

An Improved Affinity Tag Based on the FLAG[®] Peptide for the Detection and Purification of Recombinant Antibody Fragments

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ABSTRACT

The commercially available monoclonal antibodies M1 and M2 were raised against and bind the FLAG[®] sequence DYKDDDDK with high specificity. Using the calcium-dependent M1 antibody and the FLAG tag attached to the N terminus of various fragments of the antibody McPC603 expressed in *Escherichia coli*, we found that the M1 antibody binds with almost the same affinity to a much shorter version of this sequence (DYKD). Since most antibody light chains start with an aspartate, the addition of only three additional amino acids to the N terminus is sufficient to detect and quantify the expressed antibody fragments using standard immunological methods. Similarly, the heavy chain can be detected specifically with the sequence DYKD, which requires four additional amino acids since most heavy chains do not start with Asp. The signal sequence of both chains that is necessary for the transport of the chains to the periplasm of *E. coli* is processed correctly. Furthermore, we investigated the influence of the amino acid at the fifth position of the FLAG sequence on the binding affinity of the M1 antibody and found that a glutamate at this position increased the sensitivity in Western blots sixfold over the original long FLAG sequence containing an aspartate residue at this position. Together, the improved FLAG is a versatile tool for both sensitive detection and one-step purification of recombinant proteins.

INTRODUCTION

In many experiments involving the expression of secreted proteins, such as antibody fragments, in *Escherichia coli* (9,17), it is desirable to detect, quantify or purify the products using standard immunological methods. Furthermore, there is often the need to investigate different expression strategies or mutated proteins or to construct various types of fragments or hybrid proteins. For these reasons a system that allows highly sensitive and specific detection regardless of the particular antibody fragment or hybrid protein expressed would be necessary to allow direct quantitative comparisons. The costly and time-consuming production of antisera against particular fragments can thus be avoided.

The FLAG[®] epitope was originally described as consisting of a highly charged and therefore soluble eight-amino acid peptide (DYKDDDDK) that is recognized by the commercially available monoclonal antibodies M1 and M2, raised against this peptide. The M1 antibody binds this peptide in a calcium-dependent manner (11). The fusion of this peptide sequence to antibody fragments allows the rapid and sensitive detection of the expressed protein by immunoblotting or enzyme-linked immunosorbent assay (ELISA) (10) and even one-step purifications using an anti-FLAG affinity column with the antibodies M1 (see below) or M2 (6). There is no observable cross-reactivity with *E. coli* proteins present in the crude extract (see below).

While the FLAG peptide can be fused to either the N or C terminus of

one or both chains of a given antibody fragment, the N-terminal fusion has several advantages: First, it was found by inhibition ELISAs that the anti-FLAG antibody M1 binds three to four orders of magnitude better under conditions where the α -amino group of the first amino acid is freely accessible (11). The expression of antibody fragments in native form by transporting them to the periplasmic space of *E. coli* generates such a free N terminus, since the signal sequence is cleaved off after transport. We found by peptide sequencing that the FLAG sequence, fused between the signal sequence and the mature part of the antibody fragment, maintains correct processing of the signal peptide after transport, leading to the desired free N-terminal FLAG peptide (see below). Therefore, all protein detected with the M1 antibody is correctly transported and processed, a feature that is important for the development or for the comparison of expression strategies of various antibody fragments or fusion proteins.

Second, the FLAG sequence at the N terminus is stable and is not removed by *E. coli* proteases, which we confirmed by N-terminal sequencing of purified antibody fragments produced in *E. coli*. Furthermore, at least the short FLAG sequence does not interfere with the binding of the antibody, regardless of which particular fragment is used (Fab, Fv or scFv) and regardless of which chain carries the FLAG (light chain or heavy chain or both).

Third, after cloning and successful expression of an antibody fragment in *E. coli*, there is often the need to construct several variants of this antibody,

for example, connecting the two chains to a single-chain Fv (scFv) constructing an Fab fragment from an Fv fragment by insertion of the constant domains or vice versa, or fusing the antibody gene to other genes [protein III of phage M13 (3), alkaline phosphatase (16), or toxins (1)] or peptides [his-tag for purification (7) or helix peptides for dimerization (8)]. All of these constructions involve one or both C termini of the antibody chains. A FLAG peptide at the N terminus does not interfere with additional cloning steps at the C terminus and facilitates direct comparisons of these constructs.

Since the N termini of the antibody variable domains are on the same side as the antigen binding pocket, the possibility exists, at least for large anti-

gens, that the attachment of the eight-amino acid, original FLAG peptide on one or both chains will sterically influence the binding of the antigen. For the use of the FLAG tag as a general detection tool in antibody expression, a shorter detection peptide would be desirable.

The C-terminal four amino acids of the FLAG tag sequence (DDDK) were originally designed as an enterokinase cleavage site to allow the specific removal of the tag after purification (4). Since for many applications there is no need for the removal of the FLAG peptide, we investigated several variants of the FLAG sequence to minimize the length of the peptide without loss in sensitivity. Fortunately, we found a shorter variant, which is even recog-

nized sixfold better than the original long FLAG peptide.

MATERIALS AND METHODS

Vectors and Strains

All experiments were done with various fragments of the antibody McPC603 (13). The Fab vector pHJ290 and the Fv vector pHJ300 (Figure 1) are derivatives of the vectors pASK29 and pASK30 (5,14,15), respectively, and contain additional restriction sites to permit the easy exchange of antibody domains between these vectors (Knappik and Plückthun, unpublished). The vectors pLisc_SE and pResc_03 (Figure 1) contain a single-chain

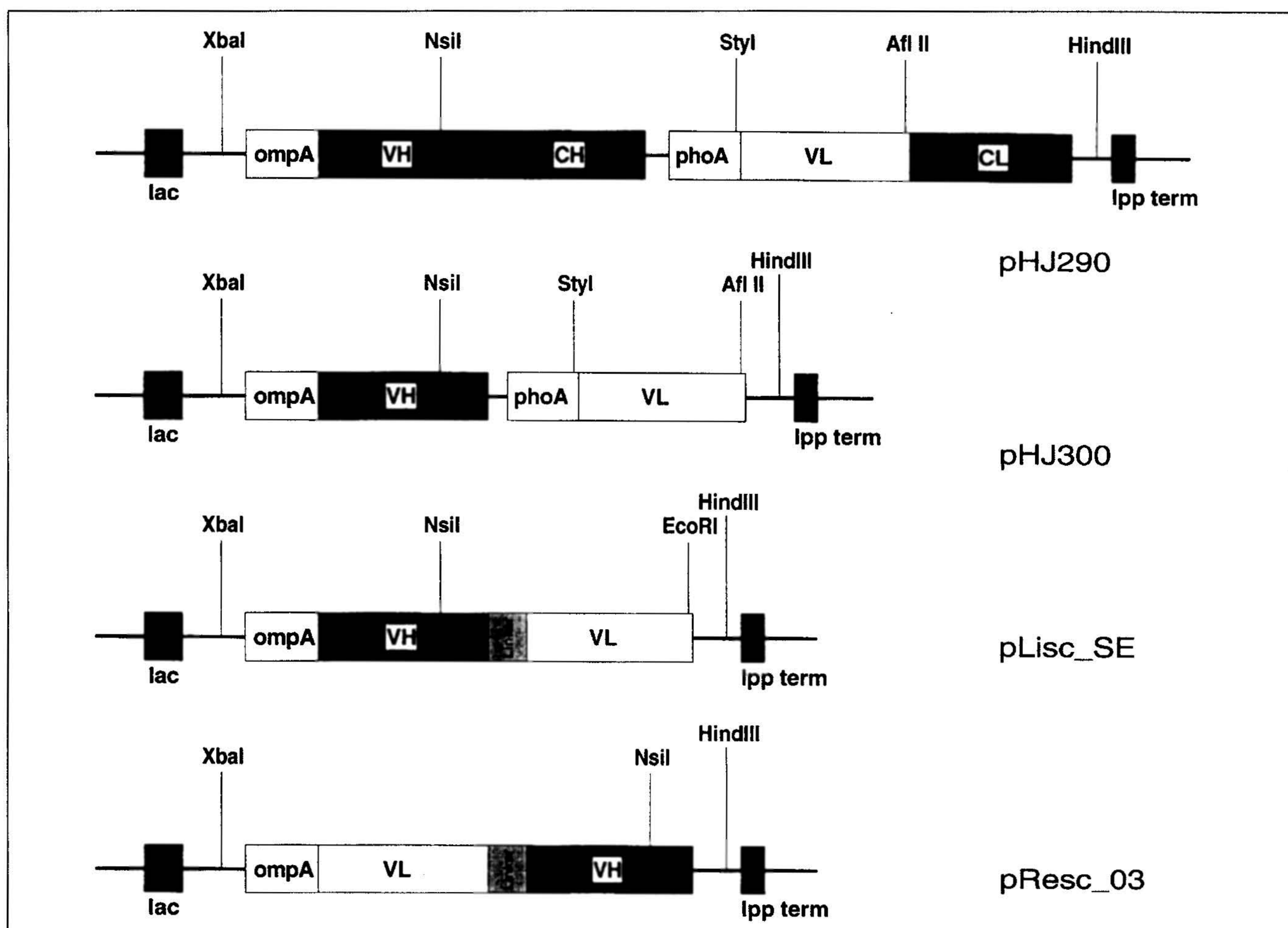


Figure 1. Vectors used to construct the FLAG derivatives. The genes for the different fragments of the antibody McPC603, used as an example in these experiments, are shown together with the regulatory elements and some unique restriction sites. The Fab vector pHJ290 and the Fv vector pHJ300 were used to insert the long and the short FLAG sequence between the *phoA* signal sequence and the *VL* gene sequence (see Figure 2). The single-chain vector pLisc_SE was used to insert the short FLAG sequence between the *ompA* signal sequence and the heavy chain. The vector pResc_03 was used to construct several variants of the FLAG sequence (see Figure 3).

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version of the McPC603 antibody in the orientations VH-linker-VL and VL-linker-VH, respectively (5). The *E. coli* K12 strain JM83 (*ara* Δ (*lac-proAB*) *rpsL* ϕ 80 *lacZ* Δ M15) was used for all cloning and antibody expression experiments.

Construction of the FLAG Variants

Vectors were constructed using standard protocols for cloning and site-directed mutagenesis. In a first step, the FLAG sequence was introduced between the *phoA* signal sequence and the light chain of the Fab and the Fv fragment of the antibody McPC603. The long and the short versions of the FLAG peptide (DYKDDDDDK and DYKD, respectively) were created by inserting appropriate oligonucleotide cassettes into the vector pHJ290 and pHJ300 between the unique *StyI* and *Tth111I* sites (see Figure 2). The resulting vectors that include the long FLAG were named pHJ290FE and

pHJ300FE; the vectors containing the short FLAG were designated as pHJ290F and pHJ300F.

In a second step, the short FLAG sequence was inserted between the *ompA* signal sequence and the heavy chain of the McPC603 single-chain vector pLisc_SE using site-directed mutagenesis (Figure 2). The resulting vector was designated pLisc_SF. For the construction of vectors containing the short FLAG peptide on both antibody chains, the vector pLisc_SF was cut with *XbaI* and *NsiI*, and the DNA fragment containing the *ompA* signal sequence, the FLAG and part of the VH sequence were inserted into the *XbaI*/*NsiI* vector fragment of the plasmids pHJ290F and pHJ300F; this led to the double-FLAG vectors pHJ290FF and pHJ300FF where the light chain carries three additional amino acids (DYK) and the heavy chain carries four additional amino acids (DYKD).

In a third step, the short FLAG se-

quence and various variants of it were inserted between the *ompA* signal sequence and the light chain of the vector pResc_03 using site-directed mutagenesis. The resulting vectors were named according to Figure 3. All constructs were verified by dideoxynucleotide sequencing.

Expression, Fractionation and Purification

The expression of the antibody fragments and the preparation of periplasmic fractions were carried out as described elsewhere (8). Functionally expressed antibody fragments were purified from crude extracts of *E. coli* with phosphorylcholine-Sepharose affinity chromatography (5).

For the purification using the FLAG tag as affinity handle, an anti-FLAG-M1 affinity gel (Eastman Kodak Company, New Haven, CT, USA) was used. Before loading onto the column, the periplasmic fraction was dialyzed

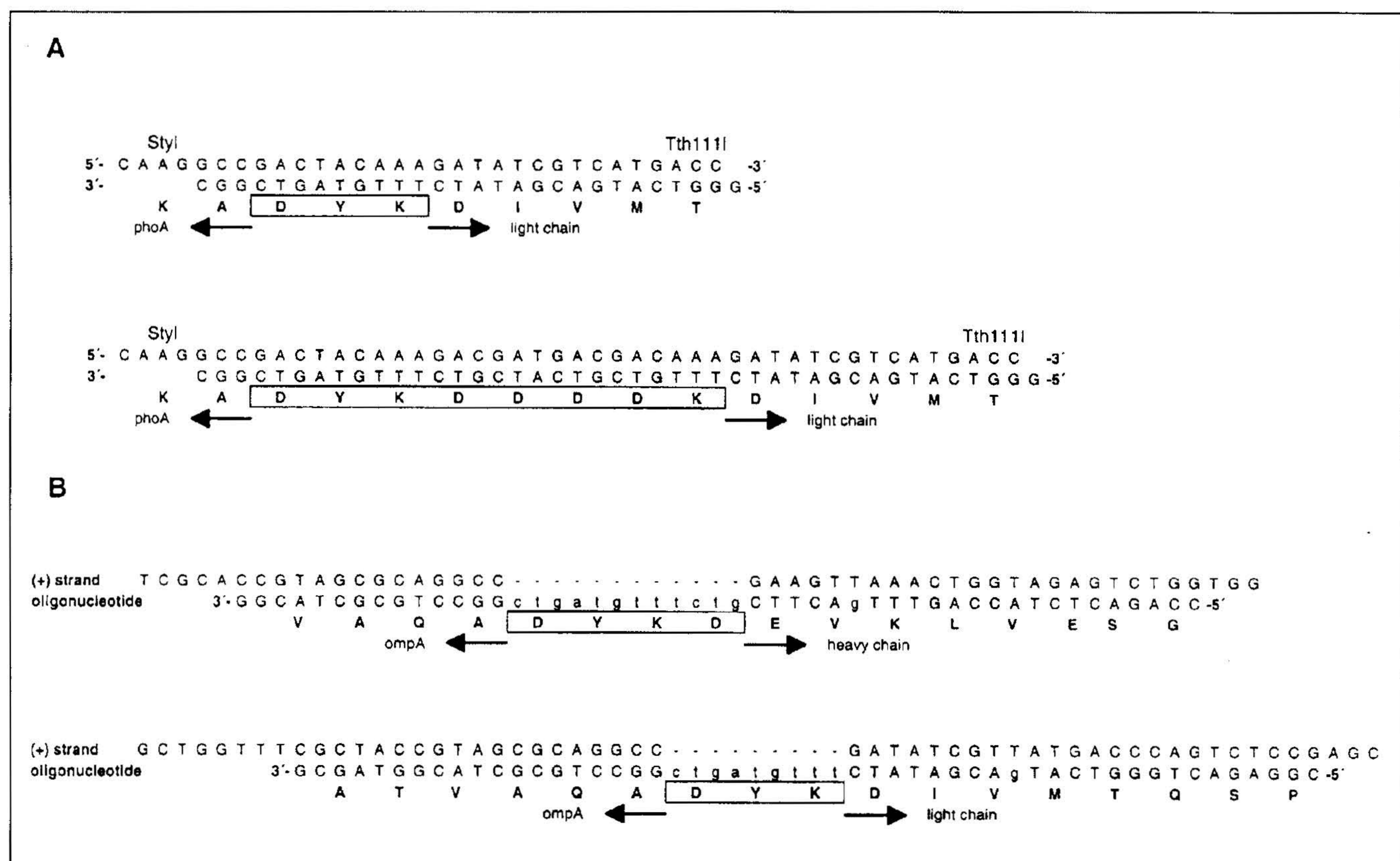


Figure 2. Oligonucleotides used for the construction of the FLAG vectors. (A) Oligonucleotides used to introduce the short and the long FLAG sequence between the *phoA* signal sequence and the VL gene sequence by cassette cloning. The additional amino acids are boxed. (B) Mutagenesis oligonucleotides used to introduce the short FLAG sequence into the single-chain vectors pLisc_SE and pResc_03. The oligonucleotides are shown together with the hybridizing part of the (+) strand of the respective vector. The additional amino acids are boxed.

against TBS (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM CaCl₂) and filter-sterilized. The chromatography was carried out at 4°C according to the instructions of the manufacturer. Briefly, 4 mL of dialyzed and filter-sterilized periplasmic extract (corresponding to about 500 mL cell culture) were loaded onto the anti-FLAG-M1 column (1 mL) under gravity flow. The column was washed three times with 5 mL of TBS. Bound protein was eluted by adding six times 1 mL of 0.1 M glycine-HCl buffer (pH 3.0). The fractions were neutralized immediately with 17 µL 1 M Tris base (pH 10.4).

N-Terminal Sequencing

The two chains of the purified Fv and Fab protein, respectively, were separated using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and blotted onto an activated glass fiber sheet. The bands corresponding to each of the four chains were individually subjected to nine cycles of gas-phase sequencing. In all four sequenator profiles, the NH₂-terminal amino acids corresponding to the expected FLAG sequence followed by the first amino acids of the antibody chains were seen exclusively, indicating precise cleavage of the signal sequence and protease resistance of the FLAG peptide.

Immunoblot

The precise protocol used for immunoblotting the antibody fragments and using the FLAG peptide as detection tag has been described elsewhere (2). Briefly, *E. coli* extracts containing the appropriate antibody fragment were separated with 15% SDS-PAGE under reducing conditions and electroblotted onto a nitrocellulose sheet. The sheet was blocked with TBST (50 mM Tris-HCl, pH 7.4, 15 mM NaCl, 1 mM CaCl₂, 0.05% Tween® 20) containing 1% (wt/vol) PVP-40 (polyvinylpyrrolidone, mol wt 40000 Da). After washing the sheet with TBST, the anti-FLAG M1 antibody was added [11 µg in 50 mL TBST/1% (wt/vol) PVP-40]. A second blocking step was performed with TBST/1.5% gelatin as blocking solution. As the second antibody, an Fc-specific anti-mouse IgG antiserum conjugated to horseradish peroxidase

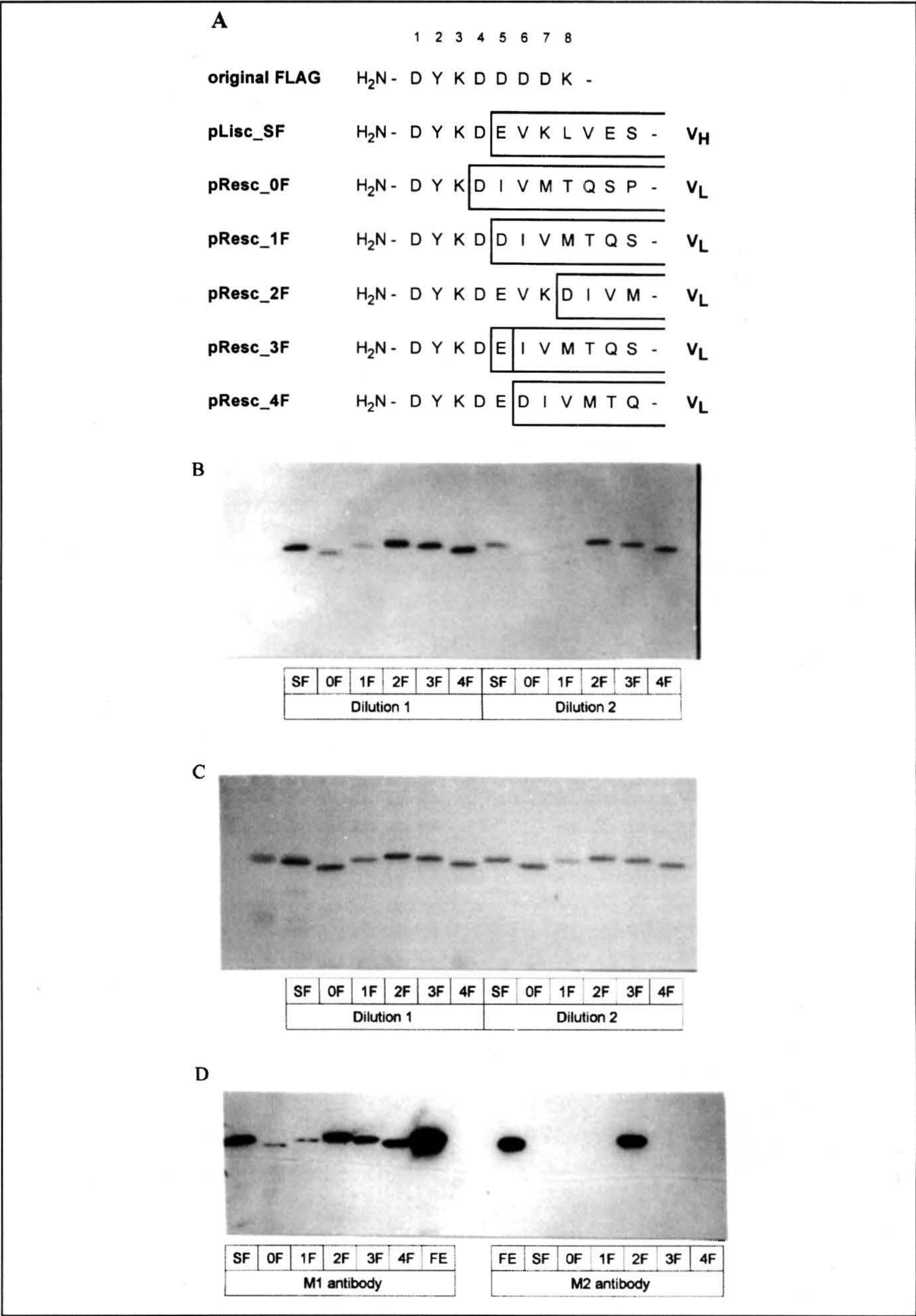


Figure 3. Western blot analysis of different FLAG variants. The FLAG sequences were inserted into the single-chain vectors pLisc_SE and pResc_03 by site-directed mutagenesis. **Panel A:** The original eight-amino acid FLAG peptide is compared with the amino-terminal eleven amino acids of the mature single-chain antibody fragments constructed. The names of the vectors are indicated in the first column. Sequences derived from the antibody are boxed. **Panels B and C:** Western blot analysis after reducing SDS-PAGE of crude extracts of *E. coli* cells expressing the single chain antibody fragments. In Panel B, the anti-FLAG antibody M1 was used to detect the antibody fragments. Panel C shows the control experiment where the antibody fragments were detected with an antiserum raised against the McPC603 Fab fragment. The lanes are named according to the respective vector names. The amount of crude extract loaded on the gel shown in Panel C was about 50 µL for “Dilution 1” and 25 µL for “Dilution 2,” whereas only 15 µL and 7.5 µL crude extract, respectively, were used for the blot B since the detection with the anti-FLAG M1 antibody is more sensitive than the detection with the anti-McPC603 antiserum. **Panel D:** Comparison of the M1 and the M2 antibody in the detection of the antibody fragments constructed. The samples were prepared and analyzed by Western blotting as described above (Panel B) except that 20 µL crude extract instead of 15 µL were used. The blot was cut into two parts. The M1 antibody was used for detection in the first half of the blot while the M2 antibody (40 µg in 50 mL) was used for detection in the second half of the blot. The blotting procedure was performed in parallel under exactly the same conditions. Purified Fab fragment containing the long FLAG sequence at the light chain (lane FE) was included to serve as a positive control.

(Sigma Chemical, St. Louis, MO, USA) was used. After extensive washing with TBST and TBS (50 mM Tris-HCl pH 7.4, 15 mM NaCl, 1 mM CaCl₂), the POD-conjugate was detected by the use of enhanced chemiluminescence with luminol (ECL[®] kit; Amersham International, Little Chalfont, Bucks, UK) as substrate and an x-ray film was the detection medium. The detection of antibody fragments using a rabbit antiserum raised against the McPC603 Fv fragment has been described elsewhere (14).

RESULTS

Only Four Amino Acids of the FLAG Tag Are Sufficient for Sensitive Detection with the Anti-FLAG M1 Antibody

In order to compare the influence of the length of the FLAG tag on the sensitivity in immunoblots, the Fab fragments containing the long and the short version of the Flag peptide at the N terminus of the light chain, respectively, were purified using phosphorylcholine-Sepharose affinity chromatography and

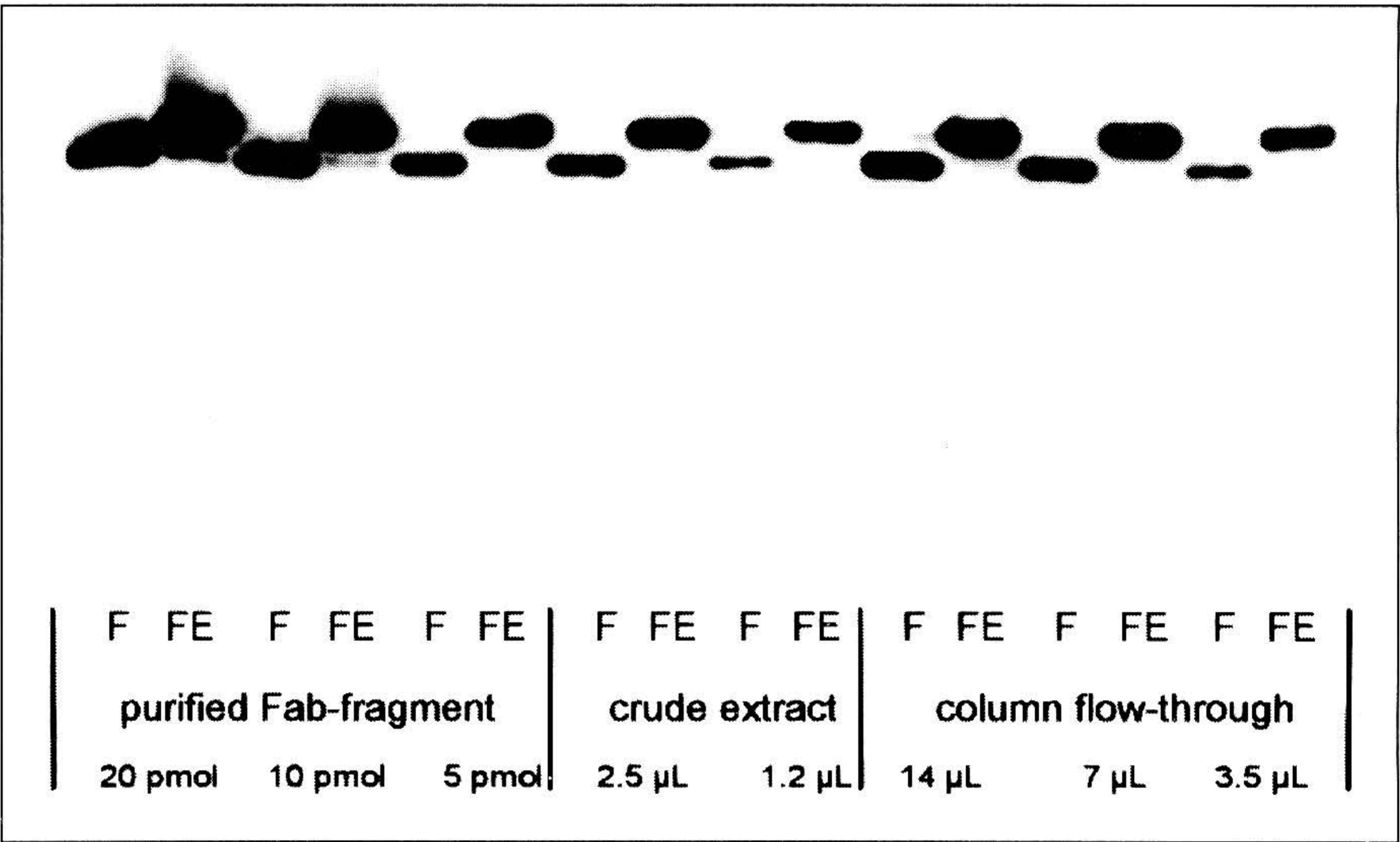


Figure 4: Western blot analysis of Fab fragments. The lanes designated as “FE” show the light chain of the Fab fragment of the antibody McPC603 with the long FLAG peptide attached to the N terminus, whereas the lanes “F” show the same fragment but with the short three-amino acid sequence DYK attached. *E. coli* cells containing the plasmid pHJ290FE and pHJ290F, respectively, were grown, induced and harvested. The cells were passed through a French press, and the antibody fragments were purified with phosphorylcholine-Sepharose affinity chromatography. The yield of purified functional material was similar to the yield obtained with the antibody fragments without the FLAG attached, regardless of which antibody fragment and which FLAG variants were used. The purified Fab proteins as well as aliquots of the crude extract (diluted 10-fold before loading; 1 µL crude extract corresponds to about 90 µL culture) and of the flow-through of the column were subjected to a 15% SDS-PAGE under reducing conditions and blotted as described in the experimental section. Since excess light chain of the antibody McPC603 is soluble but not functional without the heavy chain, it is found in the flow-through of the column and can be detected there. This behavior is always seen with the McPC603 antibody whether or not a FLAG sequence is attached.

subjected to immunoblotting (see Figure 4). To demonstrate the specificity of the anti-FLAG M1 antibody, *E. coli* crude extracts containing the appropriate antibody fragments were also blotted. The intensity of the signal produced with the short FLAG was about 60% of that produced with the long FLAG, as estimated by densitometric scanning of the film. Hence, only the

first four amino acids of the FLAG tag are sufficient for specific and sensitive detection in immunoblots. Because the antibody light chain already starts with an aspartate, the sequence needed to create this signal consisted of only three additional amino acids (DYK) attached to the N terminus of the light chain (see Figure 2). Interestingly, the apparent molecular weight of the Fab

light chain containing the long FLAG was about 3 kDa higher than the light chain containing the short FLAG peptide, although the real difference in molecular weight was only 588 Da.

The Four-Amino Acid Tag Gives a Tenfold Higher Signal for the Heavy Chain Than for the Light Chain Because of Differences in Sequence Following the Tag

The antibody Fv fragment containing the short FLAG on both the heavy and the light chain was purified in functional form and subjected to immunoblotting (Figure 5). Although the first four amino acids were the same for both the heavy and the light chain, the signal intensity was about ten times higher for the heavy chain. Since the antibody fragment was purified using phosphorylcholine-Sepharose affinity chromatography, only functional and therefore heterodimeric forms should be present with a molar ratio of 1:1 of the two chains. This was verified by performing SDS-PAGE with the purified fragments and Coomassie staining the gel; which showed the expected

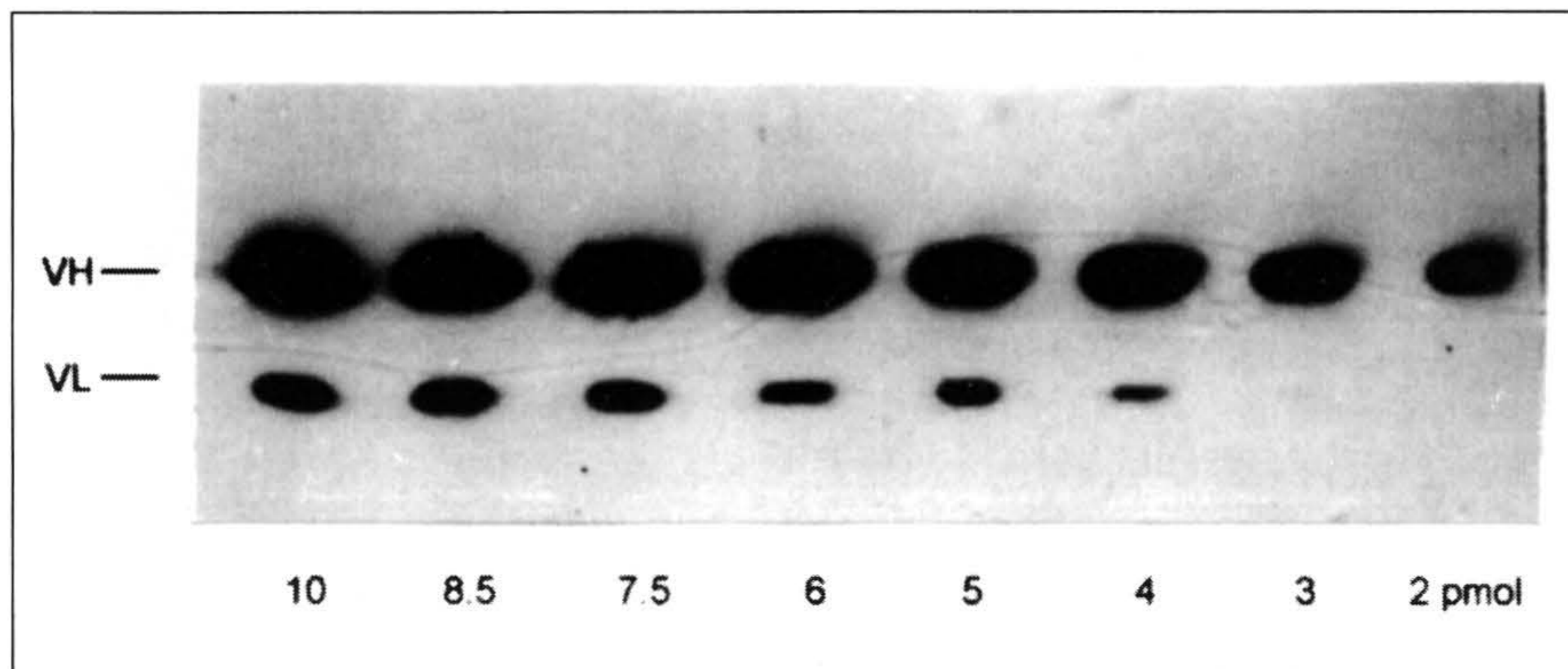


Figure 5. Western blot analysis of the Fv fragment of the antibody McPC603 bearing the short FLAG peptide on both chains. *E. coli* cells carrying the plasmid pHJ300FF were grown, induced and harvested. The cells were passed through a French press and the Fv fragment was purified on a phosphorylcholine-Sepharose column. Decreasing amounts of Fv fragment were separated on a 15% SDS-PAGE under reducing conditions, blotted as described in the text and detected with the anti-FLAG antibody M1.

identical intensity for both chains (see Figure 6, lane 13). We ruled out that the difference in signal intensity is due to protease cleavage or degradation by performing an N-terminal sequencing of the purified protein chains as described in the Materials and Methods section. These sequencing results also ruled out that there is a difference in the cleavage site of the signal peptidase during transport in the periplasm, which could have caused such a result since it is known that the anti-FLAG M1 antibody needs a free α -amino group at the N terminus for sensitive recognition of the FLAG sequence. Both chains start with the expected FLAG peptide sequence. Hence, the differences in signal intensity must be due to the differences in the amino acids of both chains following the FLAG sequence.

A Glutamate at the Fifth Position Is Responsible for the Increase in Sensitivity

Since the signal intensity in Western blots of the short FLAG peptide attached to the N terminus of the heavy chain is tenfold higher than if the same sequence is attached to the light chain and since the long FLAG peptide leads only to a 1.7-fold increase in sensitivity compared with the short FLAG peptide, the short FLAG attached to the heavy chain can be detected sixfold more sensitively than the long original FLAG peptide. To account for this increase in sensitivity, we constructed several variants of the FLAG sequence and attached them to the N terminus of the light chain of the single-chain Fv fragment encoded by vector pResc_03 (see Figure 3A). The resulting antibody constructs were expressed, and the crude extracts were subjected to immunoblots using the anti-FLAG M1 antibody (Figure 3B). The plasmid pLisc_SF containing the single-chain antibody with the short FLAG attached to the heavy chain was included for comparison. In a control experiment, a second immunoblot was performed with the same fractions, but detected with an antiserum raised against the McPC603 antibody Fv fragment to verify that all fractions contain the same amount of single-chain antibody (Figure 3C). Surprisingly, the change of the amino acid at the fifth position from

aspartate to glutamate is sufficient for the increase in sensitivity (compare fraction 1F with fraction 3F in Figure 3). Therefore, the FLAG sequence DYKDE increases the sensitivity in immunoblots tenfold compared with the sequence DYKDD and sixfold compared with the original long FLAG DYKDDDDDK, which was used as the hapten for the production of the anti-FLAG M1 monoclonal antibody (4).

In an additional experiment, we tested the ability of the anti-FLAG M2 antibody to bind to the different FLAG versions constructed (Figure 3D). While the original long FLAG sequence was detected by the M2 antibody, M2 was not able to bind to the short versions of the FLAG sequence. Unexpectedly, however, we found that the M2 antibody binds to the peptide provided by the pResc_2F antibody construct. The N-terminal seven amino acids of the construct are identical to the pLisc_SF construct, which is not recognized by the M2 antibody. The molecular reasons for this difference in recognition is yet unclear, because the M2 epitope is not precisely known.

The Short FLAG Is Sufficient for One-Step Purification Using the Anti-FLAG M1 Affinity Column

To test the short improved FLAG as an affinity handle for antibody purification, we prepared a periplasmic extract of induced *E. coli* cells containing the plasmid pLisc_SF. The extract was divided into three parts. One part was subjected to purification with phosphorylcholine-Sepharose affinity chromatography and used as a control. The two other parts were subjected to affinity chromatography using the anti-FLAG M1 and the anti-FLAG M2 affinity column, respectively. While the single-chain antibody containing the short FLAG did not bind to the M2 affinity column (data not shown), the use of the anti-FLAG M1 affinity column resulted in highly purified antibody (see Figure 6). The yield and purity were similar to the purification with phosphorylcholine-Sepharose affinity chromatography. Therefore, the short FLAG is useful for both detection and one-step purification of antibody fragments expressed in *E. coli*.

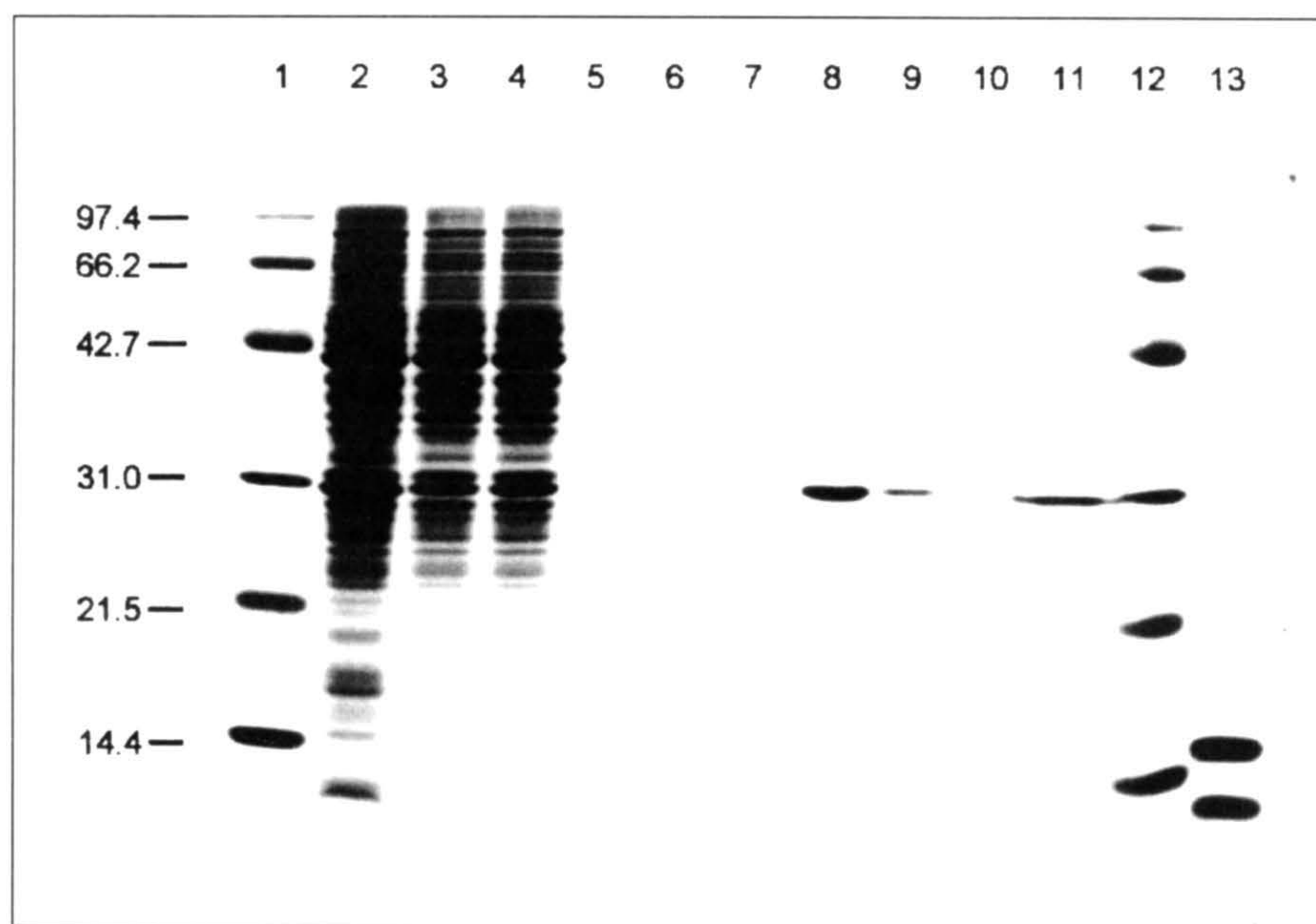


Figure 6. Purification of the scFv fragment pLisc_SF containing the short FLAG with the anti-FLAG M1 affinity column. Coomassie-stained 15% SDS-gel (PAGE performed under reducing conditions) showing the fractions obtained with the purification protocol described in the Material and Methods section. Lane 1: molecular weight marker. Lane 2: periplasmic extract prior to loading onto the column. Lane 3: column flow through. Lanes 4-6: washing fractions. Lanes 7-10: fractions during elution with 0.1 M glycine-HCl buffer (pH 3.0). Lane 11: control (3.5 µg of pLisc_SF scFv fragment purified with phosphorylcholine-Sepharose affinity chromatography). Lane 12: molecular weight marker. Lane 13: 10 µg of Fv fragment (from the vector pHJ300FF) purified with phosphorylcholine-Sepharose affinity chromatography.

DISCUSSION

The FLAG peptide has been successfully used as a detection and purification tag of antibody fragments expressed in *E. coli* (6,10). The system has been applied to other proteins as well (12). In most cases, the FLAG peptide was added to the C terminus of the protein of interest and the detection was performed with the anti-FLAG antibody M2. In the case of antibody expression, however, the addition of the FLAG sequence at the N terminus together with the use of the M1 antibody seems to be a more general and versatile tool, since the detection directly demonstrates correct processing and therefore transport of the chains into the periplasm of *E. coli*. Additionally, the C terminus is frequently used for fusion of extra modules or different affinity handles and consequently should not be blocked by a detection tag in a flexible vector system. The high charge minimizes the possibility of interacting with the recombinant protein and changing its structure. In the case of antibodies, however, the N terminus is located at the same face of the protein as the complementarity determining regions (CDRs) constituting the antigen-binding pocket.

In this paper, we demonstrated that it is possible to truncate the eight-amino acid FLAG down to four amino acids being recognized without significant loss of sensitivity in immunoblots. To our knowledge, this is the shortest affinity tag against which a monoclonal antibody is commercially available. Furthermore, in many cases it is sufficient to add only the first three amino acids of the FLAG peptide to the protein, since many antibodies already start with an aspartate in the light chain. There is no reason to doubt that the detection and purification system described here will work with other recombinant proteins as well, if the expression system leads to a free N-terminal FLAG sequence.

Interestingly, the fifth position of the FLAG sequence, although not necessary for detection, greatly enhances the affinity of the monoclonal anti-FLAG antibody M1, if occupied by a glutamate instead of the original aspartate residue. Prickett et al. (11) showed by

inhibition ELISA experiments that an alanine residue at position five increases the inhibitory ability of the peptide about twofold to threefold. The reason for this phenomenon is not yet understood. Since the M1 antibody recognizes the tag in a calcium-dependent manner, one may speculate that the metal ion is chelated by the FLAG peptide and that this particular conformation is recognized by the M1 antibody. The aspartate at position five may either disturb the chelated conformation or directly interact with the antibody less efficiently than a glutamate. Accordingly, we suggest the use of the DYKDE peptide, if very sensitive detection down to the femtomolar range is needed or if the expression behavior of antibodies differing at their N termini should be compared quantitatively. For specific detection, however, the four amino acid FLAG is sufficient and, if the chain already starts with an aspartate, only three amino acids of the FLAG sequence need to be added to the N terminus.

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