CONSTRUCTION OF A SINGLE-CHAIN ANTIBODY AGAINST INTERMEDIATE FILAMENTS

BY

SHARON ANN RUTHERFORD

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McGill University,
Montreal

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ABSTRACT

Intermediate filaments are fibrous proteins, appearing in a wide variety of tissue specific forms. The function of these proteins is poorly understood, although they are commonly believed to perform a structural role in the cell. Evidence suggests that the role these proteins play may be more dynamic than was previously believed. To gain more insight into their normal *in vivo* function, a single-chain monoclonal antibody has been constructed to serve as a specific reagent which can disrupt the intermediate filament network *in vivo*. The work presented in this thesis represents the first step in an approach which involves the use of single-chain monoclonal antibodies as specific reagents to target and disrupt the function of intracellular proteins.

The polymerase chain reaction was used for the cloning and modification of the heavy and light chain variable regions of the murine monoclonal antibody produced by the TIB 131 hybridoma. The variable regions of the light and heavy IgG chains were initially amplified from cDNA using degenerate 5' primers and 3' primers complementary to the constant region of the appropriate chain. The amplification products were cloned individually, sequenced, then modified to include restriction sites suitable for cloning into an expression vector. The two modified variable regions were cloned into an expression vector, and when expressed in either bacteria or in a rabbit reticulocyte lysate system, yielded a protein of the expected molecular weight.

ABREGE

Les filaments intermédiaires sont des protéines fibreuses se retrouvant dans une grande variété de t[illegible]. La fonction de ces protéines n'est pas vraiment connue, bien que l'on croie qu'[illegible] remplissent un rôle structural dans la cellule. Mais certaines évidences suggèrent que le rôle de ces protéines serait plus dynamique. Pour mieux comprendre leur fonction *in vivo*, une chaine unique d'un anticorps monoclonal a été construite dans le but d'être utilisée comme réactif spécifique pouvant rompre le réseau de filaments intermédiaires. Les résultats présentés dans ce mémoire représentent le premier pas à [illegible] méthode utilisant une chaîne unique d'anticorps monoclonaux comme [illegible] pouvant interrompre la fonction des protéines intracellulaires.

La réaction en chaîne à la polymérase a été utilisée pour cloner et modifier les régions variables légères et lourdes de l'anticorps monoclonal murin produites par l'hybridome TIB 131. Les régions variables des chaînes légères et lourdes de IgG d'un cADN ont été amplifiées au préalable utilisant des amorces dégénérées en 5' et complémentaires en 3' à la région constante du transcript produit. Les produits de l'amplification ont été clonés individuellement, séquencés et ensuite modifiés pour inclure des zones de restriction permettant de les insérer dans un vecteur d'expression. Les deux régions variables modifiées ont été insérées dans un vecteur d'expression et ensuite exprimées soit dans une bactérie ou un lysat de réticulocyte de lapin produisant ainsi une protéine d'un poids moléculaire escompté.

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LIST OF ABBREVIATIONS

Amino terminal	N terminal
Antibody constant region	Fc
Antibody variable region	Fv
Bovine serum albumen	BSA
5-bromo-4-chloro-3-indolyl-ß-D galactopyranoside	X GAL
Carboxy terminal	C terminal
Complementarity determinant region	CDR
Dulbecco's Modified Eagle Medium	D MEM
Ethylenediamine tetraacetic acid	EDTA
Ethylene glycol-bis(ß-aminoethyl ether) N,N,N',N'-tetraacetic acid	EGTA
Heavy chain variable region	V_H
Immunoglobulin G	IgG
Isopropyl-ß-D-thio-galactopyranoside	IPTG
Kilo Dalton	kDa
Light chain variable region	V_L
Luria-Bertani broth	LB
Monoclonal antibody	MAb
Nitrocellulose	NC
Phenylmethylsulphonyl fluoride	PMSF
Phosphate buffered saline	PBS

Polyacrylamide gel electrophoresis . . . . . PAGE

Polymerase chain reaction . . . . . PCR

Protein kinase A . . . . . PKA

Protein kinase C . . . . . PKC

Single-chain antibody . . . . . scFv

Sodium dodecyl sulphate . . . . . SDS

INTRODUCTION

Due to the complexity of biological systems, it has long been accepted that gaining more insight into the biological function of a particular protein or proteins relies on two approaches, 1) to study a protein in isolation and in pure form, or 2) to use agents that interact specifically with a given protein and inhibit its function, when in the presence of other proteins. The isolation of a protein, although difficult, is often the direction taken, due to the scarcity of reagents able to specifically interact with, inhibit or disrupt the target protein.

One of the characteristic features of recent developments in molecular biology is the emphasis on selectivity, notably, the ability to specifically disrupt the intracellular function of a targeted protein using an intracellularly expressed single-chain antibody (scFv). This approach has recently been shown to disrupt the biological activity of the human immunodeficiency virus type I (HIV-I) (Marasco *et al*, 1993).

Using a similar approach, the long term objective of the research presented herein is to disrupt specifically the intermediate filament (IF) network or cytoskeleton, and elucidate what effects this has on the morphology of cells and their signal transduction pathways.

Thus the immediate aim of this research thesis is to construct and express a full length scFv against IFs. This work represents the first essential step toward disrupting the biological function of these proteins by the intracellular expression of an anti-IF scFv.

LITERATURE REVIEW

This review describes the structure of IFs and presents a synopsis of their putative functions. A brief description of antibody structure and function precedes a review of the design, development and applications of scFvs, focusing on the intracellular expression of scFvs as specific inhibitors of protein function.

Intermediate filaments

IFs are components of the eukaryotic cytoskeleton. They are major components in many cells and are most prominent in vertebrates. Representative species from nearly all invertebrate phyla, as well as early chordates (Bartnik and Weber, 1989), higher plant cells (Hargreaves *et al*, 1989) and *Saccharomyces cerevisiae* (protein MDM1) contain IFs, suggesting that these proteins are universal components of eukaryotic cells (McConnell and Yaffe, 1993).

IF proteins share a prominent amino acid sequence which confers structural similarities, consisting of a central heptad-rich "rod" domain situated between a non-heptad N-terminal "head" domain and a non-heptad C-terminal "tail" domain (see Fig. 1). Although different types of IFs were originally classified according to their tissue and cell-type distribution, sequence analysis has justified the distinction of six, possibly seven different classes of IFs. Within each class, individual members are defined by the sequence of variable domains within the N- and C-terminal regions.

The central rod domain falls in two groups. Those, like vimentin, desmin, glial

fibrillary acidic protein (GFAP), peripherin and neurofilaments, are capable of forming homopolymers. When co-expressed in a cell, they co-polymerize with one another. Others, like the cytokeratins, obligately form heteroduplexes of an acidic and a basic keratin protein (Steinert, 1990).

The rod domain exists as four α-helical regions separated by three linkers. In IF assembly, conserved α-helical central regions align in a parallel orientation and wind together to a coiled-coil dimer (Fig. 1). Until recently, it was believed that two dimers align in an anti-parallel, in-register fashion to form a tetramer or protofilament (Fig. 1), considered to represent the actual building block of IFs. Steinert *et al* (1991) presented data suggesting that the mechanism of *in vitro* assembly, and, the dynamic *in vivo* assembly-disassembly characteristics of keratin IFs and IFs in general, are mediated through a variety of small oligomeric species ranging in size from one to several molecules rather than protofilaments (Ip *et al*, 1985; Stewart *et al*, 1989).

The N-terminal has recently been implicated in the stabilization (Potschka *et al*, 1990) and oligomerization processes (Traub *et al*, 1992), whereas the carboxy-terminal tail region appears to remain relatively inert (Eckelt *et al*, 1992). The antiparallel arrangement of the two coiled-coils has been reported to be, at least in part, determined by the two-fold, symmetrical association of the amino-terminal head region of one of the coiled-coil dimers with the carboxy-terminal halves of the α-helical rod domains of the anti-parallel coiled-coil dimer (see Fig. 1). Similar interactions occur during filament assembly and in the intact filament. Clearly, the association of the rod domains confer the stability, undoubtedly required in IF function.

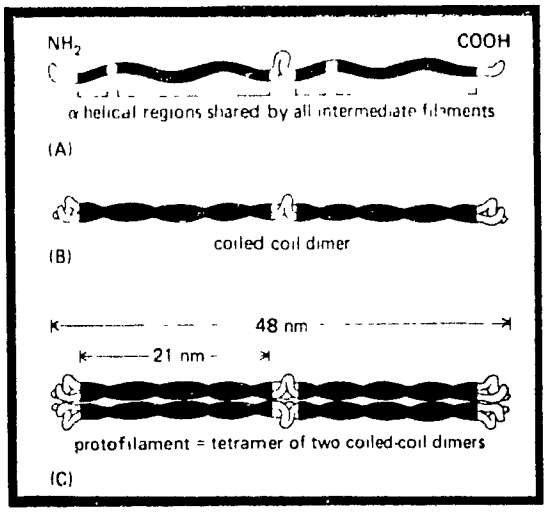

Fig. 1. Various units of intermediate filaments. (A) IF monomer, identifying the 4 conserved α-helical regions common to IFs; (B) coiled-coil dimer model formed by the parallel alignment of the α-helical rod domains, and; (C) tetramer or protofilament, generated by the anti-parallel association of 2 coiled-coil dimers. Reproduced from Alberts *et al*, (1989).

Although they have been recognized as important constituents of the cytoskeleton, it has only recently been acknowledged that the intracellular organization and assembly process of IFs is dynamic, both in intact cells during mitosis and in cell culture under experimental conditions. Two-dimensional gel analyses of proteins from [^{32}P]-labelled cells have revealed that phosphorylated vimentin is among the most prominent phosphoproteins in the cytoplasm (Tsuda *et al*, 1988). The phosphorylation of vimentin has been demonstrated to be site specific and restricted to the 9 kDa N-terminal domain (Ando *et al*, 1989; Geisler *et al*, 1989; Inagaki *et al*, 1987). This process appears to be involved in the reorganization of the vimentin filaments, which has been shown to be altered during mitosis (Chou *et al*, 1989; Franke *et al*, 1984; Evans, 1988). In fact, a near-amino-terminal nonapeptide motif, conserved from amphibia to man, located in the head domain of vimentin has been reported to be required, under physiological conditions, for the assembly of soluble subunits (Herrmann *et al*, 1992).

The dynamic aspects of vimentin and other IFs during the cell cycle has been well reviewed (Goldman *et al*, 1991). Vimentin has been reported to be a target of $p34^{cdc2}$, the catalytic subunit of the mitosis phase promoting factor, which is a protein kinase having several substrates, including the tumour repressor, p53. The vimentin sites phosphorylated *in vitro* by the vimentin/$p34^{cdc2}$ kinase have been determined (Chou *et al*, 1991). Amino terminal phosphorylation of vimentin by $p34^{cdc2}$, in BHK-21 fibroblasts, disperses the protein into numerous disassembled cytoplasmic aggregates. Upon dephosphorylation, aggregates accumulate as a filament cap near the surface of the daughter nuclei (Chou *et al*, 1990).

CLASS	COMMON NAME	ORIGIN	SIZE (kDa)	REF.
I	acidic keratins	all epithelia	40-60	1
II	neutral-basic keratins	all epithelia	50-70	1
III	vimentin	mesenchymal & cultured cells	53	1
III	desmin	myogenic cells	52	1
III	glial fibrillary acidic protein (GFAP)	glial cells and astrocytes	51	1
IV	neurofilaments NF-L NF-M NF-H	most neurons	60-70 105-110 135-150	1
V	lamins at least 4	nuclear lamina of eukaryotes	60-70	1,2
VI	nestin	neuroepithelial stem cells	200	3
VII(a)	tanabin	embryonic neural tissue	192	4

Table 1. Intermediate filaments: classes, cellular origin and molecular weights. References; (1) Steinert and Roop, 1988, (2) Dessev *et al*, 1990, (3) Lendahl *et al*, 1990, and (4) Hemmati-Brivanlou *et al*, 1992.

(a) The IF designation given to tanabin has been based on the size, sequence and percentage identity of the α-helical tracts and linkers of the central rod domain, and the size and sequence of the N- and C-terminal domains. Further information regarding introns and extent of variation that defines the neurofilament family, remains to be ascertained.

Vimentin IFs form a network which communicates with both the nuclear envelope and the plasma membrane. Two functionally distinct sets of vimentin attachment sites have been reported, reflecting a vectorial assembly process. Studies have shown that vimentin interacts in a cooperative and nonsaturatable fashion with the nuclear envelope, while under similar conditions, it associates saturably and noncooperatively with the plasma membrane (Georgatos and Blobel, 1987). One population of such receptors is located along the nuclear envelope and comprises polypeptides recognizing the carboxy-terminal tail domain. The plasma membrane contains binding sites that interact with the amino-terminal head domain (Vikstrom *et al*, 1991).

Close similarity in the general structure of desmin and vimentin heterodimers to that of members of different families of regulatory transcription factors has been reported, suggesting that IF proteins could play a role as regulatory DNA-binding proteins (Traub *et al*, 1987; Traub *et al*, 1993).

There is a considerable body of evidence implicating vimentin and other IFs as factors participating in signal transduction. In fact, IF proteins appear to be *in vitro* targets for kinases known to be activated by second messengers of both the protein kinase C (PKC) and protein kinase A (PKA) pathways (Ando *et al*, 1991; Georges *et al*, 1989; Inagaki *et al*, 1988). The association of PKC with vimentin and other insoluble cellular fractions has been well documented. The stimulation of PKC by DAG and Ca^{++} or by phorbol esters was reported to be coincident with the translocation of PKC from the cytosolic to the particulate fraction (Kraft and Anderson, 1983). Gopalakrishna *et al*, (1986) reported that activation by growth factors or hormones caused redistribution of

PKC to the particulate fraction of stimulated cells. Immunofluorescence studies demonstrated that endogenous PKC binds to cytoskeletal elements associated with the particulate fraction (Ito *et al*, 1989; Papapadopoulos *et al*, 1989).

It was also speculated that, in the PKC signal transduction pathway, the vimentin network functions as a docking site at which PKC isoforms encounter their respective substrates. Lipton *et al* (1992) reported that the homeostatic epithelial radial skeletal configuration of microvascular endothelial cells (MEC) is maintained through primary signals, using cAMP as a second messenger. Following trauma, Ca^{2+}-activated post-translational alterations of cytoskeletal components lead to rapid reorganization of the vimentin cytoskeleton, conforming to the irregular contours of the cell. The unique vimentin-associated compartmentalization of Ca^{2+} and PKC was observed in this process, suggesting that vimentin acted as a physical substrate in supporting signal transduction in activated MEC. The rapid signal transduction (100ms) in histamine-stimulated MEC suggests that the inherent electrical conduction qualities of vimentin may also participate in the process, as vimentin has been shown to bridge the plasma membrane-nuclear membrane gap.

In support of the observation of PKC localization, Spudich *et al* (1992) reported the translocation and association of the β-isoform of PKC to vimentin following antigen activation of rat basophilic leukaemia cells (RBLs), implying that such cytoskeletal arrays could serve as specific docking sites at which PKC isoforms encounter their respective substrates. The localization of PKC isoforms and their substrates to such formations could be a general mechanism to confer specificity to signal transduction pathways.

Vimentin and caldesmon (a cytoskeletal protein) have been implicated as mediators of endothelial cell contraction through PKC phosphorylation of these proteins (Stasek *et al*, 1992).

A possible physiological relationship between the cell cycle regulating Mos proteins and vimentin has been reported (Bai *et al*, 1993). c-Mos is an important cell cycle regulator and v-Mos expression induces an alteration of vimentin in transformed cells, which suggests a possible physiological relationship between Mos proteins and vimentin. Previous studies indicated that vimentin-containing IFs were involved in the transformation process resulting in reorganization of the cytoskeleton. A change in the binding pattern of various associated molecules to specific sites on the chromosomes, leading to a different pattern of gene sequestration and exposure, ensued (Chan *et al*, 1989).

Cumulative evidence thus far suggests that IFs are dynamic proteins likely to mediate a number of cellular functions. The lack of specific reagents to disrupt their function has hindered a direct study of their function *in vivo*. In one study, an IF specific MAb was microinjected into 3T3 cells. This lead to the transient disruption of the vimentin network. The reassembly or reorganization of vimentin was thought to be due to the degradation or transport of the IgG molecules (Klymkowsky, 1981).

To the present time, no attempt to transfect cells with an inducible scFv against these intracellular proteins has been reported. Such an approach would have, in this case, the potential of maintaining an intracellular scFv concentration suitable for continued IF disruption, enabling observations to be made relating to growth, morphology, protein

phosphorylation and c-*fos* expression. This methodology provides a powerful tool for targeting other cellular antigens, thus contributing to a knowledge of truly specific *in vivo* processes.

Antibody structure and function

Antibodies belong to the family of proteins known as immunoglobulins. They are multimeric glycoproteins which may be subdivided into five classes (IgA, IgD, IgE, IgG and IgM) according to the types of heavy chains they contain. Prototypic IgG is a tetramer consisting of two identical light chains (approximately 220 amino acids), and two identical heavy chains (approximately 450 amino acids), bridged by interchain disulphide bonds, stabilized by Van der Waals forces and folded to form globular domains, which are autonomously folding units (Novotny *et al*, 1983).

Each chain (see Fig. 2) consists of two principle regions. The N-terminal variable region (V) and the C-terminal constant region (C). Light chains contain one V and one C region, while IgG heavy chains have one V and three C regions. The variable domain of the antibody (Fv) is generated by the association of the variable regions of the light (V_l) and heavy (V_H) chains. The polypeptide chains are arranged so that each Ab molecule contains two identical antigen binding sites. By means of its constant domain (Fc), IgG mobilizes cellular defense mechanisms. The attachment of the Fc region to the receptors on many cells of the immune system triggers various functions such as phagocytosis, antibody-dependent cytotoxicity and the secretion of potent mediators (Kinet, 1989).

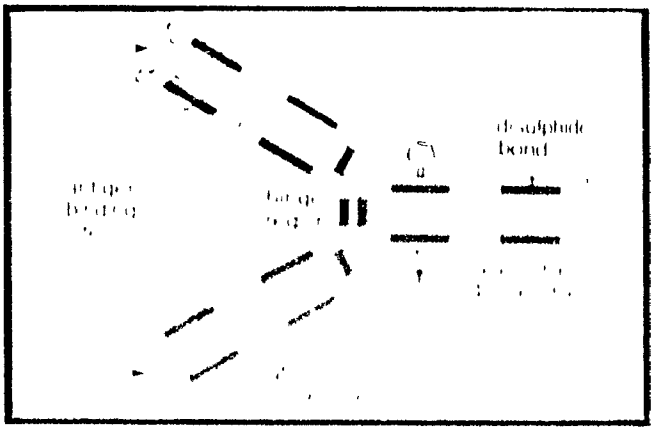

Fig. 2. The basic structure of IgG. The unit consists of two identical light polypeptide chains and two identical heavy polypeptide chains. The amino-terminal end is characterized by sequence variability in both the heavy and light chains. The light chain consists of one constant region (C_L), while the constant portion of the heavy chain is divided into three regions: C_H1, C_H2 and C_H3. Antigen binding sites are formed by the association of the variable regions of one light and one heavy chain. Reproduced from Roitt *et al*, 1989.

Each V region is encoded by two or three different elements, V_l and J_l (joining) for the light chains, and V_H, D (diversity) and J_H for heavy chains. Multiple copies of these elements generate a great number of variants, the number of which increases as a result of somatic changes in the individual germ line genes (Tonegawa, 1983).

The variable regions of each chain are characterized by three areas of hypervariablity, referred to as complementarity determinant regions (CDRs), which alternate with less variable framework areas (Kabat *et al.* 1977; Kabat and Wu, 1978). The antigen-binding region is composed of 6 CDRs, three contributed by each chain. The specificity and affinity of the binding site are determined by the structure and sequence of the six CDRs.

X-ray crystal analyses of immunoglobulins have been carried out on several intact antibodies revealing the structures of typical variable and constant domains. Each has the form of two approximately anti-parallel ß-sheets, connected by a disulphide bridge between cysteine residues at conserved positions in the sequence. The strands of the ß-sheets are connected by loops, six loops or CDRs - three from the V_l domain and three from the V_H domain - form the antigen binding site. This pattern is conserved from one antibody to another, even though there is variation in sequence of the variable domains, notably in the hypervariable regions. The less variable framework sequences determine the folding of the variable domain (Alzari *et al*, 1987; Amit *et al*, 1986; Clothia *et al*, 1985; Clothia *et al*, 1986; Clothia *et al*, 1989).

In mammalian cells, the heavy and light chains are cotranslationally translated across the membrane and into the lumen of the endoplasmic reticulum (ER). Like all

proteins destined for synthesis in the ER immunoglobulins are characterized by an N terminal stretch of hydrophobic amino acids, the signal sequence. In the lumen of the ER, several events occur such as immunoglobulin folding, formation of disulphide bonds, and the association of light and heavy chains into heteromeric molecules In addition to these critical steps, antibodies are glycosylated in the lumen of the ER and Golgi apparatus as they transmit to the cell surface (Hong and Tang, 1993)

Development, Design and Applications of scFvs

The development of scFvs resulted largely from attempts to improve clinical applications of MAbs. Size and antigenicity (most MAbs being rodent derived) of the native MAb, as well as that of the proteolytic products of the native MAb, hindered many potentially useful applications (Jain, 1987). Ethical considerations hampered the isolation of human MAbs, and attempts to secure stable hybridomas from peripheral blood lymphocytes proved difficult due to the limited number of cells involved in the immune response (Borrebeack *et al*, 1989).

Initially, to circumvent this problem, MAbs were "humanized" Original chimeric MAbs substituted human constant regions for murine constant regions. The murine hypervariable region was retained, preserving the binding specificity of the murine antibody (Jones *et al* 1986). More sophisticated approaches led to the transplanting of the 6 CDRs, rather than the whole variable region, from the rodent MAb directly into human MAb. The new MAb was as effective as the native rodent MAb with respect to effector function; however, binding affinity was reduced (Reichmann *et al*, 1988) Both

approaches eventually elicited some degree of anti-idiotype responses (Brüggemann, *et al*, 1989).

The first scFv was developed by Huston *et al* (1988). This 26 kDa polypeptide comprised the V_H and V_L of the anti-digoxin MAb 26-10, connected by a 15 amino acid linker. X-ray coordinates from the 26-10 Fab fragments were computer analyzed to determine the distance between the C terminus of the V_H and the N terminus of the V_L. Avoiding both an ordered secondary structure as well as a tendency to interfere with protein folding, a 15 residue sequence $(Gly\text{-}Gly\text{-}Gly\text{-}Gly\text{-}Ser)_3$ was designed to bridge the determined gap. The construct was assembled from 58 synthetically generated oligonucleotides and expressed as a fusion protein [(leader)-Asp-Pro-V_H-(linker)-V_L] in *Escherichia coli*, under the control of the *trp* LE sequence (Miozzari and Yanovsky 1978). Once cleaved at the Asp-Pro site, the isolated and renatured protein exhibited a similar specificity for digoxin and a binding affinity that was 6 folds lower than that of the Fab fragments.

Several aspects of scFv construction, expression and applications have been improved upon. One of the first improvements with respect to construction was the independent introduction of the polymerase chain reaction (PCR) (Saiki *et al*, 1988). Heretofore, genes encoding the V regions for scFvs were assembled from overlapping synthetic oligonucleotides of various lengths, a process considered to be the rate limiting step in scFv construction. General PCR methods to isolate the V region sequences from any immunoglobulin secreting cell were described by Larrick *et al* (1989), Orlandi *et al* (1989) and Songsivilai *et al* (1990). Conserved sequences at each end of the nucleotide

sequence encoding the V domain of immunoglobulins were used to design the primers. The 3' primers (VFOR) were constructed from conserved sequences within the Fc region of the light and heavy chains. Each of these sequences was unique and 100% homologous with germ line Fc regions. The 5' primers (VBACK), designed using the database of Kabat *et al* (1983), were suitably degenerate (implying that more than one nucleotide could exist at a particular position in the primer sequence), to accommodate differences in sequences. The versatility of database designed primers was demonstrated in investigations by Orlandi *et al* (1989), in which an identical set of primers successfully amplified the variable regions of five different hybridoma cell lines.

Another refinement focused on linker design. Criteria for linker design required that it be long enough to span the distance between the V_L and the V_H in the Fv structure, flexible enough to allow association of the V regions, and suitably hydrophilic, as it is exposed to the aqueous surface of the molecule. Several linkers have been successfully used in scFv construction. The 3-dimensional structure of the myeloma protein MCPC603 (Segal *et al*, 1974) was used by Bird *et al* (1988) to model the linker, EGSKSSGSGSESKST, which was used successfully to link the V_L to the V_H. The original $(G_4S)_3$ linker has demonstrated the usefulness of small amino acids in linker design. It has been successfully used both to link the V_H to the V_L (Huston *et al*, 1988; Marasco *et al* 1993; Nicholls *et al*, 1993) and the V_L to the V_H (Chaudary *et al*, 1989; Glockshuber *et al*, 1989). One notable linker, a flexible peptide (28 amino acids) of a naturally secreted fungal cellulase, was used in the construction of the secretable scFv (Takkinen *et al* 1991). Despite the success with different linkers, binding affinity matching that of the

native Ab was difficult to achieve. Several groups reported a reduction in the scFv binding affinity, ranging from a 2-10 fold reduction (Bedzyk *et al*, 1990; Bird *et al*, 1988; Condra *et al*, 1990; Huston *et al*, 1988; Pantoliano *et al*, 1991), compared to that of the Fab. Recently, enzymatic inverse PCR was used to generate a library of scFv mutants in which the V_l was linked to the V_H by a 15 amino acid peptide of variable composition, yielding a binding affinity equal to that of the native Fv (Stemmer *et al*, 1993).

Several different expression systems for scFvs have also been developed. Pioneering work in the production of Abs generated by means of recombinant techniques, involved the individual cloning and expression of murine heavy and light chain genes in *E. coli*. (Boss *et al*, 1984). Expression of complete immunoglobulin genes took place in myeloma cells, which appeared to offer the preferred environment since the transfected genes were faithfully transcribed, translated and glycosylated (Morrison, 1985). Wood *et al* (1985) investigated the expression of immunoglobulin genes in yeast and demonstrated the synthesis, processing and secretion of the light and heavy chains; however, the efficiency of assembly of soluble MAb was low. The first scFvs were expressed in *E. coli*; however, the proteins could only be produced in non-native state as inclusion bodies (Huston *et al*, 1988; Miozzari and Yanofsky, 1978). Skerra and Pluckthun (1988) were able to express, in *E. coli*, completely functional antigen binding fragments of the McPC603 MAb, having the same affinity constant as the intact antibody, by linking the V_H and V_l genes to the signal sequence of outer membrane protein A (*ompA*). ScFvs were secreted thus into the periplasm and did not require *in*

vitro manipulations. More recently, the pectate lyase gene (*pelB*) from *Erwinia carotovora* has been used for secretion of the scFv into the culture medium (Ayala *et al*, 1992; Marks *et al*, 1991). A major advantage of the use of a secretion system over the production of scFvs and larger immunoglobulin fragments as inclusion bodies in *E. coli*, is that the secretion of the recombinant protein in soluble form avoids the need for protein solubilization from the isolated inclusion bodies. The latter procedure generally results in some loss of activity.

Novel PCR applications have resulted in methodologies which have expedited and improved scFv construction (Davis *et al*, 1991; Horton *et al*, 1989), largely through the elimination of the need for a 3-piece V_H - linker - V_L ligation. Single-chain antibody technology is associated with an abundance of other applications ranging from clinical uses to catalytic functions. The development of scFvs has significantly reduced three hurdles associated with the clinical or therapeutic uses of MAbs or their fragments: 1) potential toxicity caused by the circulation of unbound, uncleared radiolabelled MAbs (Colcher *et al*, 1990), 2) inability of MAbs to penetrate large tumour masses, (Milenic *et al*, 1991; Yokota *et al*, 1992), and 3) appearance of human anti-mouse Abs (Huston *et al*, 1993).

ScFvs have been modified to become immunotoxins, recombinant proteins in which a toxin is linked to either the amino terminus or carboxy terminus of the scFv (Batra *et al*, 1990(a); Chaudary *et al*, 1990; Condra *et al*, 1990). The use of a scFv interleukin-2 fusion protein to target IL-2 activity to cells of the immune system in the area of tumours, has been the focus of recent work (Savage *et al*, 1993). Catalytic scFvs

have also demonstrated potential (Gibbs *et al*, 1991; Hilvert *et al*, 1989; Iverson and Lerner, 1989; Tramontano *et al*, 1986; Schultz, 1989). Efficient *in vivo* expression of functional catalytic scFvs could target selective chemical transformations, such as complementation of a deficient metabolic pathway (Bowdish *et al*, 1991).

Intracellularly expressed scFvs as inhibitors of protein function

The strategies designed to eliminate the function of a single protein without relying on genetic alterations in its structure or level of synthesis, have been based on the inducible cellular expression of a neutralizing antibody to selectively inactivate the protein. Evidence demonstrating the ability of microinjected MAbs to induce phenotypic effects *in vivo* have been reported. The introduction of MAbs specific for the oncogenic v-Ki-*ras* protein into cells transformed by this protein, induced a transient reversion of the cells to a normal phenotype (Feramisco *et al*, 1985). Affinity purified anti-actin Mabs, when microinjected into *Xenopus* oocyte nuclei, perturbed mitotic spindle formation (Rungger-Brandle *et al*, 1979). In an experiment particularly germane to this project, anti-IF MAbs were able to transiently disrupt the IF network, resulting in the collapse of the IF network onto the nucleus (Klymkowsky, 1981).

The model system selected to test the feasibility of inducible MAb expression was the yeast enzyme alcohol dehydrogenase (ADH), due to its comprehensive characterization (Jornall, 1977) and the existence of three characterized neutralizing MAbs against the ADH activity (Carlson and Weissman, 1988). Carlson (1988) was able to confer some degree of allyl alcohol resistance upon yeast *in vivo*. Isolated heavy and

light cDNAs, from which signal sequences had been removed to ensure cytoplasmic location, were expressed concomitantly, in diametric orientation, under the control of a galactose inducible promoter. This resulted in the synthesis of stable heavy- and light-chain polypeptides, with an ensuing limited degree of neutralization *in vivo*.

Other specific cellular compartments have been targeted as location sites for independently expressed antibody chains. Biocca *et al* (1990) substituted the hydrophobic amino acid core of the signal peptide for secretion with hydrophillic residues, resulting in the association of the mRNA to free (as opposed to bound) polysomes. The same group substituted the signal peptide with the nuclear localization signal of the large T antigen of the SV40 virus, leading to the nuclear localization of the heavy and light chains.

The addition of the ER KDEL retention peptide (Lewis and Pelham, 1992) to a scFv recognizing the gp120 HIV-1 envelope protein, secured the co-location and subsequent interaction of the scFv with the precursor gp160 in the ER (Marasco *et al*, 1993), marking the first example of the disruption of an intracellular mechanism by a scFv. Direct binding of the scFv to gp160, which is normally cleaved in the ER to yield gp40 and gp120, was postulated to be the cause of reduced gp120 expression and diminished virus particle infectivity. A similar investigation was undertaken in plants. A constitutively expressed scFv against a plant virus resulted in the reduction of infection incidence and a delay in symptom development. It was speculated that this phenotype was the result of the scFv binding to the Ca^{2+} binding sites on the coat protein, abolishing a critical step in the uncoating of the virus or in the assembly of progeny virus

(Tavladoraki *et al*, 1993). Firek *et al* (1993) reported an accumulation of functional scFv protein in the cell wall continuum (apoplast) of transgenic plants following transformation with a scFv gene, suggesting that it may be feasible to use plant synthesized scFv proteins to perturb the activity of extracellularly located antigens as an approach to generate pathogen resistant plants.

Clearly, the information presented in this review establishes scFvs as potent agents for the disruption of targeted intracellular systems. The construction of an anti-IF scFv will represent the first step in addressing the function of these proteins.

MATERIALS AND METHODS

Reagents

Tissue culture supplies and antibiotics were purchased from Gibco BRL (Grand Island, NY) and Hyclone (Logan, UT). Bacterial media supplies were furnished by Difco (Detroit, MI). The TIB 131 hybridoma cell line was purchased from the American Type Culture Collection (Rockville, MD), and DH5α *E. coli* were purchased from Gibco. Plasmid expression (pRSET) and cloning vectors (pCR™1000 and pCR™)II, accompanied by JM109 and InvαF' *E. coli* strains respectively, were purchased from Invitrogen (San Diego, CA). RNAzol B was purchased from Cinna/Biotecx Laboratories International Inc. (Houston, TX) and the RNA "Fast Track" kit was furnished by Invitrogen. RNAsin ribonuclease inhibitor and Muloney Murine Leukaemia Virus (M-MLV) reverse transcriptase were purchased from Gibco. Oligonucleotides were synthesized by either

Institut Armand Frappier (Laval, Que.), Sheldon Biotechnology (Montreal, Que.) or the Ontario Cancer Institute (Toronto, Ont.). PCR reagents were obtained from Promega (Madison, WI) or Pharmacia (Uppsala, Sweden) and the PCR reaction was performed on a GeneAmp 9600 Perkin Elmer Cetus Thermocycler (Norwalk, CT). Isopropyl-ß-D-thio-galactopyranoside (IPTG) and 5-bromo-4-cholor-3-indolyl-ß-D galactopyranoside (X-GAL) were purchased from ICN Biomedicals Canada (Mississauga, Ont.) DNA restriction enzymes and T4 ligase were purchased from either Gibco, Promega or Pharmacia. DNA was purified from agarose gels using a Sephaglas BandPrep Kit from Pharmacia. DNA templates were sequenced using either a Sequenase II kit (United States Biochemical Corp., Cleveland, OH) or a Pharmacia T7 Sequencing kit. All isotopes were purchased from Dupont-New England Nuclear (Boston, MA), and autoradiography was performed using X-OMAT AR2 film (Eastman Kodak, Rochester, NY).

Ultrapure SDS-PAGE and Western blot analysis reagents were products of Bio-Rad Laboratories (Anaheim, CA), as were protein molecular weight standards. Nitrocellulose for protein transfer was supplied by Amersham International (Amersham, England) as were ECL Western blot detection reagents. Bovine serum albumen (BSA) used for blocking was supplied by BDH Inc. (Toronto, Ont.). Horseradish peroxidase conjugated goat-anti-mouse IgG was supplied by Gibco. Methionine-free rabbit reticulocyte lysate-based coupled-transcription/ translation reagents, were supplied by Promega (Madison, WI). Protease inhibitors were purchased as follows; phenylmethylsulfonyl fluoride (PMSF), α-macroglubulin and pepstatin from Boehringer Mannheim Canada (Laval, Que.), leupeptin from ICN Biochemicals (Cleveland, OH),

EDTA from BDH and EGTA from Sigma (St. Louis, MO).

Apparati used for SDS-PAGE was supplied by Hoeffer (San Francisco, CA); sequencing was performed on a Stratagene (La Jolla, CA) Base Ace, and; DNA gel electrophoresis was executed on Bio-Rad sub apparatus.

Plasmids and E. coli strains

Plasmids pCR™1000 and pCR™II, designed for cloning PCR products, were in the form of a TA Cloning Kit (see Appendices A and B). Plasmid pCR™1000 (version 1.1) was used initially to clone the V_H. Due to difficulties encountered with sequencing, V_L was cloned into the improved pCR™II (version 1.3). Both systems take advantage of the non-template dependent activity of thermostable *Taq* polymerase used in PCR, that add single deoxyadenosines to the 3' end of all duplex molecules. These A overhangs are used to insert the PCR product either TA cloning vector, which provides single 5' T overhangs at the insertion sites. The prokaryotic expression vector, pRSET is a PUC-derived expression vector designed for high level protein expression and purification from cloned genes in *E. coli*, although the sequences coding for the purification option were removed for our purposes (see Appendix C). This vector is propagated in an *E. coli* strain which does not contain the T7 polymerase necessary for expression. Infection with an M13 phage containing the T7 RNA polymerase gene driven by the *E coli lac* promoter, induces expression of the recombinant protein.

Two standard *E. coli* strains were used for transformation, DH5α and InvαF'. JM109 was used in expression experiments. Bacterial cultures were generally propagated

in LB broth or on LB agar (15% agar) supplemented with the appropriate antibiotic (ampicillin 100 μg mL^{-1} or kanamycin 50 μg mL^{-1}). Unless otherwise stated, all antibiotic resistance selection was achieved using the aforementioned concentrations. Transformed JM109 cultures were maintained on M9 minimal media agar plates, supplemented with thiamine (0.5%) and ampicillin. SOC broth was used for scFv expression in JM109.

Tissue culture and cell lysate preparation

Murine hybridoma (ATCC TIB 131) and human lymphoma (CEM) cells were cultured at 37° C in a 5% CO_2 humidified atmosphere, in Delbecco's Modified Eagle Medium (D-MEM) supplemented with 10% fetal calf serum (characterized). The TIB 131 hybridoma secretes an IgG_1 MAb which reacts with all classes of IFs (Pruss *et al*, 1981). It was generated by the fusion of the mouse myeloma line NS-1 with spleen cells from C3H/He mice which were immunized with human GFAP. It is noteworthy that the myeloma fusion parent, the nonimmunoglobulin-secreting myeloma cell line, P3/NS1-Ag4-1, derived from Balb/C myeloma P3 (Kohler *et al*, 1976), expresses, but does not secrete, a kappa chain. Hybridoma supernatant was used after 3 days of culturing.

Total cell lysate from human lymphoma CEM cells (a gift from Dr. W. Beck at St. Jude's Children's Research Hospital, Memphis, TE) was prepared from cells in log growth. Briefly, 1.0 x 10^7 cells were washed 3 times in ice cold PBS and resuspended in 2 X Laemmli sample buffer (0.123 M Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, 10% ß-mercaptoethanol) containing protease inhibitors (2 mM EDTA, 4 mM EGTA, 4 mM PMSF, 2 μM leupeptin, 2 μM pepstatin, 0.2 U mL^{-1} α-macroglobulin). The lysate

was passed 15 times through a 25 5/8 guage needle then centrifuged to pellet insoluble particles (10 min., 4°C, 16,000g). An equal volume of sterile water was added to the lysate supernatant, which was subsequently boiled for 5 min. and stored at -20°C until used.

SDS gel electrophoresis and Western blotting

Aliquots of CEM total cell lysate (0.25×10^6 - 0.5×10^6 cells) were resolved on a 10% acrylamide gel as described by Laemmli (1970). Fractionated proteins were visualized by silver staining as described by Harlow and Lang (1988). For Western blotting, resolved proteins of a determined cell number were transferred to a nitrocellulose membrane (Hybond-C) as described by Towbin *et al* (1979). The membrane was blocked overnight at 37°C with a 3% solution of BSA in phosphate buffered saline (PBS) containing sodium azide (0.015 M), then exposed (overnight, 4°C) to a 1:25 dilution (5% powdered Carnation skim milk in PBS and 0.015 sodium azide) of either TIB 131 supernatant or D-MEM, or 5% milk alone. Following three PBS washes, a 1:1000 dilution of horseradish peroxidase conjugated goat-anti-mouse IgG: 5% milk (PBS) was used to probe the membrane (2 hr., 25°C). Following three PBS washes, the membrane was patted dry, washed in ECL Western blot detection reagents for 1 min., exposed to film for 1 min. then developed.

RNA isolation and reverse transcription

Total RNA was extracted from 2.5×10^7 late-log phase TIB 131 hybridoma cells

as described by Chomczynski and Sacchi (1987) using RNAZol B. Spectrophotometric analysis (λ260) revealed a concentration of 3 μg/μL, and a $\lambda_{260}/\lambda_{280}$ ratio of 1.74. First strand cDNA synthesis for the V_H PCR amplification was based on a protocol by Sambrook et al (1990). A 20 μL reaction mixture containing 10 μg of total RNA, 250 μM dNTPs, 50 pmoles of oligo-T primer, 5 mM $MgCl_2$, 15 mM KCl, 10 mM Tris-HCl (pH 8.3), 20 U RNAsin ribonuclease inhibitor and 50 U M-MLV reverse transcriptase, was incubated at 37°C for 60 min., 75°C for 5 min. and 10°C for 10 min.

For the V_L, a "Fast Track" mRNA isolation kit was used. First strand synthesis protocol differed somewhat from that used for the heavy chain. A 20 μL reaction volume containing 1 μg mRNA, 40 U placental ribonuclease inhibitor, 50 pmoles oligo-T primer, 0.1 M dithiolthreitol, 50 U M-MLV reverse transcriptase, 5x buffer and 200 mM dNTPs was incubated at 37°C for 60 min. and at 95°C for 5 min..

PCR and cloning

Amplification of the V_H with thermostable *Taq* polymerase, was performed in a 50 μL reaction volume, containing 4 μL of the cDNA reaction, 300 pmoles each of the V_HFOR1 (5'- TGG AGC TGT TTT GGC - 3') and V_HBACK1 primers (5' AGG T(C/G)(C/A) A(G/A)C T(G/T)C TCG AGT C(T/A)G - 3') (Orlandi *et al* 1989, and Sastry *et al*, 1989), 200 μM of each dNTP, 2 mM $MgCl_2$, 10 mM Tris HCl (pH 8.3), 15 mM KCl and 5 U *Taq* polymerase, which was subject to 40 rounds of temperature cycling. A cycle consisted of denaturation at 94° C - 1 min., annealing at 37° C - 1 min., elongation at 72° C - 2 min.. An extended elongation period (72° C - 30 min.) completed

the reaction.

Amplification of V_l was carried out as previously described with the following exceptions: 200 μM of each dNTP, 1 U *Taq* polymerase, 3 μl of the cDNA product and 50 pmoles of V_lFOR1 (5'- TGG TGG GAA GAT GG - 3') (Hamlyn *et al*, 1978) and V_lBACK1 (5'- GGT CGA CCG A(C/T)A T(C/T)G T(G/C)C TNA CNC AAT CNC CAG C - 3') (Huston *et al*, 1989) were used. It should be noted that amplification with an 18 nt primer (5'- ACC ACG TCG TAG TCG GGC - 3') immediately 5' to the latter sequence failed to amplify the target region.

As previously stated, the two PCR products were ligated into different versions (1.1 and 1.3) of the pCR TA cloning vector. Ligation reactions were executed according to the recommended 1:1 to 1:3 molar ratio of vector: PCR product, using 50 ng of the vector per reaction. Specifics of the reaction were followed as described in the manufacturer's protocol. Competent *E. coli* were transformed and plated on LB agar with kanamycin and X-GAL (0.4 mg mL^{-1}). Selected white colonies were used to inoculate 3 mL of LB broth containing kanamycin. The cells were incubated overnight at 37°C in a gyratory shaker-incubator. Plasmid DNA was isolated using an alkaline lysis method as described by Sambrook *et al* (1990), with the addition of a final precipitation using PEG and NaCl.

DNA sequence determination

Double stranded dideoxynucleotide chain termination sequencing was performed, based on the method described by Sanger *et al* (1977). Sequences of both the heavy and

light chains were determined using the M13 Forward (5'-GTA AAA CGA CGG CCA GT-3') or M13 Reverse (5'-CAG GAA ACA GCT ATC AC-3') primers. One V_H clone and 6 complete and 5 partial V_L clones were sequenced (see RESULTS). Translations of the sequences were compared to GenBank amino acid sequences (computation performed at the NCBI using the BLAST network service).

Modification of the V_H and V_L

Modifications of both variable regions were necessary for the following reasons, 1) two distinct light chains were being amplified by the PCR reaction (see RESULTS) and it was necessary to enrich for the one believed to be that recognizing IFs, and 2) no V_H restrictions sites, suitable for the construction of a scFv were present.

Modification of the V_L

Given that two distinct light chains were being amplified by the PCR reaction (see RESULTS), selection for and modification of anti-IF V_L was performed using the original V_LBACK1 primer and a new 3'primer:

Apa I ____ tag ____ Nhe I

V_LFOR2 5' - GGG CCC ACC CTC CAA AGT ATT AGG GCT AGC GTT TCC AGC TTG GTC CCC CTC -3. This new primer incorporated nucleotides coding for a tag, a P-glycoprotein epitope (PNTLEG), recognized by the C494 MAB (Georges *et al*, 1990, a gift from Victor Ling, O.C.I). This primer took advantage of the additional 30 bases present at the 3' end of only the Class II clone. The 3' end of V_LFOR2 was complimentary to the positions 313-333 of the Class II V_L sequence. An *Nhe*I site was

inserted between the V_l and the tag in order to render the vector and tag amenable to accepting other scFvs. The *ApaI* restriction site was redundant for use in the pRSET vector. It had originally been included for the scFv insertion into another plasmid. PCR was performed as previously described for the V_l, using newly reverse transcribed cDNA. The PCR product of anticipated size was excised from a 2% agarose gel, purified according to manufacturers directions, then ligated into pCR™II as previously described. Transformation, plasmid purification and sequencing procedures were repeated.

Modifications of the V_H

Two new PCR primers were designed. The new 5'primer,

Nde I — Sal I

V_HBACK2 5' - CAT **ATG** GAG GTC CAG CTG CAG GAG TCA GGA CC -3'

incorporated an *Nde* I site for insertion into the pRSET expression vector and an in frame translation start site. The new 3' primer V_HFOR2 5' - GTC AGT CGA CTT AGA TTC GGA ACC AGA ACC GGA AGA CAA GCT TGA GGA GAC TGT GAA AGT GGT GCC TTG -3' was complimentary to positions 343-364 of the V_H, coded for a flexible linker (SSSLSSGSGSESKST) and included an overlapping 3' *Sal* I site to link to the V_l. Amplification was carried out as previously described, using the new primers and the original pCR™1000-V_H plasmid as the template. The PCR product was run on 2% agarose gel and visualized with ethidium bromide.

Construction of the scFv

A diagrammatic representation of the procedure used to construct the scFv in the pRSET expression vector is shown in Fig. 10. Digestion of two plasmids, pCR™II-V_l

containing V_L, and the expression pRSET, as well as the V_H PCR modification product took place in 50 μL reaction volumes. A total of 4 μg of PCR[TM]II-V_L was digested (3 hr., 37°C) with 20 U *Sal* I, precipitated with 1/5 volume 7M NH_4CH_3COO and 2 volumes 100% ethanol, washed with 1 mL 70% ethanol, dried, then digested with 20 U *Eco* RI (3 hr., 37° C). In a similar fashion, 4 μg of pRSET were digested with 20 U *Eco*, then 20 U of *Nde* I and finally, 1 μg of the V_H PCR product was digested with 20 U *Sal* I and 20 U of *Nde* I. The digests were run on a 2% agarose gel (with ethidium bromide) and the appropriate bands were excised and purified. The yields of the purified DNA were estimated to be approximately 500ng for each preparation.

A 20 μL triple ligation reaction contained approximately 100 fmoles of digested vector, 35 pmoles of each insert (V_H and V_L), ligation buffer, and 1 U T4 ligase (overnight, 15° C). Competent DH5α cells were transformed with 10 μL of the ligation reaction. Colonies were randomly selected for plasmid purification, and those plasmids appearing to contain an insert, upon visualization on a 1% agarose gel (results not shown), were digested with the appropriate restriction enzymes to determine whether they were candidates for sequence verification. Four plasmids were selected for sequencing. All four appeared to be candidates for a full length scFv, and all four were used to transform JM109 cells, for maintenance on minimal salts agar with thiamine (0.5%) and ampicillin. Clone 18a was selected for expression experiments.

scFv expression

The pRSET prokaryotic expression vector was used to express the scFv. It

contains a T7 promoter from which the recombinant protein can be transcribed. Once infected with the M13 phage, which carries the T7 polymerase gene under control of the *lac* promoter, F' cells (JM109) transformed with the pRSET-scFv provide the environment for protein synthesis from the T7 transcribed RNA template.

Following manufacturers instructions, the phage stock accompanying the kit was titered (8 X 10^{10} pfu/ mL). To induce expression of the recombinant protein, IPTG (1 mM final concentration) was added to a 50 mL culture of log phase transformed JM109 (OD_{260} = 0.3) cells. These were infected after 1 hour (OD_{260}= 1.0) with 5 pfu/cell. One mL samples were removed at 1.5 hr. intervals, centrifuged (4 min. 16,000g), the supernatant removed, and the pellet frozen at -20° C. When all the time points had been collected, the pellets were resuspended in 100 μL of 0.5 M phosphate buffer, then subjected to three freeze/thaw cycles between a dry ice/methanol bath and a water bath of 42° C. The samples were centrifuged (10 min., 4° C, 16,000g). An equal volume of 2x Laemmli sample buffer containing previously mentioned protease inhibitors, was added to the extracted supernatant, and the remaining pellet was resuspended in equal amounts (200 μL total) of 0.5 M phosphate buffer and 2x Laemmli buffer, and passed 15 times through a 25 5/8 gauge needle. All samples were boiled 5 min. then stored at -20°C.

A 20 μL volume of each time point of both the supernatant and pellet preparations were resolved on two separate 10% acrylamide gels as previously described. Half of each gel was stained with Coomassie blue, and the other half used to transfer the proteins to a nitrocellulose membrane for Western blot analysis using the C494 MAb.

The TNT™ T7 Coupled Reticulocyte Lysate System was used to express the scFv under eukaryotic conditions. The protocol accompanying the manufacturer's instructions were followed. The 50 μL lysate reaction contained the TNT rabbit reticulocyte lysate, reaction buffer, T7 polymerase, amino acid mixture minus methionine (1 mM), ^{35}S-methionine (1,000Ci mmol^{-1}) at 10mCi mL^{-1} (translation grade), RNasin ribonuclease inhibitor (40 U μL^{-1}), 1 μg pRSET-scFv plasmid DNA containing the T7 promoter, and nuclease-free H_2O. The reaction was incubated at 30° C for 90 min. A 2 μL aliquot was removed and resolved on a 10% acrylamide gel.

RESULTS

MAb production by TIB 131 Hybridoma cells

TIB 131 MAb binding to IF proteins in CEM cells was determined by Western blot analysis. Figs. 3 and 4 illustrate the SDS-PAGE resolution of total CEM cell lysate in Laemmli sample buffer (see METHODS). The resolved proteins were either silver stained, to demonstrate the heterogeneous population of proteins in CEM cells (Fig. 3), or transferred to a nitrocellulose membrane and probed with a 1:25 dilution of hybridoma supernatant (Fig. 4). The Western blot results (Fig. 4) in lanes 3 and 4 (0.25 x 10^6 cells and 0.5 x 10^6 cells respectively) demonstrate a specific binding of TIB 131 MAb to a 53 kDa protein. CEM cells are of mesenchymal origin, and as such produce vimentin (53 kDa). The cell lysate resolved in lanes 1 and 2 (0.5 x 10^6 cells) was probed with a 1:25 dilution of D-MEM media (without TIB 131 MAb) or with a second antibody

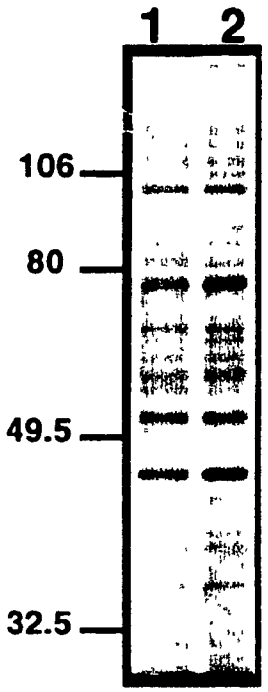

Fig. 3. Total cell lysate of CEM cells. Molecular weight markers in kDa, are shown on the left. Lanes 1 and 2 contain the silver stained lysate of 0.5 x 10^6 and 1.0 x 10^6 CEM cells respectively.

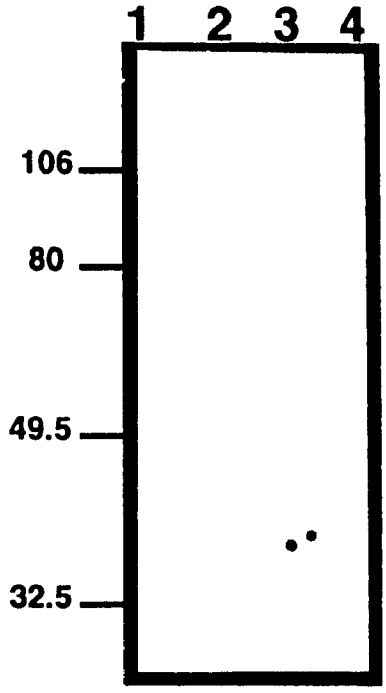

Fig. 4. TIB 131 MAb binding to vimentin in CEM cell lysates. (A) SDS-PAGE and Western blot analysis of CEM total cell lysate. Molecular weight standards in kDa, are shown on the left. Lane 3 contains 0.25 X 10^6 cells, lanes 1, 2 and 4, contain 0.5 X 10^6 cells. A band of 53 kDa, corresponding to the molecular weight of vimentin, is visible in lanes 3 and 4. No bands are visible in lanes 1 and 2, which were respectively exposed to a 1:25 dilution of D-MEM:5% milk and the second antibody (horseradish peroxidase conjugated goat-anti-mouse IgG), and the second antibody only. All lanes were equally exposed to a 1:1000 dilution of the second antibody and developed simultaneously with ECL Western blotting detection reagents.

(horseradish peroxidase conjugated goat-anti-mouse IgG), respectively. No 53 kDa protein band was detected under these conditions in Lanes 1 and 2 of Fig. 3. Taken together, these results confirm that the TIB 131 hybridoma cells produce, as expected, an IF specific MAb (Pruss *et al*, 1981).

Isolation and cloning of TIB 131 variable domains

The variable heavy and light chain regions of TIB 131 MAb were isolated by PCR using FORWARD (VFOR) and BACK (VBACK) primers. The VFOR primers for the IgG heavy and light chains were complementary to 15 and 14 nucleotide sequences respectively, in the antibody constant regions. The VBACK primers encoded the first 21 and 34 nucleotides of the heavy and light chains, respectively. Following a PCR reaction using two appropriate primers, the products of the amplification reactions were resolved on a 2% agarose gel and visualized with ethidium bromide. The results in Fig. 5. show two DNA fragments of approximately 360 bp and 345 bp for TAHP4 heavy chain and TIBNL1 light chain fragments respectively. The molecular sizes of both the TAHP4 and TIBNL1 DNA fragments were consistent with the expected sizes of the V_H and V_L domains, taking into account the positions of the VFOR primers. Moreover, the obtained sizes of V_H and V_L of the TIB 131 MAb were consistent with previous reports using a similar approach (Orlandi *et al*, 1989; Sastry *et al*, 1989).

To identify the nucleotide sequences of the 356 and 345 bp PCR fragments, aliquots of the above amplification reactions were ligated directly into PUC derived

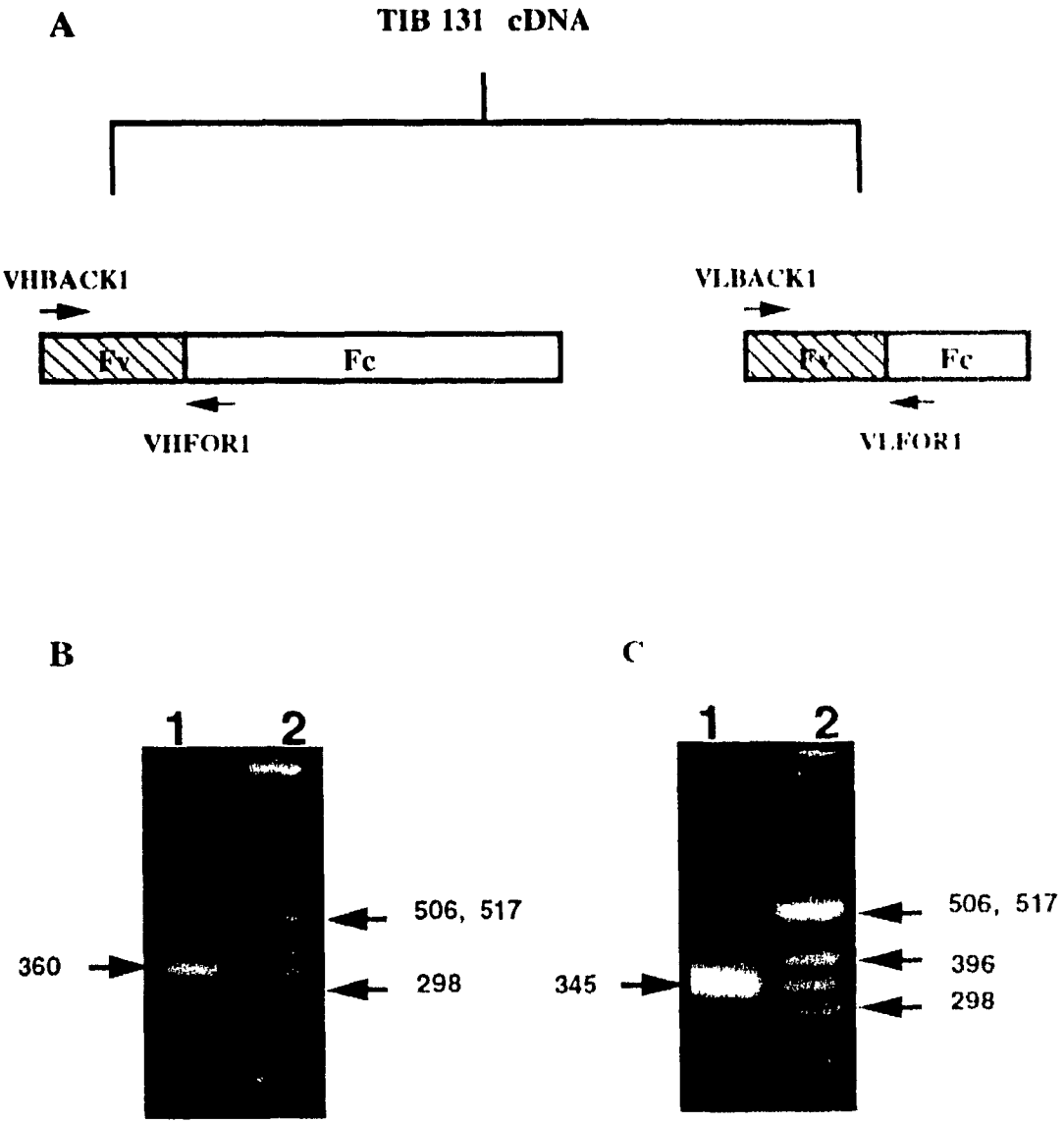

Fig. 5. Isolation of TIB 131 MAb variable domains using PCR. (A) Diagramatic representation of the amplification of the TIB 131 variable regions from a cDNA template. Using PCR, chain specific FORWARD and BACK primers amplified heavy and light chain variable regions. (B) Amplified variable regions of the TIB 131 heavy and light chains were resolved on a 2% agarose gel. Lanes B1 and C1 show approximately 360 bp and 345 bp fragments, which encode the variable domains of the heavy and light chains of the TIB 131 MAb respectively. Sizes of selected fragments of a 1 kb DNA ladder are indicated on the right.

plasmids, two versions of the TA cloning vector (see METHODS). Double stranded DNA from two TA clones, TIBNL1 and TAHP4 were prepared by alkaline lysis extraction (see METHODS) and sequenced using M13F and M13R primers. Double stranded sequencing of each clone (TAHP4 and TIBNL1) using M13F and M13R primers was sufficiently long such that an overlapping of the nucleotides was obtained from these flanking primers. The nucleotide sequences for each of the two clones were entered, aligned and translated using the DNASIS computer program (Pharmacia). The sequence identity of the 356 bp and 345 bp DNA fragments as V_H and V_L was established by comparison to other IgG sequences in the GenBank database. Fig. 6 shows the complete nucleotide and amino acid translated sequences of V_H and V_L of TIB 131 MAb. The V_H translation contained the two conserved cysteine residues at positions 22 and 95, and the translation was entirely in frame; however, the 3' end of the V_L translation appeared to contain a frameshift in framework 4. This was considered to be a potential problem for scFv binding given that the framework regions function to position the CDRs in the antigen binding domain. Also, the generally conserved cysteine residue at position 26 was believed to have mutated to a tyrosine residue, possibly as a PCR artifact. GenBank comparison, however, revealed that the tyrosine residue is present in this position in a few murine kappa chains (pir/JL0073, pir/PN0446 and pir/S24288).

To determine if the observed frame shift in V_L sequence was due to cloning or PCR amplification, an additional 10 V_L clones were sequenced. Five complete (clones 2,3,4,7 and 8) and 5 partial (data not shown) sequences were obtained, the DNA sequence of the complete clones, although similar to the previously sequenced V_L clone

A

VHBACK1

```
  1  CAG GTC CAG CTG CTC GAG TCA GGA CCT GGC CTG GCA AAA CCT TCT      45
  1   Q   V   Q   L   L   E   S   G   P   G   L   A   K   P   S      15

 46  CAG ACT CTG TCC CTC ACC TGT TCT GTC ACC GGC TAC TCC ATC ACC      90
 16   Q   T   L   S   L   T   C   S   V   T   G   Y   S   I   T      30

 91  AGT GAT TAC TGG AAC TGG ATC CGG AAA TTC CCA GGG AAT AAA CTT      135
 31   S   D   Y   W   N   W   I   R   K   F   P   G   N   K   L      45

136  GAG TAC ATG GGG TAC ATA AAC TAC AGA GGT AAC ACT TAC TAC AAT      180
 46   E   Y   M   G   Y   I   N   Y   R   G   N   T   Y   Y   N      60

181  CCA TCT CTC AAA AGT CGA ATC TCC ATC ACT CGA GAC ACA TCC AAG      225
 61   P   S   L   K   S   R   I   S   I   T   R   D   T   S   K      75

226  AAC CAG TAT TAC CTG CAG TTG ATT TCT GTG ACT ATT GAG GAC ACA      270
 76   N   Q   Y   Y   L   Q   L   I   S   V   T   I   E   D   T      90

271  GCC ACT TAT TAT TGT ACA AGA CTG GGG TCC TTC TAC TTT GAC TAC      315
 91   A   T   Y   Y   C   T   R   L   G   S   F   Y   F   D   Y     105
                                                VHFOR1
316  TGG GGC CAA GGC ACC ACT TTC ACA GTC TCC TCA GCC AAA ACA ACA      360
106   W   G   Q   G   T   T   F   T   V   S   S   A   K   T   T     120

361  GCT CCA                                                         366
121   A   P                                                        122
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B

VLBACK1

```
  1  GTC GAC CGA TAT CGT GCT CAC GCA ATC GCC AGC TTC CTT AGC TGT      45
  1   V   D   R   Y   R   A   H   A   I   A   S   F   L   S   C      15

 46  ATC TCT GGG GCA GAG AGG GCC ACC ATC TCA TAC AGG GCC AGC AAA      90
 16   I   S   G   A   E   R   A   T   I   S   Y   R   A   S   K      30

 91  AGT GTC AGT ACA TCT GGC TAT AGT TAT ATG CAC TGG AAC CAA CAG      135
 31   S   V   S   T   S   G   Y   S   Y   M   H   W   N   Q   Q      45

136  AAA CCA GGA CAG CCA CCC AGA CTC CTC ATC TAT CTT GTA TCC AAC      180
 46   K   P   G   Q   P   P   R   L   L   I   Y   L   V   S   N      60

181  CTA GAA TCT GGG GTC CCT GCC AGG TTC AGT GGC AGT GGG TCT GGG      225
 61   L   E   S   G   V   P   A   R   F   S   G   S   G   S   G      75

226  ACA GAC TTC ACC CTC AAC ATC CAT CCT GTG GAG GAG GAG GAT GCT      270
 76   T   D   F   T   L   N   I   H   P   V   E   E   E   D   A      90

271  GCA ACC TAT TAC TGT CAG CAC ATT AGG GAG CTT ACA CGT TCG GAG      315
 91   A   T   Y   Y   C   Q   H   I   R   E   L   T   R   S   E     105

316  GGG GAC CAA GCT GGA AAC AAA ACG GGC GGA TGC TGC ACC AAC TGT      360
106   G   D   Q   A   G   N   K   T   G   G   C   C   T   N   C     120
       VLFOR1
361  ATC CAT CTT CCC ACC G                                           376
121   I   H   L   P   T                                            125
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Fig. 6. Nucleotide and deduced amino acid sequences of the variable regions of the heavy and light chains of TIB 131 hybridoma cells. (A), V_H and (B), V_L. Nucleotides within the amplification primers are underlined.

(Fig. 6B) was noticeably distinct. Thus two distinct kappa chains were being amplified by the same PCR primers. Interestingly, only the original TIBNL1 clone was unique. The abundant clone (10 in total) was arbitrarily designated the Class I clone, and the unique clone, designated Class II. The complete sequences of 5 complete V_L Class I clones aligned with the original Class II clone (TIBNL1) are shown in Fig. 7. Since the P3-NSI/1-Ag4-1 myeloma cells were used in the original fusion protocol (Kohler *et al*, 1976, Pruss *et al*, 1981), the observed second kappa chain, other than that of the TIB 131 kappa chain, would appear to be that which is endogenously expressed, but not excreted, by the P3-NSI/1-Ag4-1 myeloma cells.

A decision on which of the two classes of the murine IgG kappa chain is likely to encode the TIB 131 light chain was based on the fact that continuous culturing of the myeloma cells would likely result in a higher number of point mutations, as was found among the Class I clones. Thus, the Class I clones were designated as representative of the endogenously expressed myeloma light chain, and the singular Class II clone was presumed to be the excreted and functional anti-IF kappa chain.

Modification of the V_H and V_L

Modifications to both the isolated variable regions were necessary due an apparent 3' frameshift in the V_L (around amino acid 106) as well as the absence of suitable restriction cloning sites in the V_H. This opportunity was taken to extend the V_H into a V_H - linker hybrid, suitable for connecting to the V_L.

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CLONE2: GACATCGTGCTCACACAATCACCAGCTACTTTGGCTGTGTCTCTAGGGC
CLONE3: GATATCGTGCTCACGCAATCGCCAGCTACTTTGGCTGTGTCTCTAGGGC
CLONE4: TTTATCGTGCTCACACAATCACCAGCTACTTTGGCTGTGTCTCTAGGGC
CLONE7: GACATCGTGCTAACACAATCTCCAGCTACTTTGGCTGTGTCTCTAGGGC
CLONE8: GATATTGTCCTAACGCAATCACCAGCTACTTTGGCTGTGTCTCTAGGGC
TIBNL1: GATATCGTGCTCACGCAATCGCCAGCTTCCTTAGCTGTATCTCTGGGGC

CLONE2:AGAGGGCCACCATCTCCTGCAAGGCCAGCCAAAGCTTTGATTATGAT-
CLONE3:AGAGGGCCACCATCTCCTGCAAGGCCAGCCAAAGATTTGATTATGAT-
CLONE4:AGAGGGCCACCATCTCCTGCAAGGCCAGCCAAAGCTTTGATTATGAT-
CLONE7:AGAGGGCCACCATCTCCTGCAAGGCCAGCCAAAGCTTTGATTATGAT-
CLONE8:AGAGGGCCACCATCTCCTGCAAGGCCAGCCAAAGCTTTGATTATGAT-
TIBNL1:AGAGGGCCACCATCTCATACAGGGCCAGCAAAAG-TGTCAGTAC-ATC

CLONE2:-GGTCC-TCA-TC-TATGC--TGCATCCAATCTAGAATCTGGGATC--
CLONE3:-GGTCC-TCA-TC-TATGC--TGCATCCAATCTAGAATCTGGGATC--
CLONE4:-GGTCC-TCA-TC-TATGC--TGCATCCAATCTAGAATCTGGGATC--
CLONE7:-GGTCC-TCA-TC-TATGC--TGCATCCAATCTAGAATCTGGGATC--
CLONE8:-GGTCC-TCA-TC-TATGC--TGCATCCAATCTAGAATCTGGGATC--
TIBNL1:TGG-CTAT-AGTTATATGCACTGGATCCAA-C-AGAAACCAGGA-CAG

CLONE2:CCAGCCAGGTTAAGTGGCAGTGGGTCTGGGA-C-AGACTTC-ACCCTC
CLONE3:CCAGCCAGGTTAAGTGGCAGTGGGTCTGGGA-C-AGACTTC-ACCCTC
CLONE4:CCAGCCAGGTTAAGTGGCAGTGGGTCTGGGA-C-AGACTTC-ACCCTC
CLONE7:CCTGCCAGGTTAAGTGGCAGTGGGTCTGGGA-C-AGACTTC-ACCCTC
CLONE8:CCAGCCAGGTTAAGTGGCAGTGGGTATGGGA-C-AGACTTC-ACCCTC
TIBNL1:CCTCCCAGACTCC-T--CA-TGTATCTTGTATCCA-ACCTAGAATCTG

CLONE2: AA--CA-T-CCA--TCCTGTGG-AGGAGGAGGATGCTG-CA-ACC-TAT
CLONE3: AA--CA-T-CCA--TCCTGTGG-AGGAGGAGGATGCCG-CA-ACC-CAC
CLONE4: AA--CA-T-CCA--TCCTGTGG-AGGAGGAGGATGCCG-CA-ACC-CAC
CLONE7: AA--CA-T-CCA--TCCTGTGG-AGGAGGAGTATGCCG-CA-ACC--AC
CLONE8: AA--CA-T-CCA--TCCTGTGG-AGGAGGAGTATGCCG-CA-ACC-CAC
TIBNL1: GGGTCCCTGCCAGGTTCAGTGGCAGT-GC-GTCTGG-GACAGACTTCAC

CLONE2: -T--ACTGTC-AGCACATTA-GG--GAGC-T--TACA-CGT-TCGGAGG
CLONE3: -T--AATGTC-AGCAAA-GAA--ATGAGGATCCT-CA-CGT-TCGGAGG
CLONE4: -T--ACTGTC-AGCAAA-GAA--ATGAGGATCCT-CA-CGT-TCGGAGG
CLONE7: -T-AAATGTC-AGCAAA-GAA--ATGAGGATCCT-CA-CGT-TCGGAGG
CLONE8: -T-AAATGTC-AGCAAA-GAA--ATTGAGGATCTTCA-CGT-TCGGAGG
TIBNL1: CTCAAACATCCATCCTGTGGAGGAGGAGGATGCG-CAACCTATTACTG-
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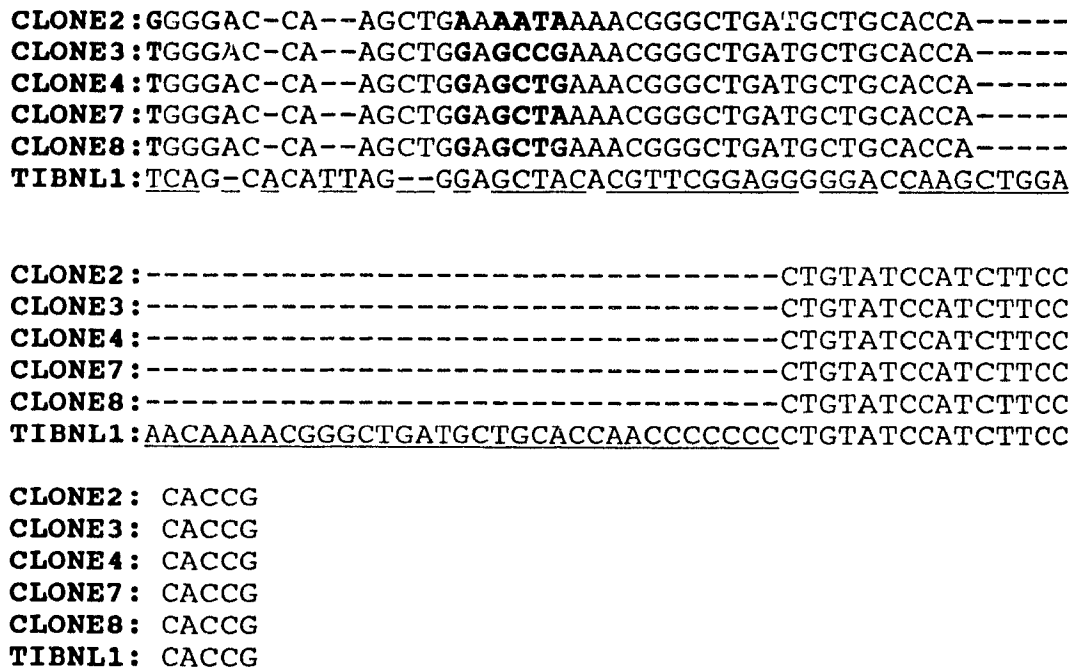

Fig. 7. Aligned nucleotide sequences of 6 selected clones of the variable region of the light chain. Clones 2,3,4,7 and 8 represent the Class I light chain. TIBNL1 represents the Class II light chain. Differences among Class I sequences are designated by bold print. Differences between Class I and Class II sequences are indicated by a line under the TIBNL1 sequence.

PCR Modification of V_H

A PCR reaction was performed using two new primers V_HBACK2 and V_HFOR2, which modified the N- and C-termini of the V_H chain. The V_HFOR2 was 69 nucleotides long and its sequence encoded for a SSSLSSGSGSESKST linker in addition to a 3' *Sal* I to overlap with the 5' *Sal* I site in the sequence of the V_L chain. The V_LBACK2 was 32 nucleotides long and included a vector compatible *Nde* I site at the 5' end, followed by an translation start site. Following the PCR reaction a band of an expected size of 398 bp was visualized on a 2% agarose gel as shown in Fig. 8a. The product was digested with *Nde* I and *Sal* I, resolved on a 2% agarose gel, excised, purified, then ligated, with the V_L into the digested (*Nde* I - *Eco* RI) pRSET expression vector.

Modification of V_L

A PCR reaction was performed using the original V_LBACK1 primer and the new V_LFOR2 primer, the latter of which incorporated a six amino acid peptide tag (PNTLEG) recognized by the C494 MAb raised against P-glycoprotein (Georges and Ling, 1991). Following the PCR reaction a band of an expected size of approximately 375 bp was visualized on a 2% agarose gel as shown in Fig. 8b. This PCR product, which was ligated into PCR™II, as previously described, yielded the sequence shown in Fig. 9. The translation of the sequence confirmed that the tyrosine residue 23 was probably the correct one (rather than a PCR artifact). The incorporation of the tag eliminated the premature stop codon (a consequence of the apparent frameshift) at the 3' end of the chain.

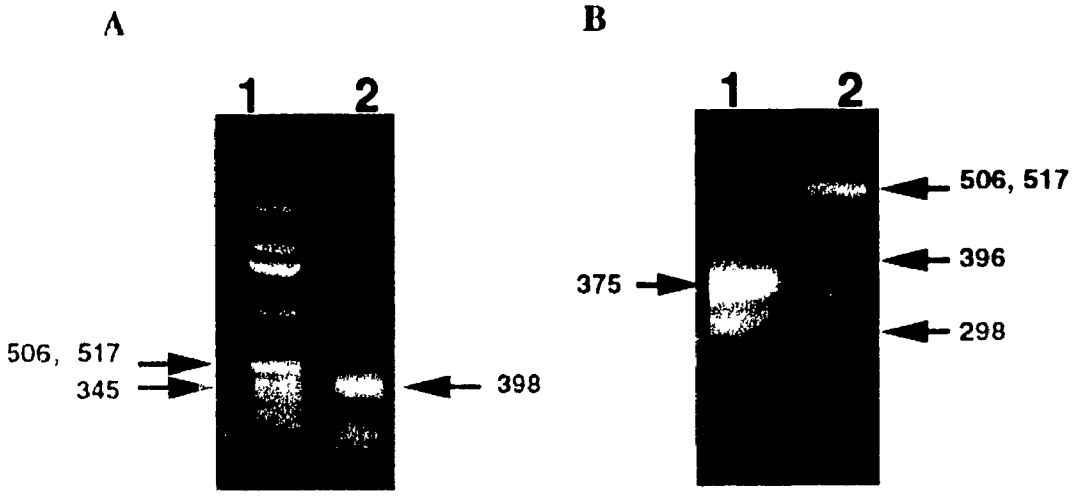

Fig 8 Modification of V_H and V_L termini using PCR. DNA from V_H and cDNA (for V_L) were separately amplified using new primers to allow for the modification of the V_H termini and the addition of a linker peptide between the V_H and V_L domains. Lanes A2 and B1 show the 398 bp and 375 bp fragments amplified with the V_HBACK2 - V_HFOR2 and V_LBACK1 - V_LFOR2, respectively. The PCR products were resolved on a 2% agarose gel Sizes of selected fragments of a DNA ladder are indicated in lanes A1 abd B2

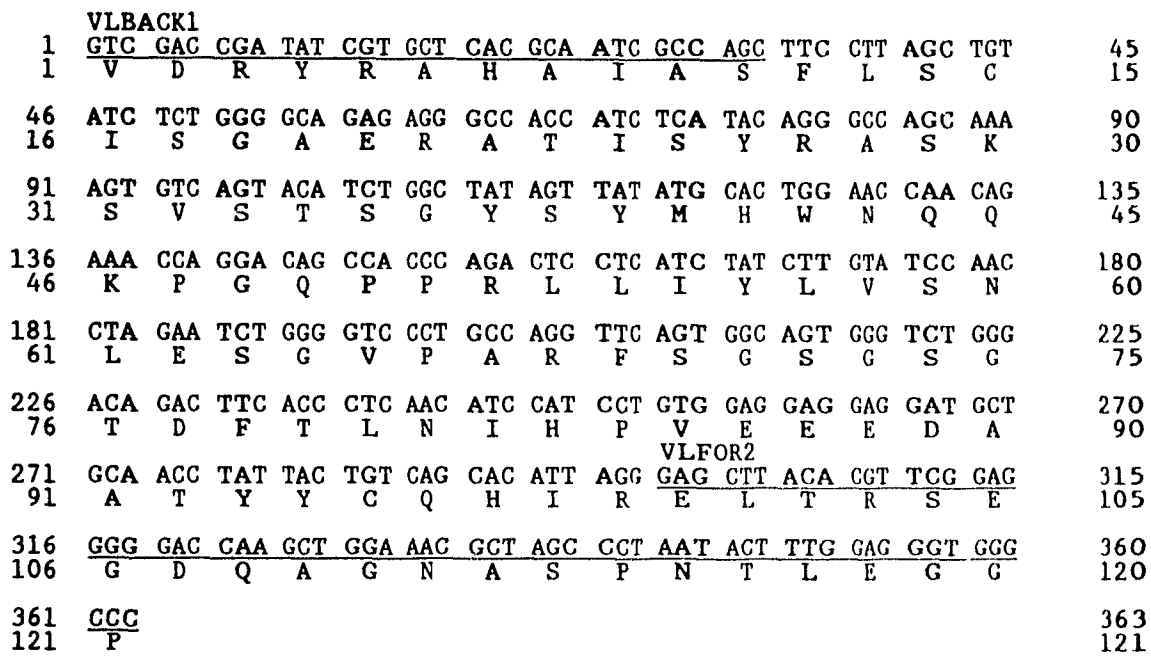

Fig. 9. Nucleotide and deduced amino acid sequences of the modified V_l of the TIB 131 hybridoma. Nucleotides within the amplification primers are underlined.

Construction of the scFv

The full length anti-IF 756 bp scFv construct consisting of the V_H and V_L joined by a linker of 15 amino acids, and followed by a peptide tag recognized by the C494 MAb, was ligated into the *Nde* I and *Eco* RI sites of the pRSET expression plasmid (see MATERIALS AND METHODS, p.28) as shown in Fig. 10. The full length construct was flanked by a ribosomal binding site and an ATG start site 5' to the V_H sequence. The pRSET also contained a stop codon 78 nucleotides from the C494 MAb tag located at the 3' end of the sequence (see Fig. 9). Following the ligation, the DH5α bacteria were transformed with pRSET-scFv plasmid and plated on LB plates with ampicillin. Viable bacterial colonies were selected and propagated in LB media with ampicillin and plasmid DNA was isolated from each clone. Of the 54 clones selected for plasmid extraction, when resolved on a 1% agarose gel, 4 clones appeared to contain an insert of the expected size of approximately 750 bp. Digestions of these four clones yielded fragments of expected sizes, as shown in lanes 2, 3, 4 and 5 in Fig. 11 (clone 18a is shown), indicating that the two variable regions were in the desired order in the vector. Partial sequence analysis of the four clones confirmed that the variable regions were in the correct orientation. The complete sequence of clone 18a, the putative scFv appears in Fig. 12.

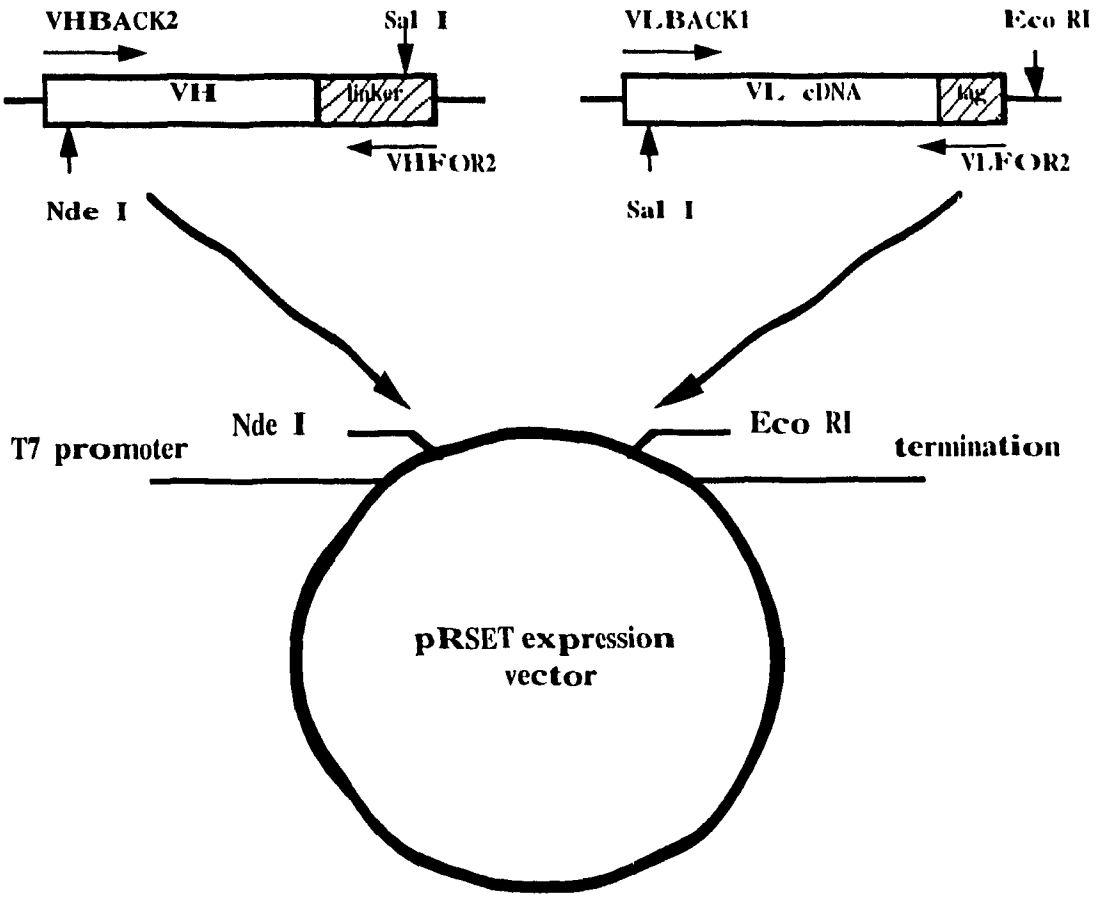

Fig. 10. Construction of an anti-IF scFv. Variable regions of the heavy and light chains from the TIB 131 MAb were amplified and modified using PCR. An *Nde* I restriction site was incorporated into the 5' end of the V_H to make it compatible with the same site in the vector. An *Eco* RI site, convenient for cloning into the pRSET vector, was already present in the TA-V_L plasmid. The two variable regions and the pRSET plasmid were digested with the appropriate enzymes, then ligated to form the pRSET-scFv.

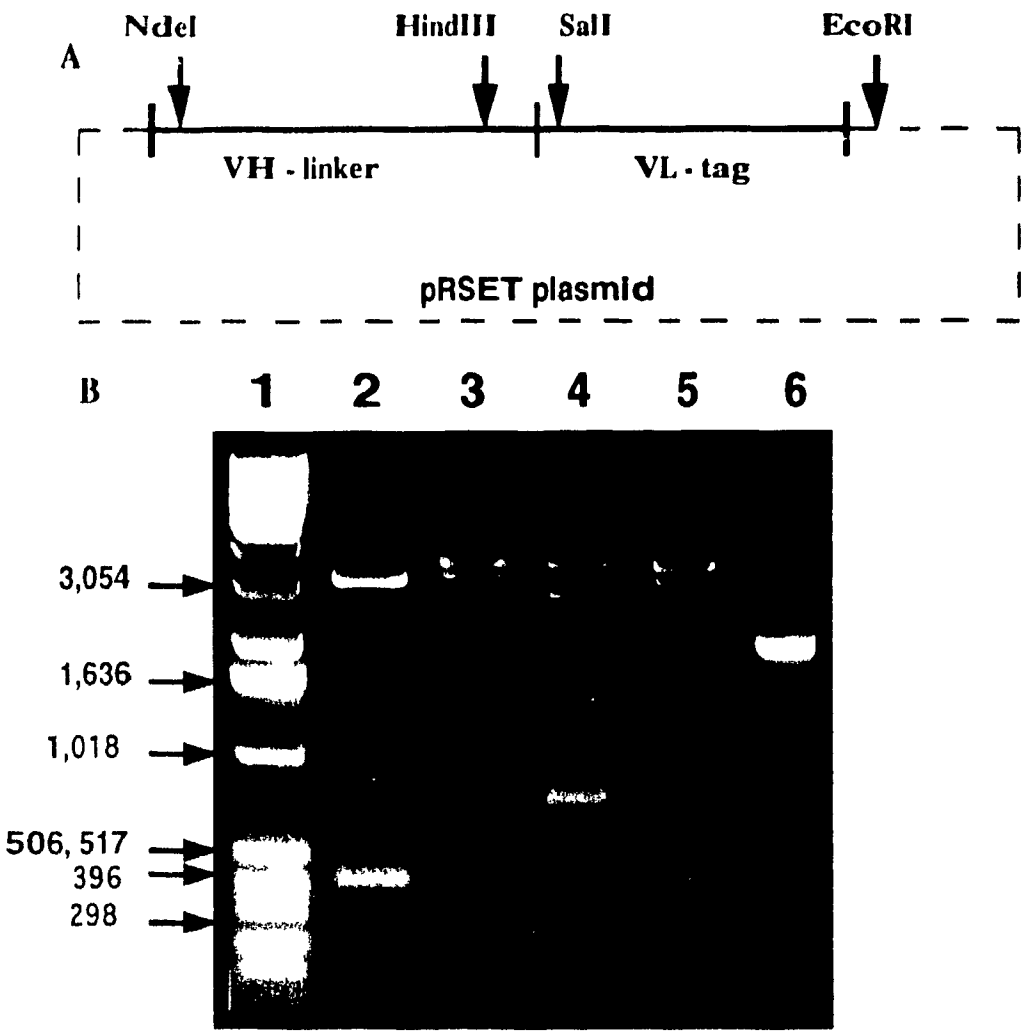

Fig. 11. Restriction pattern of pRSET-scFv clone 18a. (A) Restriction map of the pRSET-scFv plasmid. The solid line represents the scFv, and the broken line depicts the pRSET vector. Restriction sites are indicated, with expected fragment sizes appearing on the right. (B) Ethidium bromide-stained agarose (2%) gel electrophoresis of restriction digests of scFv clone 18a. Sizes of selected fragments of a 1 kb DNA ladder appear on the left. Lane 1 contains the 1 kb ladder. Double restriction digests appear as follows: lane 2, *Hind* III - *Eco* RI; lane 3, *Eco* RI - *Sal* I; lane 4, *Nde* I - *Eco* RI; lane 5, *Sal* I - *Nde* I. Undigested clone 18a appears in lane 6. (C) Expected sizes of digest fragments

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     VHBACK2
 1   CAT ATG GAG GTC CAG CTG CAG GAG TCA GGA CCT GGC CTG GCA AAA      45
 1    H   M   E   V   Q   L   Q   E   S   G   P   G   L   A   K      15

46   CCT TCT CAG ACT CTG TCC CTC ACC TGT TCT GTC ACC GGC TAC TCC      90
16    P   S   Q   T   L   S   L   T   C   S   V   T   G   Y   S      30

91   ATC ACC AGT GAT TAC TGG AAC TGG ATC CGG AAA TTC CCA GGG AAT     135
31    I   T   S   D   Y   W   N   W   I   R   K   F   P   G   N      45

136  AAA CTT GAG TAC ATG GGG TAC ATA AAC TAC AGA GGT AAC ACT TAC     180
46    K   L   E   Y   M   G   Y   I   N   Y   R   G   N   T   Y      60

181  TAC AAT CCA TCT CTC AAA AGT CGA ATC TCC ATC ACT CGA GAC ACA     225
61    Y   N   P   S   L   K   S   R   I   S   I   T   R   D   T      75

226  TCC AAG AAC CAG TAT TAC CTG CAG TTG ATT TCT GTG ACT ATT GAG     270
76    S   K   N   Q   Y   Y   L   Q   L   I   S   V   T   I   E      90

271  GAC ACA GCC ACT TAT TAT TGT ACA AGA CTG GGG TCC TTC TAC TTT     315
91    D   T   A   T   Y   Y   C   T   R   L   G   S   F   Y   F     105

316  GAC TAC TCG GGC CAA GGC ACC ACT TTC ACA GTC tcc tca agc ttg     360
106   D   Y   W   G   Q   G   T   T   F   T   V   S   S   S   L     120

361  tct tcc ggt tct ggt tcc gaa tct aag tcg act GTC GAC CGA TAT     405
121   S   S   G   S   G   S   E   S   K   S   T   V   D   R   Y     135

406  CGT GCT CAC GCA ATC GCC AGC TTC CTT AGC TGT ATC TCT GGG GCA     450
136   R   A   H   A   I   A   S   F   L   S   C   I   S   G   A     150

451  GAG AGG GCC ACC ATC TCA TAC AGG GCC AGC AAA AGT GTC AGT ACA     495
151   F   R   A   T   I   S   Y   R   A   S   K   S   V   S   T     165

496  TCT GGC TAT AGT TAT ATG CAC TGG AAC CAA CAG AAA CCA GGA CAG     540
166   S   G   Y   S   Y   M   H   W   N   Q   Q   K   P   G   Q     180

541  CCA CCC AGA CTC CTC ATC TAT CTT GTA TCC AAC CTA GAA TCT GGG     585
181   P   P   R   L   L   I   Y   L   V   S   N   L   E   S   G     195

586  GTC CCT GCC AGG TTC AGT GGC AGT GGG TCT GGG ACA GAC TTC ACC     630
196   V   P   A   R   F   S   G   S   G   S   G   T   D   F   T     210

631  CTC AAC ATC CAT CCT GTG GAG GAG GAG GAT GCT GCA ACC TAT TAC     675
211   L   N   I   H   P   V   E   E   E   D   A   A   T   Y   Y     225
                                              VLFOR2
676  TGT CAG CAC ATT AGG GAG CTT ACA CGT TCG GAG GGG GAC CAA GCT     720
226   C   Q   H   I   R   E   L   T   R   S   E   G   D   Q   A     240

721  GGA AAC GCT AGC CCT AAT ACT TTG GAG GGT GGG GCC                 756
241   G   N   A   S   P   N   T   L   E   G   G   P                252
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Fig. 12. Nucleotide and deduced amino acid sequences of the complete scFv, derived from the TIB 131 hybridoma. Nucleotides within the terminal amplification primers are underlined; the linker appears in lower case letters, and; the C494 epitope tag is shown in italics.

Expression of the scFv

To demonstrate the expression of the scFv construct into a full length protein, JM109 cells containing the pRSET-scFv were infected with the M13 phage for 0 - 6 hr. Given that the pRSET plasmid contains the T7 promoter from which transcription may occur, and that the JM109 strain doesn't express T7 polymerase, it was necessary to provide T7 polymerase to induce transcription of the scFv. Infection by the M13 phage provided the T7 polymerase. The results in Fig. 13 show an incremental expression of a protein of the predicted molecular size of 28 kDa, which is recognized by the C494 MAb. The expression of the scFv protein was estimated to be less than 1% of the total bacterial proteins. Lane 1 represents T_0, before the addition of IPTG to the culture, a full hour before induction of expression. Lanes 2, 3, 4 and 5 represent T_{15}, T_{30}, T_{45} and T_{60}, where the subscript indicates the time (in hours) after JM109 infection by the M13 phage. The incremental expression of a protein of the expected weight of 28 kDa, and which is recognized by the C494 anti-PNTLEG MAb would suggest that the scFv construct is being expressed, and that it is likely to be expressed in frame.

In vitro scFv expression

To further confirm expression of a full length scFv encoding the TIB 131 scFv encoding protein, a TNT *in vitro* expression system was used to translate the DNA insert in the pRSET-scFv clone 18a. The results in Fig. 14 (Lane 1) show a translated protein with a molecular mass of 28 kDa. The size of the translated protein is in agreement with the predicted size of the light and heavy chain variable domains. In addition, these results

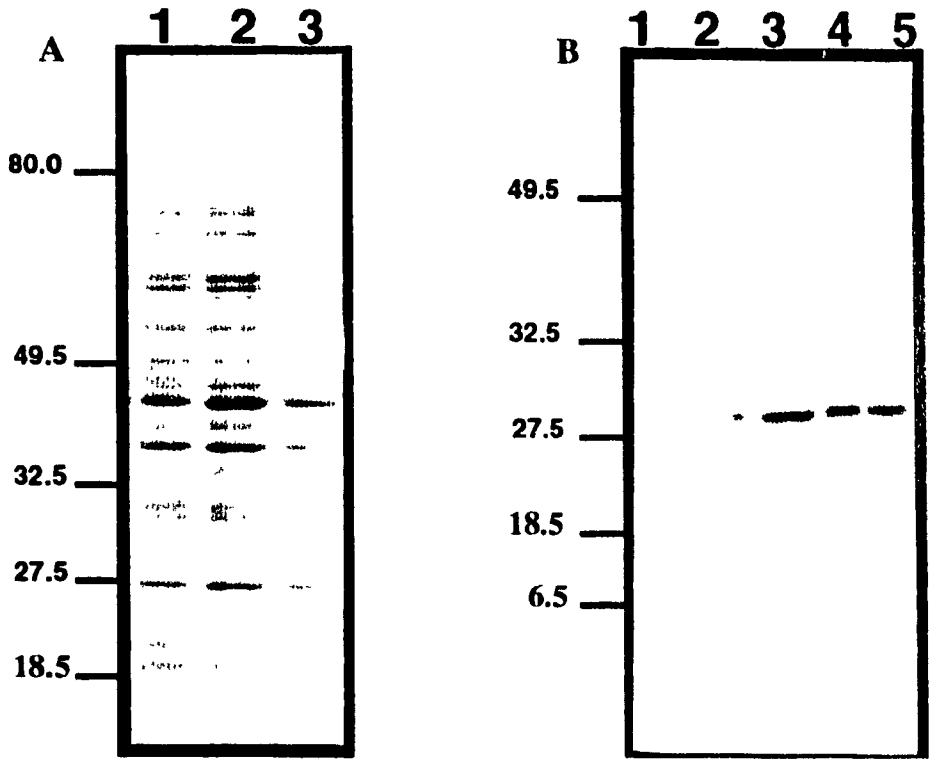

Fig. 13. scFv expression in JM109 *E. coli*. (A) Transformed (pSET-scFv) JM109 cell lysate stained with Coomassie blue. Molecular weight standards in kDa are shown on the left. JM109 bacteria were infected with the M13 phage containing the T7 polymerase gene, necessary for transcription of the scFv from the T7 promoter. Samples were removed at 1.5 hour intervals. A 20 μL aliquot of the solubilized (with 2x Laemmli buffer) lysate pellet sample of T_0, T_4 and T_5, was resolved on a 10% acrylamide gel, as shown in lanes 1, 2 and 3 respectively. The subscript indicates the time (in hours) after infection. Lane 1 represents T_0, the sample taken 1 hr. before M13 infection. (B) SDS-PAGE and Western blot analysis of scFv expression in *E. coli*. Molecular weight standards in kDa are shown on the left. A 20 uL aliquot of the lysate supernatant (with 2x Laemlli buffer) sample of each time point was resolved on a 10% acrylamide gel. Lane 1 represents T_0, the sample taken 1 hr. before M13 infection. Lanes 2, 3, 4 and 5 represent T_{15}, T_{30}, T_{45} and T_{60}. The blot was probed with the anti-tag C494 MAb. All lanes were equally exposed to a 1:1000 dilution of the horseradish peroxidase conjugated goat-anti-mouse IgG and developed simultaneously wit' ECL Western blotting detection reagents. The incremental expression of a protein of the expected weight of 28 kDa, and which is recognized by the C494 anti-PNTLEG MAb would suggest that the scFv construct is being expressed, and that it is likely to be expressed in frame.

confirm previous results in Fig. 13 (Lanes 2, 3, 4 and 5) which suggest that a full length scFv protein has been synthesized and is in frame, as demonstrated by the C494 MAb.

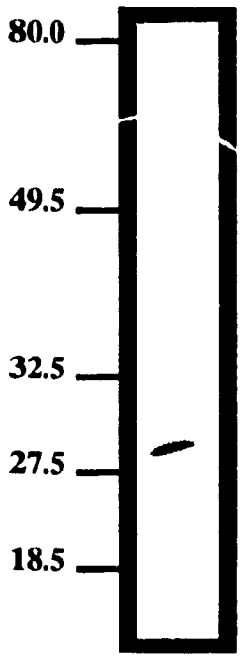

Fig. 14. Autoradiograph of SDS-PAGE resolution of the eukaryotically translated scFv. A 2 μL volume of the rabbit reticulocyte lysate reaction, which incorporates [^{35}S]-methionine into translated proteins, was resolved on a 10% acrylamide gel. Molecular weight markers appear on the left. A band of the expected molecular weight is visible at 28 kDa, suggesting that the scFv is being expressed as a full length construct.

DISCUSSION

In this study we have constructed a single-chain monoclonal antibody using PCR. The choice of the VFOR primers sequence was based on Kabat analysis of 3' sequences for conserved amino acids. The VBACK primers encoded a linker sequence coding for a 15 amino acid peptide (SSSLSSGSGSESKST) and an epitope tag for the V_H and V_L chains respectively. The identification of two classes of V_L sequences (Classes I and II) is consistent with the presence of an endogenously expressed kappa light chain by P3/NS1-Ag4-1 myeloma cells. Interestingly, 10 isolates encoded the Class I sequence which was different from the Class II expressed kappa light chain. A major difference between the two classes was the presence of a 30 bp insert (in Class II) not found in the Class I sequences. In addition, the Class I sequences were identical, except for the presence of a number of point mutations. Based on the frequency of point mutations within the Class I light chains, which is likely to result from the continuous culturing of P3/NS1-Ag4-1 myeloma cells, it was assumed to represent the endogenous non-secreted kappa chain.

The resultant 28 kDa protein as demonstrated by the expression of the cloned 18a encoding for TIB 131 scFv using IPTG induction and an *in vitro* translation system is consistent with the construction of a full length scFv. In fact, given that a 28 kDa protein is detected by Western blotting with the C494 MAb (the MAb tag introduced at the 3' end of the scFv construct), this predicts the in frame ligation between the V_H-linker and V_L sequences. Further studies will determine the binding specificity and affinity of this scFv towards IF proteins. If the results of these studies demonstrate similar binding

specificity and affinity to the native IgG TIB 131, then the 18a scFv construct will be introduced into a eukaryotic vector and transfected into 3T3 cells. The stable transfectants of scFv which will be isolated, will be examined for morphological and growth characteristics when the anti-IF scFv is expressed constitutively or inducibly Initially, the integrity of the IF cytoskeleton will be examined (using the C494 MAb against the tag on the anti-scFv) when high levels of anti-IF scFv are expressed. The observed changes in the IF cytoskeleton will be compared to those seen when the native anti-IF was micro-injected into 3T3 cells and led to a transient collapse of the IF network. However, in that study, the collapse of the IF network had no effect on the microtubule or microfilament networks (Klymkowsky, 1981). This latter finding is interesting since it has been postulated that the IF network interacts with microtubules and microfilament networks. Furthermore, the knowledge gained from the selective disruption of the IF cytoskeleton would likely increase our understanding of the *in vivo* role these proteins play in mammalian cells.

The availability of an agent, such as a functional anti-IF scFv, able to specifically and directly induce the disruption of the IF network would be particularly useful, since there are no other agents able to achieve this. Some small-molecular-weight compounds, however, do exert influence on IF organization, but the mechanism of this process is unknown. Moreover, constitutive or inducible expression of an anti-IF scFv would have a decisive advantage over injection of larger MAb variants, since studies have indicated that the IF network recovers from induced collapse before any significant observations relating to the phenotype can be made (Emerson, 1988).

Aside from inquiries relating to effects of total IF collapse, a growing number of reports have associated activation of cells to PKC stimulation and to cytoskeletal changes (Apgar, J.R. 1991; Huang *et al*, 1988; Mochly-Rosen *et al*, 1990). Intracellular expression of a disruptive anti-IF scFv could be useful in interpreting reports relating the translocation of various PKC isoforms to IFs. Reported colocalization of PKC ßII with an isolated nuclear membrane fraction (Hocevar and Fields, 1991) could be perturbed by the presence of anti-IF scFv directed into the nucleus. The integrity of the nuclear lamin network could potentially be disrupted by the scFv, compromising the suspected association with the ßII PKC isoform. The apparent colocalization of the PKC ß-isoform with vimentin filaments (Spudich *et al*, 1992), could also be disturbed using the scFv. Given that the colocalization of PKC and vimentin is speculated to provide specific docking sites where PKC isoforms encounter their specific substrates, and considering that PKC activation is often assessed by the expression of the c-*fos* gene, it would be possible to observe the effects of the scFv by evaluating c-*fos* expression.

Added to the list of "unknown functions" ascribed to IFs is their role in mitosis and associated phosphorylation events. Given that phosphorylation and dephosphorylation induces great changes in the IF network, their role in mitosis could be directly addressed by the specific disruption of these proteins with the described anti-IF scFv. The presence of the affixed tag would offer a convenient way to trace the patterns of these proteins during mitosis.

Although the binding specificity and affinity of the constructed anti-IF scFv remains to be established, this work has nonetheless accomplished the first step in

establishing a direct and explicit mechanism for disrupting and hence observing the functions of IFs.

CONCLUSIONS

The objective of constructing and expressing a scFv derived from a hybridoma cell line producing a MAb that recognizes IFs, has been accomplished. The 28 kDa protein was identified using the C494 MAb against the incorporated peptide tag.

Future work could be undertaken to demonstrate the ability of the scFv to bind to IFs *in vitro*. Once this has been established, the scFv would be inserted into a eukaryotic expression vector, transfected into cells such as 3T3 cells (since preliminary work was done using this cell line), and expressed in either a constitutive or inducible manner. Information gleaned from this direct approach of specifically disrupting these proteins of ambiguous function, would help to elucidate their role in the intracellular biological system.

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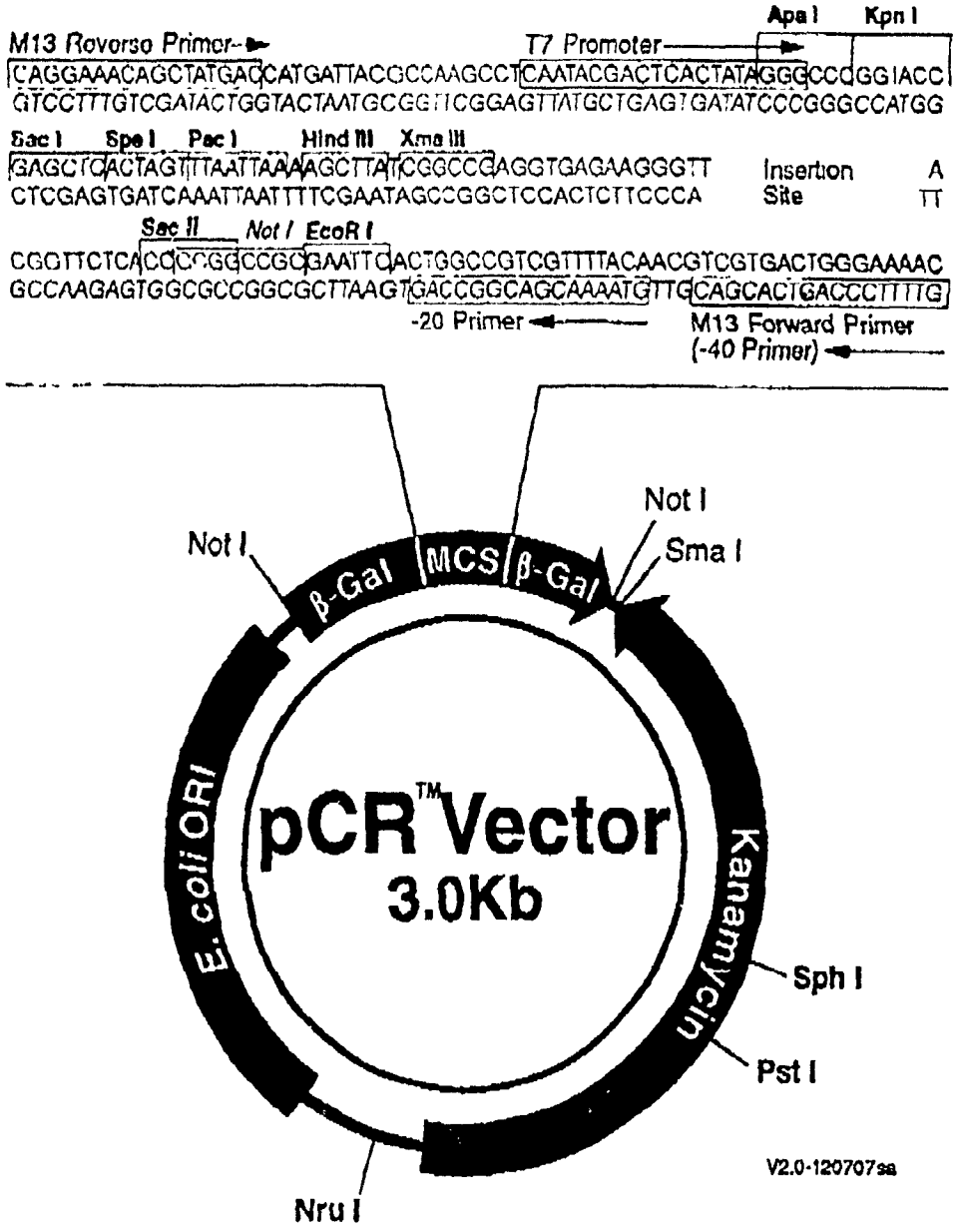
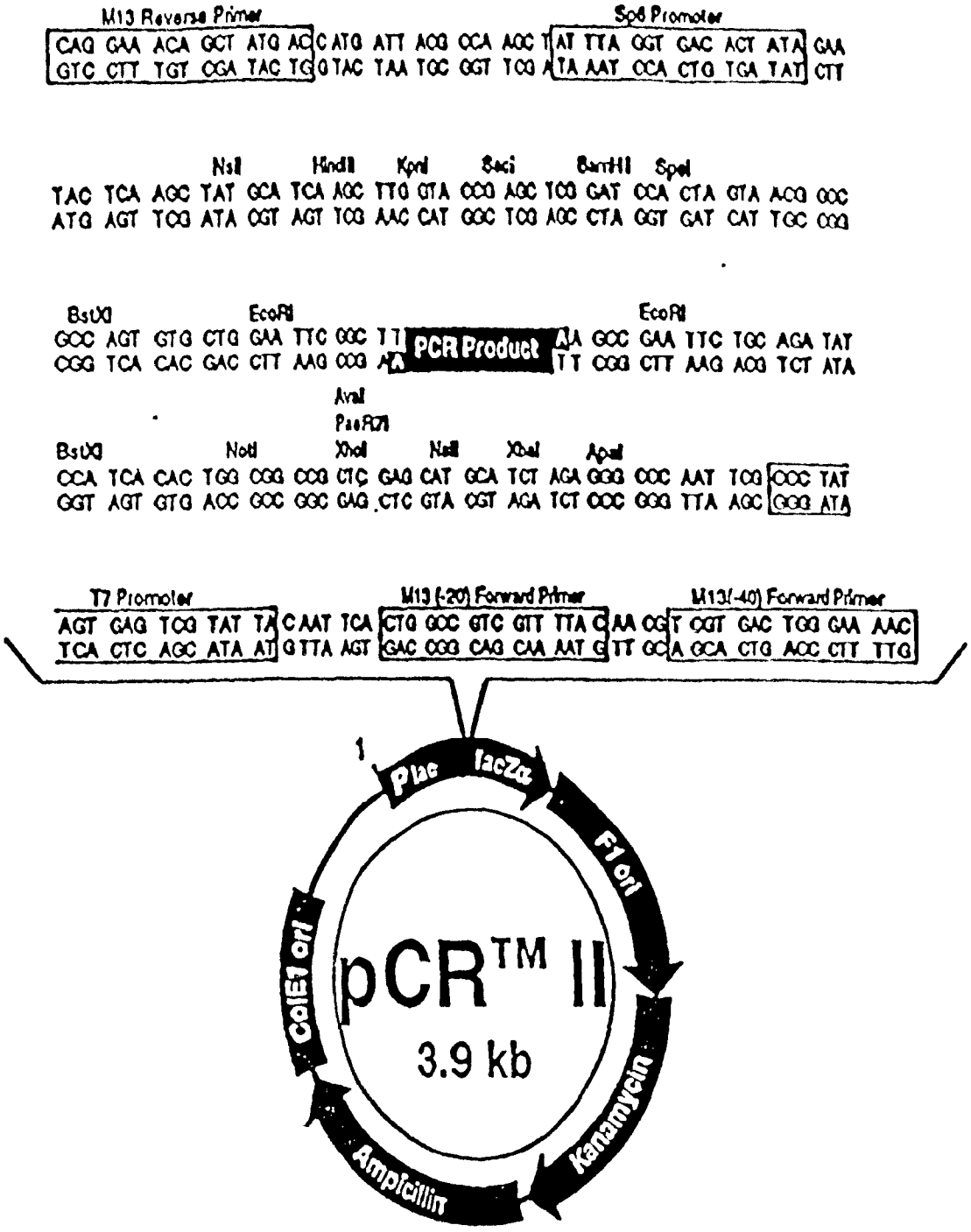
APPENDIX A: TA cloning vector pCR™ 1000 (Invitrogen)



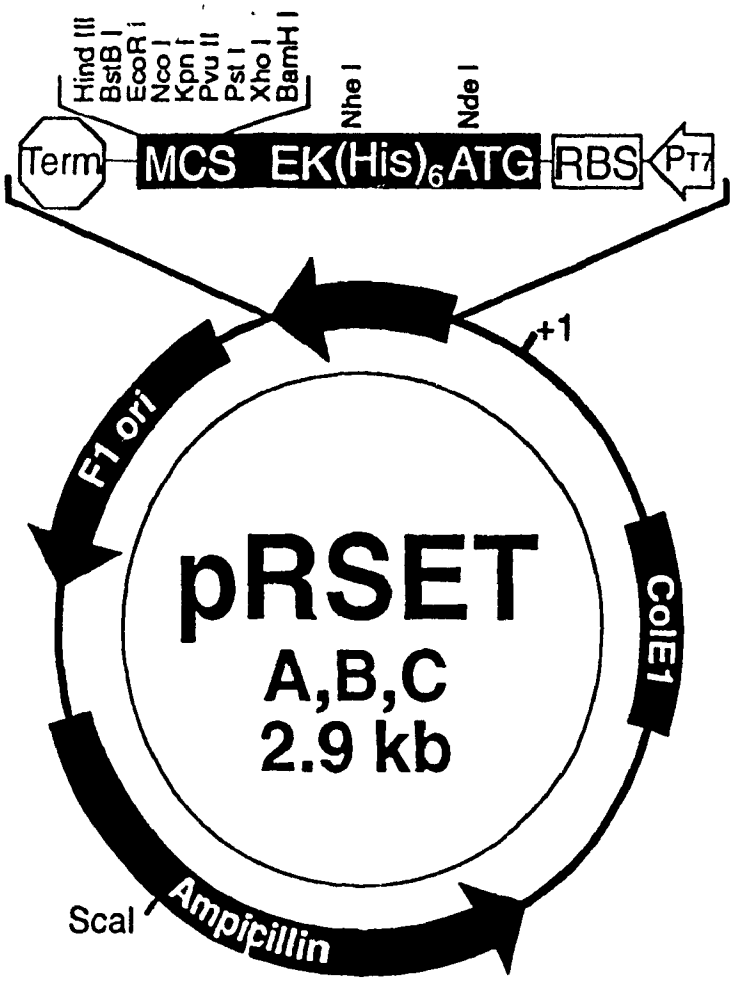
Appendix A: pCR™1000 was used to clone and sequence the PCR amplification product using VHBACK1 and VHFOR1 primers.

APPENDIX B: TA cloning vector pCR™II (Invitrogen)



Appendix B: pCR™II was used to clone and sequence the PCR amplification product using VLBACK1 and VLFOR1 primers, as well as the modified product generated by the VLBACK1 and VLFOR2 PCR primers.

APPENDIX C: pRSET prokaryotic expression vector (Invitrogen)



APPENDIX C: pRSET was used to express the scFv in the bacterial strain JM109, as well as in a rabbit reticulocyte lysate system. The T7 promoter present in this vector made this possible. The *Nde* I and *Eco* RI sites were used to insert the scFv. The in intermediate sequences coding for the histidine residues, designed for purification, were