned in the laboratory, as contamination in the PCR reaction is all too easy.
Following the appropriate sequence analysis in the M13 vectors the V-region and the relevant flanking sequences have been cloned into mammalian expression vectors. pSV2 based vectors are used (see fig.5b) which provide the second half of the V-C intron, the human IgG1/K constant regions, immunoglobulin enhancers, a SV40 polyadenylation site, the SV40 origin of replication, and selectable markers.

Sequence Range: 1 to 369

VH

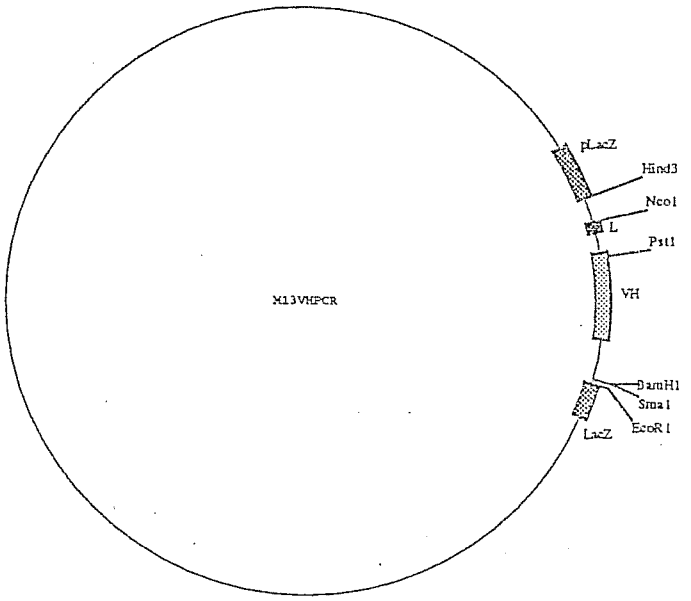
```
                    20                  40                  60
    *    *    *    *     *    *    *    *     *    *    *    *
GGTGTCCACTCCCAGGTCCA ACTGCAGGAGTCTGGGCTGA GCGGTGTAGGCCTGGGCTCA
 G  V  H  S  Q  V  Q   L  Q  E  S  G  L   S  G  V  G  L  G  S>

                    80                  100                 120
    *    *    *    *     *    *    *    *     *    *    *    *
GTGAAGCTGTCCTGCAAGGC TTCTGGCTACATCTTCACCA GCCACTGGATAAACTGGGTG
 V  K  L  S  C  K  A   S  G  Y  I  F  T   S  H  W  I  N  W  V>

                    140                 160                 180
    *    *    *    *     *    *    *    *     *    *    *    *
AAGCAGAGGCCTGGACAAGG CCTTGAGTGGATCGGAAATA TTTATCCTTCTGATAGTTAT
 K  Q  R  P  G  Q  G   L  E  W  I  G  N   I  Y  P  S  D  S  Y>

                    200                 220                 240
    *    *    *    *     *    *    *    *     *    *    *    *
ACTAACTACAATCAAAAATT CAAGGACACGGCCGCATTGA CTGTAGACAAATCCTCCAGC
 T  N  Y  N  Q  K  F   K  D  T  A  A  L   T  V  D  K  S  S  S>

                    260                 280                 300
    *    *    *    *     *    *    *    *     *    *    *    *
ACAGCCTACATGCAGCTCAG CAGCCCAACATCTGAGGACT CTGCGGTCTATTACTGTACA
 T  A  Y  M  Q  L  S   S  P  T  S  E  D   S  A  V  Y  Y  C  T>

                    320                 340                 360
    *    *    *    *     *    *    *    *     *    *    *    *
AGAGGGGCCTATGGTAACTA CTGGTACTTCGATGTCTGGG GCCAAGGGACCACGGTCACC
 R  G  A  Y  G  N  Y   W  Y  F  D  V  W   G  Q  G  T  T  V  T>

    *
GTCTCCTCA
 V  S  S>
```

Sequence Range: 1 to 325

VK

```
                    20                  40                  60
    *    *    *    *     *    *    *    *     *    *    *    *
CAGCTGACCCAGTCTCCAGC AACAAAGGCTGTGTCTCTAC CCACGAGGGCCACCATCTCC
 Q  L  T  Q  S  P  A   T  K  A  V  S  L   P  T  R  A  T  I  S>

                    80                  100                 120
    *    *    *    *     *    *    *    *     *    *    *    *
TGCAAGGCCAGTCAAAGTGT TGATTATGATGGTGATAGTT ATATGAACTGGTACCAACAG
 C  K  A  S  Q  S  V   D  Y  D  G  D  S   Y  M  N  W  Y  Q  Q>

                    140                 160                 180
    *    *    *    *     *    *    *    *     *    *    *    *
AAACCAGGACAGTCACCCAA ACTCCTCATCTATGCTGCAT CCAATCTAGAATCTGGGATC
 K  P  G  Q  S  P  K   L  L  I  Y  A  A   S  N  L  E  S  G  I>

                    200                 220                 240
    *    *    *    *     *    *    *    *     *    *    *    *
CCAGCCAGGTTTAGTGGCAG TGGGTCTGGGACAGACTTCA TCCTCAACATCCATCCTGTG
 P  A  R  F  S  G  S   G  S  G  T  D  F   I  L  N  I  H  P  V>

                    260                 280                 300
    *    *    *    *     *    *    *    *     *    *    *    *
GAGGAGGAGGATCGTGCAAC CTATTACTGTCAGCAAAGTA ATGAGGATCCGTGGACGTTC
 E  E  E  D  R  A  T   Y  Y  C  Q  Q  S   N  E  D  P  W  T  F>

                    320
    *    *    *    *     *
GGTGGAGGGACCAAGCTGGA GATCT
 G  G  G  T  K  L  E   I  X>
```

Fig. 4: Nucleotide and deduced aminoacid sequences of anti NT-3 light (VK) and heavy (VH) variable regions



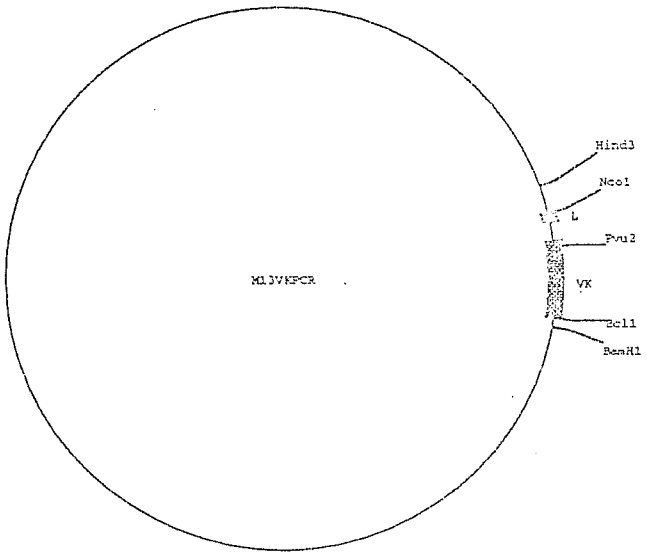


Fig. 5a: maps of M13VPCR plasmids

The expression of functional recombinant immunoglobulins from these plasmids was assayed by transient transfection in COS cells. This exploits the SV40 origin of replication, which in the presence of the large T antigen allows the plasmid to be amplified to high levels. The advantages of this system is that sufficient functional antibody to perform ELISAs can be produced after only three days, whereas the analysis of recombinant immunoglobulin from stable transfected clones can take weeks.

Thus, transient transfections were performed. These were usually done in parallel, for immunofluorescence, and for supernatant harvesting. This allows one to be sure that the supernatant used for assays and determination of antigen binding activity is derived from cultures which have a sufficient number of positive cells present. The presence of single positive cells as well as double positive cells (expressing both the heavy and the light immunoglobulin chain) serves as a good internal control.

When 1-10% of cells are positive one can expect up to 50ng/ml immunoglobulin in the supernatant three days after the transfection, which is sufficient for most tests of specificity.

The day before testing the antibody specificity, 48 hours after transfection, the production of immunoglobulin by COS cells was tested by indirect immunofluorescence (fig. 6)

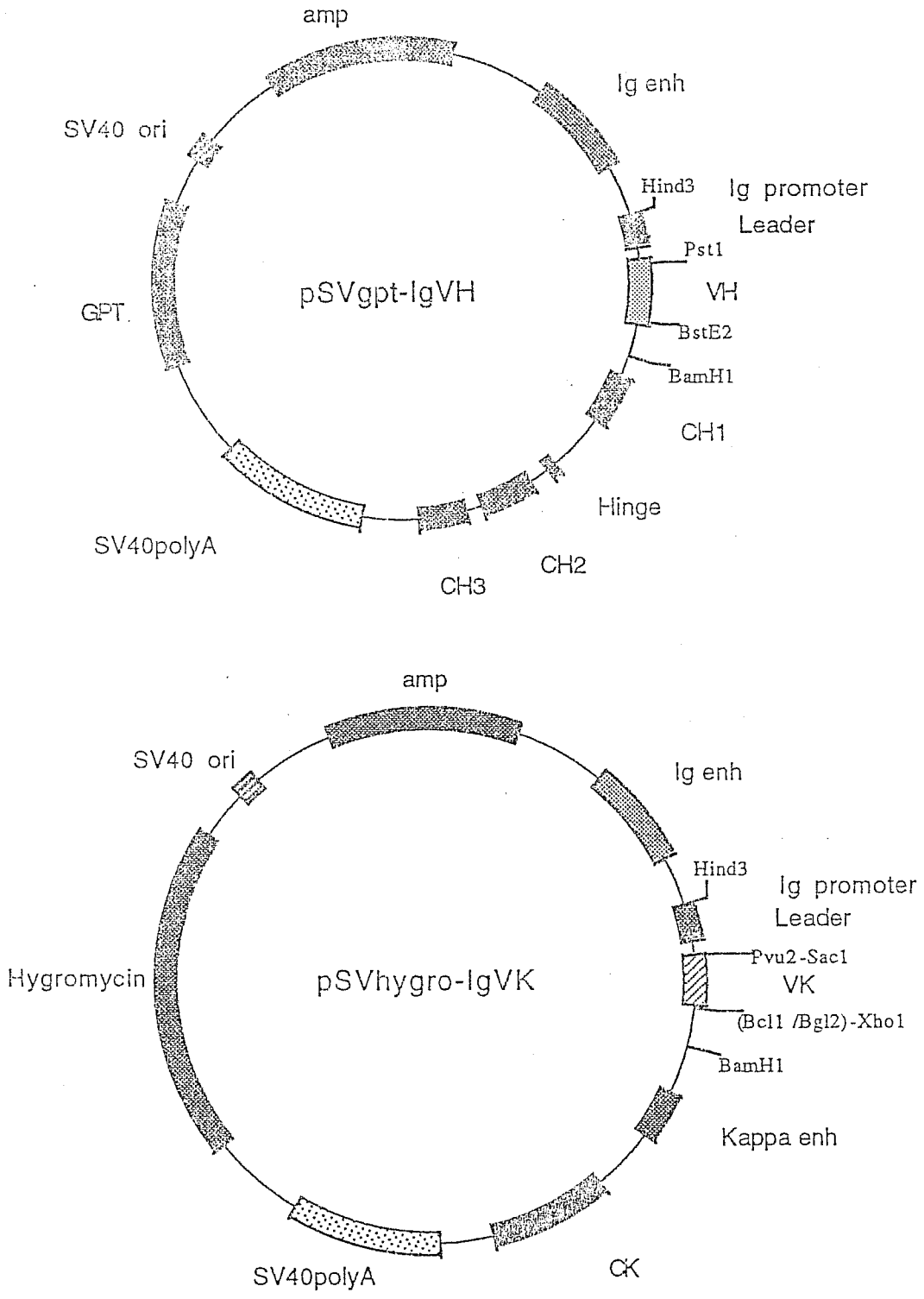


Fig. 5b: maps of pSVhygro-IgVK and pSVgpt-IgVH plasmids

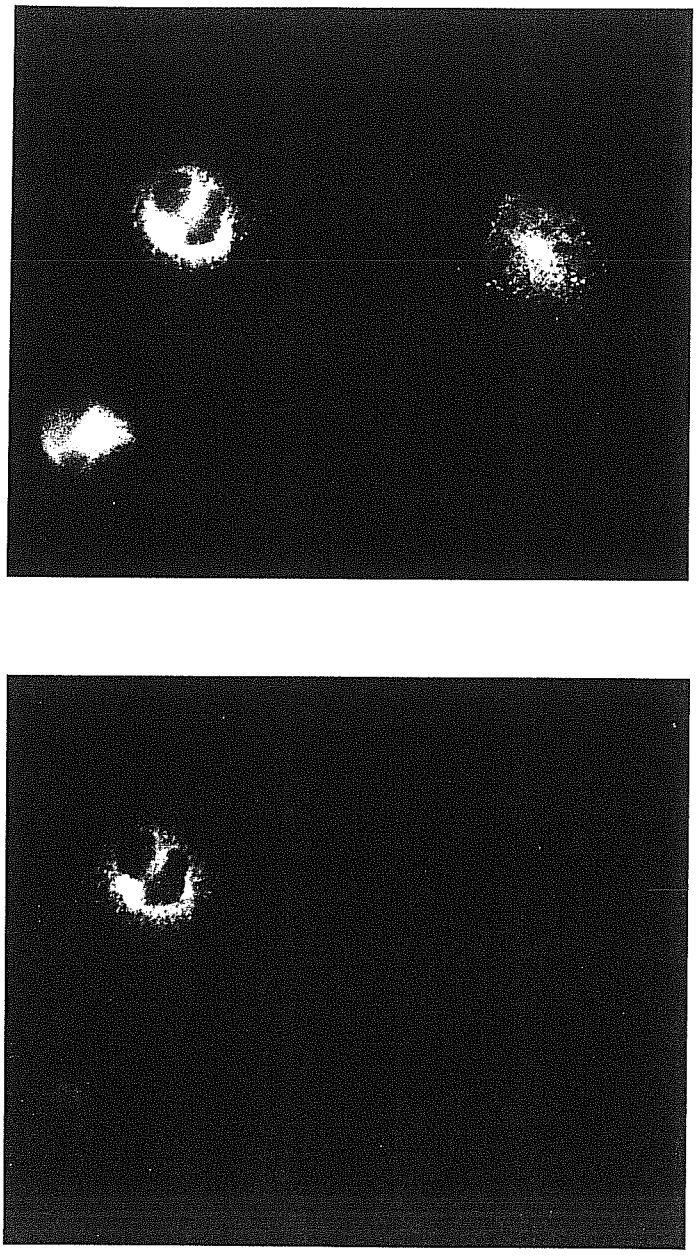
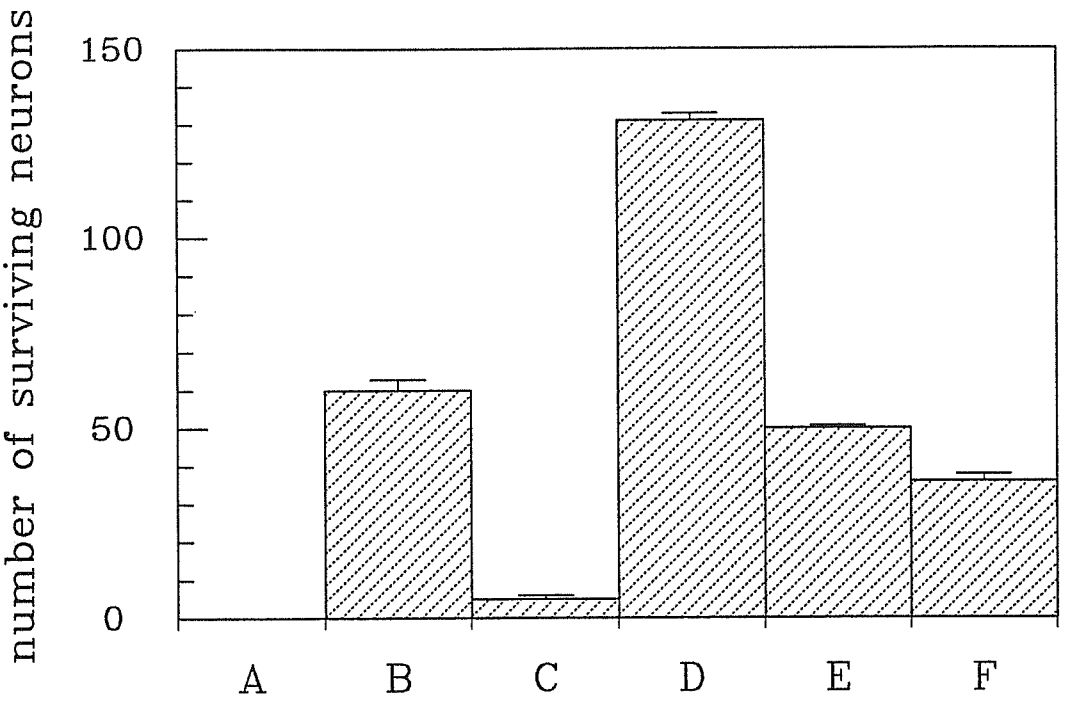


Fig. 6: Coexpression of heavy and light chain anti Nt-3 recombinant antibody in COS cells transfected with pSVgpt-IgVH and pSVhygro-IgVK. 48 hours after transfection the cells were stained by double indirect immunofluorescence with FITC labelled anti human heavy chain (up) with biotinylated anti human light chain (down) antibodies, followed by Texas Red Streptavidin. The same field is shown for two fluorochromes used.

Verifying the binding activity and the specificity of the recombinant antibody

Different assays can be done to verify the binding activity of the cloned antibody, depending on the availability and nature of the antigen.
In our case we performed specific biological assays on chick nodose ganglia. These assays allow to verify the anti NT-3 specificity of our recombinant antibody, as these neuronal populations are also responsive to BDNF. Thus it offered us the possibility to test any weak crossreaction of the recombinant antibody with other neurotrophic factors. The striking structural similarity between the neurotrophins, if on one hand has allowed to localise some peculiar loops involved in the receptor specificity of each neurotrophin, on the other can constitute a real problem, because the cloned antibody could recognise some common structural epitope that is present in all the neurotrophic factors. Therefore, for in vivo studies, it is mandatory that the recombinant antibody is not only able to bind to NT-3, but also that it neutralises in a specific way its biological action. This was verified by performing bioassays on embryonic nodose ganglions in the presence of supernatants from transiently transfected COS cells

Bioassays have been performed on chick embryonic ganglions (E6-E9). The collection, cleaning, trypsinization, and dissociation of ganglia was carried out as described previously (Barde et al 1980; Lindays and Rohrer 1985). The dissociated cell suspension were enriched for neurones by the preplating technique of McCartly and Partlow (1976). Cultures were maintained in F-14 medium (Barde et al 1980) which was supplemented with 10% (v/v) heat-inactivated horse serum (GIBCO). Neuron-enriched suspensions were plated at 2000 neurones/well. Prior to plating of neurones, cell culture dishes were coated with the basement membrane protein laminin (10mg/ml) according to the methods of Edgar et al 1984. Laminin was purified from the mouse EHS sarcoma (Timp et al, 1979). The number of process-bearing neurones was determined at least 48 hr after plating.
Nodose ganglions neurones, especially those from E6 embryos, rapidly adhered to laminin-coated culture dishes and neurite outgrowth was seen as soon as 2 hr after plating in control cultures, as well as those containing NT-3, and BDNF.
The following figure (7) shows the inhibitory effect of the supernatant from COS cell transfectants containing the recombinant antibody against NT-3.



	TREATMENT	NG [a]
A	Control	0
B	NT-3 400μg/ml	60.0 $\pm$ 2.9
C	NT-3 100μg/ml + sup hyb. #12 1μg/ml	5.0 $\pm$ 0.9
D	NT-3 100μg/ml + sup cos untransfected	131.0 $\pm$ 1.8
E	NT-3 100μg/ml + sup cos anti NT-3	50.0 $\pm$ 0.7
F	NT-3 100μg/ml + sup cos anti NT-3 + sup hyb. #12 1μg/ml	36.0 $\pm$ 1.8

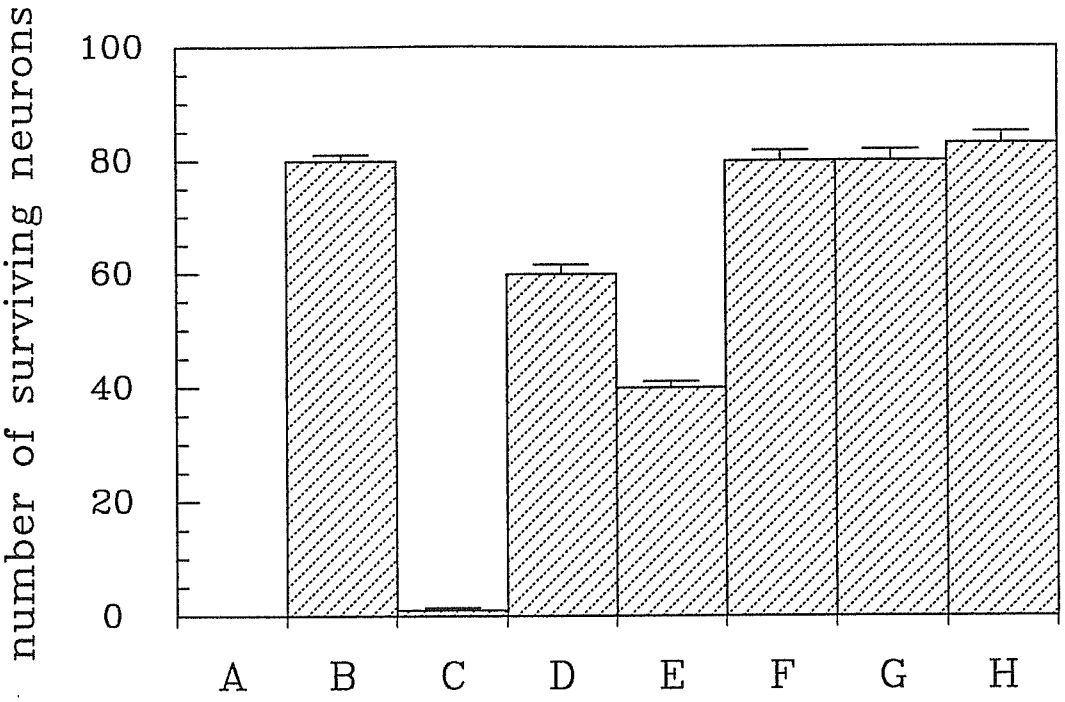
[a] : NG = nodose ganglions (surviving)

Fig. 7: Results of bioassays from COS supernatans performed on nodose ganglions (NG)

The comparison of the number of surviving nodose ganglions (see table) in the control (D=131, performed with a COS supernatant of untransfected cell), with the numbers obtained using COS supernatant containing antiNT-3 recombinant antibody alone (E=50), and plus the supernatant from parental hybridoma (F=37) allows us to conclude that the recombinant antibody maintain the same inhibitory activity as the parental monoclonal antibody
The COS supernatant of untransfected cells shows a higher survival action on the nodose ganglions, than the control with only the neurotrophic factor (B well), perhaps due to some undefined molecule(s) present in the COS medium that exerts an additive effect on the NT-3 action. The significantly lower values of E and F wells are indicative that in these cases the NT-3 survival action is neutralised by the antibody present in the medium. Thus, the residual number of surviving neurones counted in the E and F wells is due to the aspecific survival action mediated by untransfected COS supernatant (D well). This is also confirmed by comparing the effect of the parental hybridoma with or without the COS cell supernatant (not shown). The neutralising effect of the recombinant anti NT-3 antibody is thus somewhat underscored by the aspecific survival action due to the COS supernatant.
Experiments in progress are aimed at solving this problem by purifying the recombinant antibody secreted by transfected COS cells.
The results obtained from these experiments allow to conclude that the recombinant anti NT-3 antibody maintains the antigen specificity of the parental antibody.

We also tested the activity of the ScFv antibody fragment produced by E.coli cells, and obtained a significant, although incomplete inhibition (see fig. 8 and compare C D and E wells) of the NT-3 action.

The binding activity of the anti NT-3 ScFv was further assayed by ELISA with solid phase bound NT-3 which confirms that also the ScFv fragment recognised NT-3
The following figure (Fig.9) shows the results.



	TREATMENT	NG [a]
A	Control	0
B	NT-3 100μg/ml	80.0 $\pm$ 1.2
C	NT-3 100μg/ml + sup hyb. #12 1μg/ml	1.0 $\pm$ 0.4
D	NT-3 100μg/ml + ScFv $\bar{\alpha}$NT-3 0.2μg/ml	60.0 $\pm$ 1.6
E	NT-3 100μg/ml + ScFv $\bar{\alpha}$NT-3 0.45μg/ml	40.0 $\pm$ 1.2
F	BDNF 100μg/ml	80.0 $\pm$ 1.8
G	BDNF 100μg/ml + ScFv $\bar{\alpha}$NT-3 0.2μg/ml	80.0 $\pm$ 1.9
H	BDNF 100μg/ml + ScFv $\bar{\alpha}$NT-3 0.45μg/ml	83.0 $\pm$ 2.0

[a] : NG = nodose ganglions (surviving)

Fig. 8: Results of bioassays from bacterial supernatant containing anti NT-3 ScFv performed on nodose ganglions (NG)

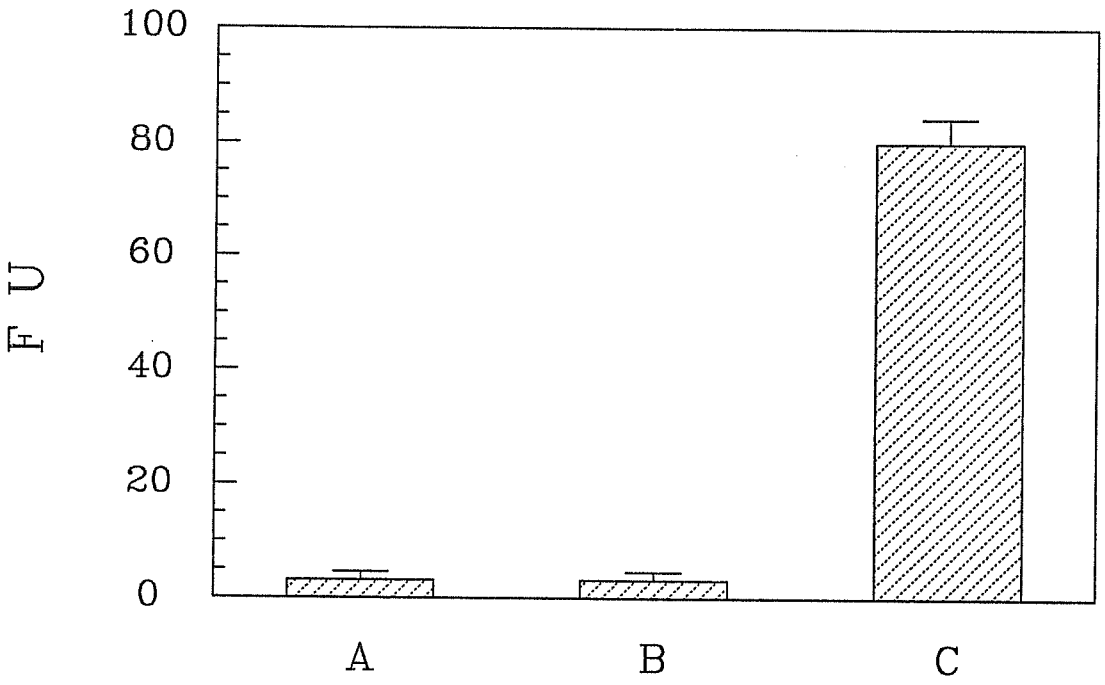


Fig.9: FU (fluorescence unit); A(3.10±1.4)=blank; B(3.10±1.5)=10ng BDNF; C(80.0±4.3)=10ng NT-3. The plates were coated 50µl per well of protein antigen, usually at 200ng/ml 50mM sodium hydrogen carbonate, pH9.6. The unreached sites were blocked with PBS + 2% BSA, 30 min at room temperature. Anti NT-3 ScFv (50µl of periplasmic extraction) was added and incubate for 1 hour at room temperature. The wells were washed out three times with PBS + 0.05% Tween-20. Purified 9E10 antibody (1:5000), in PBS + 2% BSA, was added and incubated at room temperature for 1 hour. Then, after washing out wells three times with PBS +0.05% Tween20,an antimouse IG, B-galactosidase conjugated (1:400), in PBS + 1% BSA + 0.05% Tween20 was added and incubated at room temperature for 1 hour. Substrate solution: Methyl-Umbiliferyl-Galactoside in 0.1 M sodium phosphate buffer pH7.3

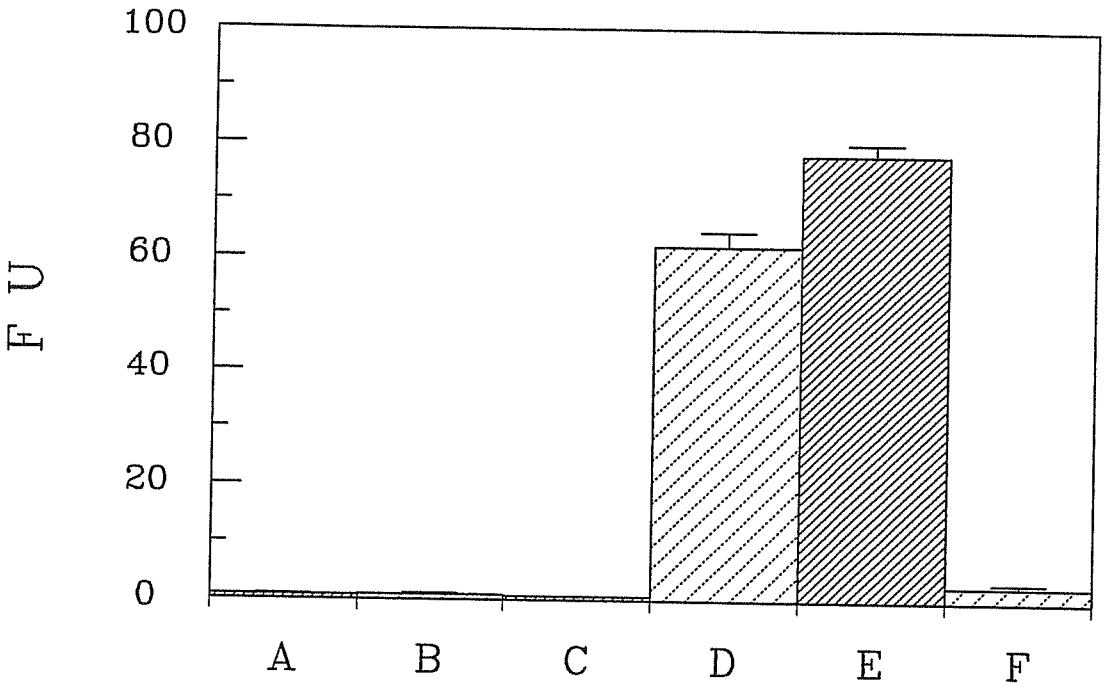


Fig.10: FU (fluorescence unit); **A**(0.7±0.1)= blank (αD11: anti NGF hybridoma supernatant); **B**(0.9±0.1)= blank (12#: anti NT-3 hybridoma supernatant); **C**(0.7±0.2)=10ng NT-3 + αD11 hybridoma supernatant; **D**(62.0±2.5)=10 ng NT-3 + 12# hybridoma supernatant; **E**(78.0±2.0)=10ng NGF + αD11 hybridoma supernatant; **F**(2.7±0.6)=10 ng NGF + 12# hybridoma supernatant. Coated plate with 50 µl per well of protein antigen, usually at 200ng/ml 50mM sodium hydrogen carbonate, pH 9.6. Blocking with PBS + 2% BSA, 30 min. at room temperature. Both hybridoma supernatants (1:5000) were incubated 1 hour at room temperature. Discarded test solution and washed out wells 3 times with PSB + 0.05 % Tween-20. Anti-mouse Ig, B-galactosidase linked (1:400), in PBS + 1% BSA + 0.05 % Tween-20 was incubated at room temperature for 1 hour. Substrate solution: Methyl-Umbiliferyl-Galactoside in 0.1 M sodium phosphate buffer pH 7.3.

The specificity of this ELISA procedure was confirmed by determining the crossreaction between two hybridoma supernatants, one against NGF (αD11) and the other against NT-3 (12#). As the figure (10) shows, there is no crossreaction whatsoever between these monoclonal antibodies.

Conclusions and outlooks

The most characteristic biological effect of the neurotrophins is their ability to promote survival of embryonic neurones. The responsiveness of neurones and neuronal precursors to the various member of the neurotrophin family is much more complex than previously assumed, and far from being understood. In order to unravel the complexity of the neurotrophic actions , it is necessary to introduce selected and specific alterations in the developing and adult CNS system. For this reason I undertook a new experimental strategy developed in the laboratory, which is based on the ectopic expression of recombinant antibodies directed against specific trophic factors.
To this aim we chose a monoclonal antibody raised against neurotrophin-3, which specifically and completely blocks the neuron survival activity of NT-3 in vitro, but not that of NGF, BDNF or Xenopus NT-4 (unpublished results). In this work the variable regions of this antibody were cloned and inserted into vectors for the expression of the recombinant antibody in mammalian cells. The activity of the recombinant antibody was determined in biological assays performed on chick embryonic nodose ganglions cells, and shown to inhibit the survival action of NT-3 on these cells. Furthermore, the cloned variable regions were inserted into vectors for the expression of single chain Fv fragments (ScFv), and the resulting ScFv protein was shown to maintain the antigen binding properties of the parental antibody. This will facilitate the use of retroviral or viral vectors for the expression of the recombinant anti NT-3 antibody in vivo.

This work was devised to perform a series of studies (in vivo-transgenic mice) in which engineered antibody genes, against specific neurotrophic factors, can be introduced into cells of an otherwise intact tissue, or secreted in the extracellular space, in order to alter its phenotype. Signalling by neurotrophic molecules seems not to be restricted to the retrograde messenger mode but include local trophic interactions, autocrine signalling, anterograde trophic signals, therefore the potential of some perturbation effects in the neurotrophic net appears to be an incisive tool.
The availability of anti NT-3 monoclonal antibody in recombinant form represents the first and most important building block to achieve its

expression by cells of the CNS. How can we get the antibodies produced by cells of the nervous system? Two complementary strategies will be pursued with anti NT-3 antibodies:

1) the creation of lines of transgenic mice harbouring the recombinant monoclonal antibodies as transgenes, under the transcriptional control of a brain-specific or an inducible promoter, and

2) intracerebral grafting of cells engineered, with recombinant retroviral or viral vectors, to secrete the recombinant monoclonal antibody or an active derivative domain such as a single chain antibody.

Hopefully, this will allow to gain more insight into the actions of NT-3 in the development and function of central neurones and synapses.

Methods

Method 1.1: RNA preparation

A large number of commercial kits are available for RNA purification which yield high quality material for cDNA synthesis, but we prefer to make total RNA and then purify polyA+ RNA from this.

Total RNA preparation.

1. Wash 10^7 mouse hybridoma cells in PBS.
2. Resuspend the cells in 1ml buffer GTC[a].
3. Add 0.1ml NaOAc (3M, pH5.2), shake.
4. Add 0.5 ml phenol, shake vigorously.
5. Add 0.2 ml chloroform, shake vigorously; leave on ice 15 min.
6. Spin 15 min 10000xg.
7. Precipitate aqueous phase with 1 volume of isopropanol; spin 15 min 10000xg.
8. Wash pellet in 70% EtOH and allow to airdry.
9. Resuspend pellet in 100µl DEPC treated H_2O.
10. Proceed to Poly(A)+RNA preparation

[a]Buffer GTC: 4M Guanidine-Isothiocyanate, 25mM Sodium Citrate, 0.5% Sarcocyl, 0.1M ß-mercaptoethanol.

Poly(A)+ RNA preparation.

1. Suspend 1g of oligo(dT)-cellulose in 0.1 M NaOH.
2. Wash oligo(dT)-cellulose 3x with DEPC H_2O and 3x with binding buffer[a].
3. Heat the RNA sample (100µg) to 65°C for 5 min. and add 1 volume of 2x binding buffer.
4. Add 10µl of oligo(dT)-cellulose (10mg total RNA can be loaded onto 1ml of packed volume).
5. Incubate 1 hr at room temperature.
6. Wash oligo(dT)-cellulose 4x with binding buffer.
7. Elute poly(A)+ RNA with 100µl elution buffer[b].
8. Precipitate mRNA with 1/10 vol NaOAc (3M; pH5.2) and 2.5 vol ethanol.
9. Recover mRNA by centrifugation, and resuspend in DEPC H_2O.
10. Use up to 10µg mRNA per standard cDNA reaction.

[a]Binding buffer: 20mM Tris-HCl (pH7.6), 0.5M NaCl, 1mM EDTA, 0.1% Sodium Lauryl Sarcosinate.
[b]Elution buffer: 10mM Tris-HCl (pH7.6), 1mM EDTA, 0.05% SDS.

Method 1.2: cDNA preparation.

cDNA can be made simultaneously for both light and heavy chains by incorporating the appropriate primers, using the same stock for amplification of both chains. Either AMV or MuLV reverse transcriptase can be used.

1. Mix in a sterile tube on ice:

RNA (up to 10mg mRNA)	10µl
10x RT buffer[a]	5µl
DTT (100mM)	5µl
dNTP (5mM each)	5µl
Oligo(s) (10pmoles/ml each)	1µl
RNAsin (10u/ml)	5µl
RTase (100 u/ml)	5µl
H_2O	14µl

2. Incubate 1hr at 37°C.
3. Boil 3 min, and then quench on ice.
4. Use up to 10 µl per standard PCR reaction.

[a]10xRT buffer: 0.5M Tris-HCl (pH8.2), 0.1M $MgCl_2$, 1M KCl. Alternatively, use the 10x RT buffer provided by the supplier.

Method 1.3: V-Region PCR amplification

Precautions against PCR contamination (dedicated pipettes and aerosol proof tips, aliquoted reagents, a separate area of the lab for PCR, UV irradiation of samples prior to adding the template and enzyme etc.) should be observed.

1. Mix the following:

H_2O	29µl
cDNA	5-10µl
10x PCR buffer[a]	5µl
dNTP 5mM each	2.5µl
BSA (10mg/ml)	0.5µl
For primer (10pmol/ml)	2.5µl

Back primer (10pmol/ml) 2.5µl

2. Overlay with parafin oil.

3. Heat to 94°C for 5 min in PCR block, and add 1µl Vent DNA polymerase (5u/µl) (New England Biolabs).

4 Perform 30 temperature cycles as follows: 94°C 1 min, 60°C 1 min, 72°C, 2 min.

5. Analyse 5-10µl of PCR reaction on 2% agarose gel.

[a]10x PCR buffer: 100mM Tris-HCl (pH8.3), 0.5M KCl, 15mM $MgCl_2$, 5mM dNTP (each), 1mg/ml BSA.

Alternatively, use the 10x PCR buffer provided by the supplier and add the dNTPs separately if they have not been included. The concentration of $MgCl_2$ in the buffer should be checked.

Method 2.1: Assembly of ScFv fragments

The separately amplified VH and VL genes are linked in an assembly step (Method 2.2) to incorporate the linker DNA generated in Method 2.1.1. The linker DNA has regions of homology with the 3' end of the amplified VH gene and 5' end of the amplified VL gene, as well as sequence encoding the $(Gly_4Ser)_3$ linker peptide which joins the two variable chains. In the same PCR, the two outer flanking PCR primers provide restriction sites for cloning. We describe here the assembly of a ScFv fragment. V-genes can also be cloned sequentially, however, the assembly procedure allows cloning using only two restriction sites (4 are needed for sequential cloning), which reduces the risk of V-genes being cut by the cloning enzymes.

Method 2.1.1 Preparation of the ScFv linker fragment

1. For batch preparation of the murine ScFv linker fragment, set up a number of PCR reactions as follows. Mixing the following:

H_20	35.5 µl
10 x PCR buffer[a]	5 µl
5 mM dNTP[b]	2.5 µl
BSA[c] (10 mg/ml)	0.5 µl
MO-LINK-FOR (10pmol/ml)	2.5 µl
MO-LINK-BACK (10pmol/ml)	2.5 µl

pSW1-ScFvD1.3 (~10 ng/ ml)[d] 1 ml

2. Overlay the tubes with paraffin oil, heat to 94°C for 5 min using a cycling heating block. Then add 0.5 ml *Taq* polymerase (5 units /µl ; Cetus) under the oil.

3. Cycle 25 times: 94°C 1 min, 60°C 1 min, 72°C 1 min.

4. Purify the ScFv linker fragment on a 2% (low-melting-point) agarose gel.

5. Precipitate the filtrate by adding 1/10 vol. 3 M sodium acetate, pH 5.2, and 2.5 vol. ethanol. Chill on dry ice for 5 min, spin at 13000 r.p.m. for 10 min. Wash the pellet in 1 ml 70% ethanol and dry under vacuum. Dilute purified linker into 5 µl water or TE per original 50 µl PCR, measure concentration on gel, dilute to appr. 20 ng/µl and store in 5 µl aliquots at -20°C.

[a]10 x PCR buffer is 100 mM Tris-HCl, pH 8.3, 500 mM KCl, 15 mM $MgCl_2$ (for the Cetus enzyme).

[b]5 mM dNTP is a equimolar mix of dATP, dTTP, dCTP and dGTP, with a total concentration of 5 mM nucleotide at pH 7.0.

[c]BSA is acetylated BSA (New England Biolabs) at 10 mg/ml.

[d]The double-stranded ScFv linker (93 bp) can be amplified with the primers MO-LINK-FOR and MO-LINK-BACK from any ScFv gene made with the primers described in (Clackson, 1991). We use plasmid pSW1-ScFvD1.3 (McCafferty, 1990), yielding the $(G_4S)_3$ linker described by (Huston, 1988). MO-LINK-FOR is complementary to the VK2BACK primer. MO-LINK-BACK is complementary to the VH1FOR-2 primer.

Method 2.1.2: PCR assembly of primary VH and VK PCR bands in ScFv configuration

1. Make up two 50 ml PCR mixes containing :

	1	2
H_20	35.5 µl	25.5 µl
10 x PCR buffer	5 µl	5 µl
20 x dNTPs	2.5 µl	2.5 µl
BSA (10 mg/ml)	0.5 µl	0.5 µl
ScFv LINKER (~20 ng)	1 µl	1 µl
VH 1° PCR band (min. 100 ng)[a]	-	5 µl
VK 1° PCR band (min. 100 ng)[a]	-	5 µl

2. Overlay with paraffin oil , heat to 94°C for 5 min using the PCR-block.

3. At the end of this incubation, add 0.5 µl *Taq* polymerase (5 unit/µl) (Cetus) under the oil. Then cycle 7 times: 94°C 1.5 min, 72°C 2.5 min. See note b.

4. After this cycling add :

VH1BACKSFI	2.5 µl	2.5 µl
VK4FOR NOT-MIX	2.5 µl	2.5 µl

5. Cycle 25 times: 94°C 1.5 min, 55°C, 1 min, 72°C 2.0 min.

6. Analyse the PCR-assembly on a 2% agarose gel.

7. Gel-purify the assembled fragment on a 2% gel; excise the band corresponding to the assembled product (appr. 720 bp) and further purify the DNA using for example Geneclean (Bio101 Inc) or Magic PCR Preps (Promega). Resupend the product in 10-25 µl of water.

[a]The PCR products VH and Vl are to use after a preliminar gel purification to remove excess primers or primer-dimer complexes from the products of the first PCR before assembly.The starting amount of primary PCR bands seems to be critical for the assembly reaction: 50 ng or more may be required. A roughly equimolar amount of linker is used.

[b]To minimise spurious priming events, the overlaps should be made long (24 nucleotides) to allow annealing to be performed at a high temperature (72°C).

Method 2.3: Restriction and cloning of assembled PCR fragment into pHEN

1. For optimal digestion, the restriction of the PCR product is performed using the buffer supplied by the manufacturer. Large overdigestion is always necessary for efficient cutting. Mix the following together:

PCR-band	36µl
10 x NEBuffer 2[a]	5µl
BSA (1 mg/ml)	5µl
SfiI (10 units/ml)	4µl

2. Overlay with paraffin. Incubate at 50°C for 3-6 hrs or overnight.

3. To this mix, add under the oil :

water	31µl
10 x NEB-NotI[a]	5µl
BSA (1 mg/ml)	5µl

1 M NaCl	5µl
NotI (10 units/ml)	4µl

(Alternatively, the mix can be precipitated in between the restrictions).

4. Leave 4 hrs-overnight at 37°C.

5. Recover the aqueous phase and gel-purify the digested product on a 1.5 % agarose gel. Recover the DNA by extraction using Geneclean or Magic PCR preps (Promega).

6. Finally, resuspend the DNA in 10 µl water, and determine the concentration of the DNA by analysis on an agarose gel by running it with markers of known size and quantity.

7. Set up the following ligation reaction:

	1	2	3
10 x NEB-Ligation Buffer[b]	1µl	1µl	1µl
water	6 µl	5 µl	1µl
Vector pHEN1 (30 ng/ml)[c]	2 µl	2 µl	2 µl
Digested PCR band (20-50 ng/ml)	-	1µl	5µl

8. Spin for a few seconds in the microfuge. Then add :

T4-DNA ligase (400 units/ml, NEB)	1 µl	1 µl	1µl

9. Leave overnight at 16°C; store at -20°C until further use.

10. Transform E.coli cells[d] with the ligation mix, using competent cells for electroporation.

11. Plate fractions on TYE[e] plates containing 100µg/ml ampicillin and 1% glucose and growth at 30°C

[a]NEBuffer 2 is 10 mM Tris-HCl, pH 7.9, 50 mM NaCl, 10 mM $MgCl_2$ and 1 mM dithiothreitol ; NEB-NotI is 10 mM Tris-HCl, pH 7.9, 150 mM NaCl, 10 mM $MgCl_2$ and 0.1 % Triton X-100.

[b]10 x NEB-Ligation Buffer is 0.5 M Tris-HCl, pH 7.8, 0.1 M $MgCl_2$, 0.2 M DTT, 10 mM rATP and 500 µg/ml BSA.

[c]The phagemid pHEN1 is cut with SfiI and NotI and gel-purified

[d]For soluble expression of antibody fragments from pHEN1, TG1 bacterial strain can be used

[e]TYE is 20 g Bacto-tryptone, 5 g Yeast extract and 0.5 g NaCl per litre.

Method 2.3: Induction of ScFv expression

1. Pick colony into 3 ml 2xTY, 100 μg/ml ampicillin, 1% glucose grow with shaking (300 r.p.m.) overnight at 30°C.
2. Make 1:100 dilution of the overnight culture into 2xTY, 100μg/ml ampicillin, 0.1% glucose and grow at 30°C, shaking until O.D.600nm is approximately 0.9 (about 3 hrs)
3. Add IPTG (final concentration 1 mM IPTG). Continue shaking at 30°C for a further 5 hrs.
4. Harvest ScFv antibody from supernatant or from periplasmic space.

This method is based on that of (De Bellis, 1990), and relies on the low levels of glucose present in the starting medium being metabolised by the time the inducer (IPTG) is added.
During the overnight growth, the outer membrane becomes leaky and antibody fragment can be detected in the culture supernatant as well as in the periplasm. The supernatant cannot be stored because the tag is lost with prolonged incubation.

Method 2.4: WESTERN BLOTTING for detection of soluble ScFv fragments

Extraction of ScFv fragment from periplasmic space.
. spin culture for 15' at 4000 g
. harvest the supernatant
.spin again the supernatant for 45' at 4000g (this fraction can be stored at 4°C for short time)
. bacterial pellet is resuspended in TES buffer (1:100 of initial culture volume) , add then the same volume of TES buffer diluited 1:4
. put on ice for 20'
.spin at 12000-g for 20'
. take the supernatant fraction, which would be contain only periplasmic proteins, and analyse it (1/100 of the final volume is sufficient) on acrylamide 12% SDS denaturing gel
TES buffer 0.2M Tri-HCl pH 8, 0.5mM EDTA, 0.5M Sucrose

After electrophoresis, the gel has been transferred into a nitrocellulose membrane which is used during the western blot:

1. Immerse the membrane in 0.1% (v/v) Tween 20 in Tris-buffered saline (100mM Tris, 0.9% sodium chloride, pH 7.5) -TTNB- with 2% dry milk for 1 hr with gentle agitation.
2. After this incubation wash 3x in the TTNB buffer
3. Transfer the membrane to a solution of the 9E10 antibody (ascite 1:2000) in TTNB. Incubate for 1 hr with gentle agitation
4. Wash with 3-4 changes of TTNB over 15 minutes with gentle agitation
(If you use biotinylated antibody and the Vectastain detection system, you have to put sequentially together the different reagents that are included in the kit: 2 drops of reagent A to 10 ml of TTNB and then add 2 drops of reagent B, mix immediately and allow 30' for complex formation before use).
5. Transfer the membrane to a 5µg/ml solution of antimouse biotinilated secondary antibody in TTBS, incubate at room temp. for 1 hrs.
6. Wash as in the step 4
7. Transfer the membrane to the vectastain reagent solution (A+B). Incubate the membrane in this solution for 30' with gentle agitation
8. Wash as in the step 4
9. Transfer the membrane to the substrate solution (3-3'-Diaminobenzidine tetrahydrochloride tablet dissolved in 15 ml of TTNB and just before using, add 12 ml of fresh 30% hydrogen peroxide)

Method 3.1: Cloning into M13VPCR shuttle vectors

The heavy chain V-region primers incorporate a PstI site at the 5′ end and a BstEII site at the 3′ of the V-region (see figure 5a and table I). The PCR fragment and the shuttle vector M13VHPCR1 are digested with these enzymes, fragment and vector are subsequently gel purified and ligated together. Care should be taken to digest the vector completely, as it already contains a V-region fragment which produces a functional polypeptide chain. Recombinant clones are identified by sequence analysis of the M13 vector. The light chain V-region may be amplified by Pvu2/Bgl2 primer set compatible with the vector M13VKPCR1. The primers (Pvu2/Bgl2) and vector (M13VKPCR1) are associated with a few drawbacks: 1) the 3' restriction sites in the vector and the V-region are Bcl1 and Bgl2, respectively, which yield compatible overhangs but results in loss of both restriction sites upon ligation, 2) the M13VKPCR1 vector must be grown in a Dam- E.coli

strain due to the Bcl1 site, which will otherwise not be cut. Occasionally one isolates a V region which has an internal site identical to one of those used for cloning into the M13VPCR vectors (BglII, PvuII, PstI, or BstEII) or into the pSV2 eucaryotic vectors. In the case of our light chain, the cloning of VL into pSV2 (BamH1,HindIII) was been very difficult because VL presented two internal BamH1 sites. So it was necessary the extracting of VL fragment (BamH1, HindIII) by a partial digestion before cloning into the pSV2 eucaryotic vector (BamH1/HindIII digested). But the presence of these diagnostic sites within the V regions is very useful to check the success of cloning.

Method 3.2: Sequencing reactions

Preparation of single strand DNA of M13 derived vectors was made according to Sambrook and T. Maniatis.
For sequencing reactions we used the DNA Sequencing Kit 2.0 (USB):

Annealing template and primer
In a centrifuge tube combine the following
Primer 1µl
Reaction buffer 2µl
ssDNA 7µl (1µg for M13 vectors)
Warm the capped tube to 65°C for 2', then allow the temperature of the tube to cool slowly to room temperature over a period of about 30'. Once the temperature is below 35°C, annealing is complete. Place the tube on ice. Annealed template should be used within about 4 hours.

Labelling reaction
1. For standard reactions (reading sequences up to 500 or so bases from the primer) dilute the Labelling Mix (dGTP) 10-fold with distilled water. For sequencing within 30 based of the primer, diluition should be about 15 fold and the amount of template DNA must be greater than 0.5 pmol.
2. Dilute the Sequenase Version 2.0 enzyme 1:8 in ice-cold enzyme dilution buffer. Only enough enzyme for immediate use should be diluted (2µl for each reaction)
3. To the annealed template-primer add the following:
template-primer (above) 10µl

DTT 0.1M 1ml
Diluited Labeling Mix 2.0µl
a-^{35}SdATP 0.5µl
Diluited Sequenase Version 2.0µl
Mix thoroughly and incubate for 2'-5' at room temperature.

Nominally 0.5µl of mCi/ml and 10mM (1000Ci/mmol)dATP should be used. As little ad 0.1µl (1mCi) can be used for many experiments

Termination Reactions
1. Have on hand 4 tubes labelled G,A,T, and C
2. Place 2.5µl of the ddGTP Termination Mix in the tube labelled G. Similarly fill the A, T, and C tubes with 2.5µl of the ddATP, ddTTP and ddCTP termination mixes, respectively. Cap the tubes to prevent evaporation.
3. Pre-warm the tubes at 37°C at least 1'
4. When the Labelling incubation is complete, remove 3.5µl and transfer it to the tube labelled G. Mix, and continue incubation of the G tube at 37°C. Similarly transfer 3.5 µl of the labelling reactions to the A, T, and C tubes, mixing and returning them to the 37°C bath.
5. Continue the incubations for a total of 3.5'.
6. Add 4µl of Stop solution to each of the termination reactions mix thoroughly and store on ice until ready to load the sequencing gel.
7. When the gel is ready for loading, heat the sample to 75-80°C for 2' and load immediately on the gel.

We used a special sequencing gel (with a saccarose gradient) to read until >300bp, the composition of the gel is:

SOL 1 (0.5xTBE)
Acryl/Bis 40% (19:1) 4.5ml
Urea 12.7g
TBE10X 1.5ml
H_2O to 30ml
APS 10% 100µl
TEMED 30µl

SOL 2 (2.5xTBE)
Acryl/Bis 40% (19:1) 1.5ml

Urea 4.2g
TBE10X 2.5ml
BPB 1% 40ml
Sucrose 1g
H_2O to 10ml
APS 10% 40µl
TEMED 12µl

Add sequentially in a pipette of 10ml, 6ml of SOL 1 + 6ml of SOL 2, thus the gradient is formed in the pipette; 50W constant.

For sequencing directly the PCR products we used the Circum Vent ™ thermal Cycle Dideoxy DNA Sequencing Kit. This method requires much less template than does a standard reactions due to the linear amplification of labelled products, and there is no need to denature double-stranded DNA template before initiating the sequencing reactions.

Protocol for termal Cycle Sequencing with labelled dATP incorporation
Preparation of Deoxy/Dideoxynucleotide reaction tubes
1. Label 4 microcentrifuge tube, A, C, G, T
2. Using the Circum Vent deoxy/dideoxy Sequencing Mixes add 3µl of A mixture to the bottom of the A tube, and 3µl of C, G ant T mixtures to the bottom of the C, G, and T tubes, respectively.

Combining the template and primer
1. Mix together the following, in a 0.5ml microcentrifuge tube:
a) >200ng double-stranded DNA 3kb in length
b) 1.2 pmol primer
c) 1.5µl 10X Circum Vent Sequencing buffer
d) 1µl 30X TRITONX-100 solution
e) distilled water to a total volume of 12.0µl
2. Mix the solution by gentle pipetting

Addition of Label, Polymerase and Deoxy/Dideoxynucleotides
1. To the tube containing the template, primer, buffer, tritonX-100 and water, add 2µl of a-^{35}SdATP (500-1200Ci/mmol)

2. Add 2 units of Vent(exo-)DNA polymerase and mix the solution by gentle pipetting
3. Immediately distribute 3.2µl of this reaction to the deoxy/dideoxy tube labeled A and mix the solution by gentle pipetting, changing pipette tips each time, repeat this addition to the C, G and T tubes.
4. overlay each reaction with 1 drop of sterile mineral oil.
5. Place the tubes in the thermal cycler, which has been preset for reaction times and temperatures (use 20 cycles)

95°C 20sec
55°C 20sec
72°C 20sec

Terminating the reaction
1. Add 4µl of Stop/loading dye solution to each tube, beneath the mineral oil. The reactions are now complete and ready to be electrophoresed in appropriate denaturing sequencing gel

Method 3.3: Cloning into the expression vectors.

Once the V regions have been sequenced in the M13VPCR vectors, we use pSV2 based vectors (see figures 5a and 5b) which provide human C regions downstream of the cloning site for the V region, as well as a eukaryotic promoter, the SV40 origin of replication and selectable markers for expression. We use COS cells in transient transfections to demonstrate the presence of functional immunoglobulin. This exploits the SV40 origin of replication, which in the presence of the large T antigen, allows the plasmid to be amplified to high levels. The advantages of this system is that sufficient functional antibody to perform ELISAs can be produced after only three days, whereas the accumulation of equivalent amounts of antibody from stable clones can take weeks. The day before testing antibody specificity, we routinely check, by immunofluorescence for the production of immunoglobulin. This allows us to interpret a negative result as truly negative, as opposed to due to problems in the transfection efficiency.
This vector system can also be used to produce stable cell lines by transfecting into cells such as NSO and incubation in the appropriate selectable markers (G418 and hygromycin). This is very useful when the hybridoma is a rat/mouse hybrid, since it allows the production of ascites.

Double stranded DNA is made from the M13VPCR constructs. The V regions (with the associated elements) are extracted from the M13VPCR vectors by digestion with HindIII and BamHI, and cloned into the pSV2 based vectors which have also been digested with HindIII and BamHI. The pSV2 vectors grow very slowly, and often plates need to be left for two days to see colonies. DNA preparation should be done by CsCl gradient using standard methods (Sambrook, 1989)

Method 3.4: Transient transfection in COS cells with DEAE-dextran (Pelham, 1984).

Efficient transient transfection is absolutely dependent upon the quality of the cells and the quality of the DNA. Cells which have been allowed to overgrow may be irreversibly damaged (from a transfection point of view). Figures in parentheses in the protocol indicate the volumes and compositions which should be used when performing parallel transfections in order to use the supernatant for assays. Step 10 uses much less DMEM in order to concentrate the antibody, and includes HEPES to prevent acidification of the medium.

1. Plate the cells at semiconfluence in a 90mm (60mm) Petri dish the day before the experiment.
2. Add 10 mg plasmid DNA to 2.7 ml DMEM.
3. Add 0.3 ml 5 mg/ml DEAE-dextran[a] (Pharmacia, mol. wt. 500,000).
4. Wash the cells twice in DMEM.
5. Add DMEM/DNA/DEAE-dextran to the dish (final concentration = 0,5 mg/ml).
6. Incubate 30 min at 37°C.
7. Add 15 ml 100mm chloroquine[b] in DMEM/10% FCS.
8. Incubate 3 hrs at 37°C in CO_2 incubator.
9. Wash with DMEM.
10. Feed with DMEM/10% FCS (2.5ml DMEM/10%FCS containing 20 mM Hepes pH 7.2).
11. Harvest 2 (3) days later.

[a]DEAE-dextran should be autoclaved and stored at -20°C in small aliquots.
[b]Chloroquine (Sigma) should be filter sterilised and kept at -20°C in small aliquots.

Method 3.5: Indirect immunofluorescence detection of the two chains.

1. Plate COS cells on polylysine-coated glass coverslips which have placed at the bottom of the petri dish and transfect them following Method 3.2.
2. Wash three times by dipping into a beaker containing PBS.
3. Fix the cells with 3,7 % paraformaldheide in PBS for 10 min at room temperature.
4. Wash once with PBS.
5. Permeabilize the cells with Tris-Cl O,1 M pH 7.6/ 0.2% Triton X100 for 4 min at room temperature.
6. Wash three times by dipping into a beaker containing PBS.
7. Incubate with the appropriate dilution of anti-human isotype primary antibody (1-2 hours at room temperature) and subsequently with the fluorescein-labelled secondary antibody (1-2 hours at room temperature), with washes in PBS between incubations. 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. Biotin avidin based systems can also be used.

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