Expression studies of catalytic antibodies

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ABSTRACT   We have examined the positive influence of human constant regions on the folding and bacterial expression of active soluble mouse immunoglobulin variable domains derived from a number of catalytic antibodies. Expression yields of eight hybridoma- and myeloma-derived chimeric Fab fragments are compared in both shake flasks and high-density fermentations. In addition the usefulness of this system for the generation of *in vivo* expression libraries is examined by constructing and expressing combinations of heavy and light chain variable regions that were not selected as a pair during an immune response. A mutagenesis study of one of the recombinant catalytic Fab fragments reveals that single amino acid substitutions can have dramatic effects on the expression yield. This system should be generally applicable to the production of Fab fragments of catalytic and other hybridoma-derived antibodies for crystallographic and structure–function studies.

Many examples of antibody-catalyzed reactions have been reported, ranging from efficient acyl transfer and redox reactions to stereoelectronically disfavored pericyclic and cyclization reactions (1, 2). In addition to providing a strategy for generating catalysts for reactions difficult to achieve by existing enzymatic or chemical methods, antibody catalysis provides a tool to gain increased insight into the mechanisms of biological catalysis and the evolution of catalytic function. These latter aims are facilitated by the availability of three-dimensional structures of antibody active sites, coupled with mutagenesis experiments to confirm hypotheses about reaction mechanisms. Unfortunately, such studies have been hampered by difficulties in expressing many of the antibodies in recombinant form in quantities sufficient for mechanistic and crystallographic studies. In addition, the ability to express antibodies or antibody fragments in a bacterial system in soluble form, either periplasmically or cytoplasmically, is a prerequisite for attempts to improve their catalytic efficiency using random or directed mutagenesis, coupled with *in vivo* selection techniques (3, 4).

A number of systems for the production of antibody fragments have been described, most of which use bacterial signal sequences to direct the immunoglobulin chains to the periplasm or culture medium, where correct folding and disulfide bond formation are possible (for review, see ref. 5). However, reports of expression systems rarely describe their general applicability. For example, we have examined a variety of published systems by using a number of hybridoma- and myeloma-derived antibodies, including Fab and single-chain Fv fragments, various promoters [T7, *lac*, *tac*, and alkaline phosphatase (PhoA)], signal sequences (pelB and stII), fusions to maltose binding protein and ThiA (6), exchange of the constant (C) regions, and expression as a single-chain Fv fragment in the yeast *Pichia pastoris* (7) (ref. 8 and H.D.U. and P.G.S., unpublished results). In each case, the majority of the protein was found in insoluble aggregates, suggesting that the primary structure of the antibody greatly influences its correct

folding in the periplasm and remains the limiting factor for its performance in a given expression system. The extensive study of the antibody McPC603 by Knappik and Plückthun (9) supports this notion. A number of antibodies have been derived from phage display methods that express well and in soluble form (10). However, the well-behaved nature of these antibodies is likely a consequence of concurrent selection for correctly folded proteins and may not apply to hybridoma-derived antibodies.

One of the earliest reports of the expression of soluble antibody fragments in *Escherichia coli* was based on a chimeric Fab fragment (11). Carter and coworkers (12–14) have observed large increases in expression yields of three humanized Fab fragments over the corresponding murine proteins; where reported, the murine–human chimeric Fab fragment was intermediate (12). Based on these observations, we have examined the expression of a panel of catalytic antibodies in a system that exploits this beneficial influence of the human sequences on the folding of the Fab fragment in the bacterial periplasm. Herein we report the expression studies of both hybridoma-derived antibodies and artificially constructed combinations of heavy and light chains and report the effects of point mutations and growth conditions on yields. This system appears to be generally applicable for the facile production of useful quantities of active soluble mouse–human chimeric Fab fragments.

MATERIALS AND METHODS

Strains and Plasmids. *E. coli* JM109 (15) was used for cloning, and the strain 25F2 (12), obtained from D. Henner (Genentech), was used for expression. Plasmids pMY61, pMY60, and pMY55 were gifts from M. Yang and D. Henner (Genentech). pMY61 contains an expression cassette for the humanized Fab fragment of antibody D1.3 (16), similar to that of pAK19 (12), as an *Eco*RI–*Sph* I fragment in pBR322. Minor differences exist upstream of the ribosome binding sites. pMY60 and pMY55 contain the isolated heavy and light chains in the same context.

Vector Construction and Cloning of Antibody Genes. Primers H1–H12 and L1–L9 have been described by Huse *et al.* (17). The antibody 48G7 had been cloned (8). The 2E11 (18) and S107 (19) murine Fab fragments were cloned into the same vector from their cell lines analogously: total RNA was isolated from ≈10⁸ cells by standard methods (20), mRNA was purified by oligo(dT) affinity chromatography (15) with a kit from Pharmacia, cDNA was generated with primers L9 and H12 for 2E11 or L9 and IgAH3 for S107 (the latter is designed to match the 3' end of the IgA heavy chain C region C_H1 sequence: 5'-GTAATAGGACTAGTAGGAGTAGGACCAGA-3'), followed by PCR amplification with primers H2, H12, L5, and L9 for 2E11 and IgAH3, L9, and primers designed to match the published variable [κ chain and heavy chain variable region (respectively, $V_κ$ and V_H)] sequences for S107. The V_H and $V_κ$

Abbreviations: PhoA, alkaline phosphatase; V, variable; C, constant; J, joining; H (as subscript), heavy chain; K (as subscript), κ chain; CDR, complementarity-determining-region.
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regions were then moved into pMY60 and pMY55, respectively, by PCR amplification with primers that produced *Mlu* I–*Bst*EII (for 48G7: *Mlu* I–*Apa* I) fragments of V_H and *Eco*RV–*Kpn* I fragments of V_κ. Heavy and light chains were combined in an *Eco*RI–*Sac* I–*Mlu* I triple ligation; a short *Sac* I–*Mlu* I fragment of pMY61 provided the intergenic region. The plasmid containing the 48G7 sequences was designated pDEI434. It was further modified by subcloning the entire expression cassette into pGC-1 (8), yielding pDEI440, and making the following substitutions by site-directed mutagenesis (21): A *Sac* I site in the C_κ region was removed by introduction of a silent mutation. Two *Hin*dIII sites upstream of the promoter were deleted by *Hin*dIII digestion, blunting with the Klenow fragment of DNA polymerase I, and religation. The *Eco*RV site was replaced with a *Sac* I site, and unique *Hin*dIII, *Xho* I, and *Bst*EII sites were introduced as shown in Fig. 1. The expression cassette was then inserted back into pMY61 to yield p4xH. The following primers were designed to match the murine joining (J) regions and incorporate a *Hin*dIII into J_κ and a *Bst*EII site into J_H for cloning: J_H1, TGAGGAGACGGTGACCGTG-GTCCCTGCGCCCCA; J_H2, TGAGGAGACGGTGACC-GTGGTGCCTTGGCCCCA; J_H3, TGCAGAGACGGTG-ACCAGAGTCCCTTGGCCCCA; J_H4, TGAGGAGACGGT-GACCGAGGTTCCTTGACCCCA; $J_\kappa1$, TTTGATTTCAAG-CTTGGTGCCTCCACCGAACGT; $J_\kappa2$, TTTTATTTCAAG-CTTGGTCCCCCCTCCGAACGT; $J_\kappa3$, TTTTATTTCAAG-CTTGGTCCCATCACTGAACGT; $J_\kappa4$, TTTTATTTCA-AGCTTTGTCCCCGAGCCGAACGT; $J_\kappa5$, TTTCAGCTC-AAGCTTGGTCCCAGCACCGAACGT. They were used as an equimolar mixture (J_H1–J_H4 or $J_\kappa1$–$J_\kappa5$) during reverse transcription and PCR instead of H12 and L9 for cloning the V_H and V_κ regions of the hybridomas 18R.136.1 (22), 28B4.2 (23), AZ-28 (24), and 39,A11.1 (25) from their cell lines directly into p4xH. The V regions of 7G12-A10 (26) were cloned into p4x (p4xH lacking the *Hin*dIII site) by using J_H1–J_H4 and a set of J_κ primers that incorporated a *Kpn* I site into the J region. Twenty-five PCR cycles (94°C for 1 min; 50–55°C or 42°C during the first 5 cycles for V_H for 2 min; and 72°C for 1.5 min) were sufficient to amplify the desired sequences. The following primers were found to be optimal: primers H6 and L6 for 18R.136.1, H2 and L5 for 28B4.2, H2 and L3 for AZ-28, H2 and L5 for 39,A11.1, and H7 and L5 for 7G12-A10. Several clones of each chain were sequenced (27) to exclude PCR errors. We found that in some cases multiple J region primers had served to amplify the same V region, making one position in the J region ambiguous. The authentic sequence was identified by producing and PCR-amplifying cDNA encoding the whole Fab fragment and sequencing directly over the J region using a ^{32}P-labeled primer and a Vent polymerase PCR sequencing kit (New England Biolabs). The combinations of the AZ-28, 18R.136.1, 28B4.2, 2E11, and S107

chains with 48G7 were constructed by swapping *Eco*RI–*Bam*HI fragments containing the light chain sequences between pDEI434 and the respective expression vectors. The 48G7 mutants were generated by site-directed mutagenesis in pDEI440 and were expressed directly in this vector.

Expression and Purification of Chimeric Fab Fragments. For small-scale expression, 25 ml of Mops medium [30 mM Mops, pH 7.4/70 mM NaCl/10 mM KCl/1.6 mM MgSO$_4$/20 mM NH$_4$Cl/0.15% glucose/thiamine hydrochloride (1 μg/ml)/ampicillin (100 μg/ml)] supplemented with yeast extract and Casamino acids (Difco) in baffled shake flasks were inoculated with 0.5 ml of an overnight culture of 25F2 freshly transformed with the appropriate plasmid and incubated with vigorous shaking (300 rpm) at 30°C for 24 h unless otherwise noted. Optimal induction was found with yeast extract at 0.15 g/liter and Casamino acids at 0.55 g/liter. Periplasmic lysates were prepared by resuspension of the cells on ice at 200 OD$_{580}$ units/ml in lysis buffer [20% (wt/vol) sucrose/30 mM Tris·HCl, pH 8.0/1 mM EDTA], transfer to microcentrifuge tubes, incubation with lysozyme at 1 mg/ml (added from a stock solution at 10 mg/ml in lysis buffer) at room temperature for 30 min, and a 3-min centrifugation in a microcentrifuge to pellet the spheroplasts. PhoA activity was measured by diluting the crude extract into DEA buffer [9.7% (vol/vol) diethanol-amine, pH 9.8/0.5 mM MgCl$_2$/0.02% NaN$_3$] with p-nitrophenyl phosphate at 1 mg/ml and monitoring A_{405}. For a crude estimation, serial dilutions were set up in microtiter plates, and the level of induction was judged visually by the highest dilution that still produced a signal. Larger cultures (1.5 liters) were treated as described above, scaling up all volumes accordingly and pelleting the spheroplasts in an SS34 rotor (12,000 rpm, 15 min). High-density fermentations were performed in a 2.5-liter Bioflow III fermentor essentially as described by Carter *et al.* (12), but with 3.3 mM NaH$_2$PO$_4$ and 6 mM K$_2$HPO$_4$. Within 24 h, the cells were fully induced at an OD$_{580}$ of 70–100 units, depending on the clone. Periplasmic lysates were prepared in 200–300 ml of lysis buffer with incubation for 1 h. The spheroplasts were pelleted (GS3 rotor at 8500 rpm for 30 min, if necessary followed by a centrifugation at 16,000 rpm for 10 min in an SS34 rotor) and subjected to another incubation in lysis buffer as before. The supernatants were either subjected to an ammonium sulfate precipitation (80% saturation) or concentrated to ≈100 ml in an Amicon ultrafiltration cell. Purification on protein G affinity columns (Sepharose CL-6B, Pierce) was performed at room temperature in 50 mM Mes, pH 5.5/100 mM NaCl. Protein was eluted with 100 mM glycine (pH 2.8); and the eluate was neutralized immediately with 0.1 vol of 1 M Tris·HCl (pH 9.0).

Characterization of the Recombinant Fab Fragments. Proteins were analyzed by SDS/PAGE followed for purified protein by silver staining or for crude lysates by Western blot analysis (15) using an anti-human κ chain-PhoA conjugate (Southern Biotechnology Associates) for detection and nitro blue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate as substrate. The same antibody with p-nitrophenyl phosphate as substrate was used to detect hapten binding by ELISA. Wells were coated with bovine serum albumin (5 μg/ml) conjugates of the appropriate haptens (8, 18, 22–26). Catalytic activity was measured as described (8, 22–24, 26). Concentrations of purified antibody were determined by measuring A_{280} and A_{260} (28). Extinction coefficients varied between 61,500 and 74,200 M^{-1}·cm^{-1}.

RESULTS AND DISCUSSION

Vector Construction. To investigate the beneficial influence of the human sequences on the folding of the Fab fragment in the bacterial periplasm, we chose to pursue chimeric constructs as opposed to humanized versions of our catalytic antibodies. Humanization is an involved process that almost inevitably
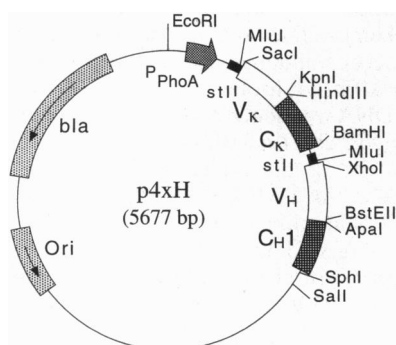

FIG. 1.   Map of the vector p4xH, indicating the restriction sites used for cloning. PphoA, PhoA promoter; stII, bacterial leader sequence; bla, β-lactamase gene; Ori, colE1 origin of replication.

results in some change in affinity for the hapten (29) that would likely be detrimental to catalysis. Due to the independence of V and C domains (30), no change in the structure of the V domains is observed when the murine C regions are exchanged for human versions (31).

The expression system is based on that described by Carter et al. (12) and this group (8). The vector from which it is derived, pMY61, is a pBR322 derivative very similar to pAK19 (12); it uses the same PhoA promoter, inducible by phosphate starvation (32), stII signal sequences for secretion into the periplasm (33), and human C regions (34, 35), excluding the Cys-Ala-Ala hinge at the C terminus of the C_H1 region. The V regions of the p-nitrophenyl phosphonate-specific antibody 48G7 were subcloned into this vector from pDE166 (8). The genes of the phosphocholine-specific myeloma S107 (19) and those of the hybridoma 2E11 (18) were derived from analogous constructs, into which they had been previously cloned from the corresponding cell lines (H.D.U., P.A.P., and P.G.S., unpublished results). The vector bearing the 48G7 Fab fragment was then modified to allow the use of the V region PCR primers H1–H10 and L1–L7, described by Huse et al. (17), to clone antibody genes of unknown sequences: a Sac I site in the C_κ sequence and a HindIII site upstream of the promoter were removed, Sac I and Xho I sites were incorporated at the N terminus of V_κ and V_H, respectively, and HindIII and BstEII sites were introduced into the J region sequences, resulting in the vector p4xH (Fig. 1).

PCR primers incorporating HindIII and BstEII sites into J_κ and J_H, respectively, were designed to complement the V region primers. Combinations of these primers were used to amplify the V regions of the antibodies 18R.136.1 (22), 28B4.2 (23), AZ-28 (24), 39,A11.1 (25), and 7G12-A10 (26) from cDNA obtained from their respective cell lines. The sequences of the light and heavy chain V regions of the cloned hybridomas along with 48G7 and S107 are listed in Fig. 2 according to their subgroups as classified by Kabat et al. (36).

Expression Studies. Small-scale shake-flask expression in Mops medium allowed for a quick estimation of relative yields, since the antibodies could easily be visualized on Western blots and their hapten-binding activity could be confirmed by ELISA assays of crude periplasmic fractions. Induction of the PhoA promoter was followed by measuring PhoA activity in

the lysate. As shown in Fig. 3, accumulation of the antibody lagged 1–2 h behind the induction of PhoA.

Under optimized expression conditions, the cells reached an OD_{580} of 2.0–2.5 units and remained stably induced for >36 h without detectable lysis. Replacement of the PhoA promoter by the lac or tac promoter resulted in significant reductions of expression levels in some cases. Yields were more rigorously determined after purification of the protein from 1.5-liter cultures. As noted by Carter et al. (12), the chimeric Fab fragments can be easily purified by protein G affinity chromatography. This method yielded the protein at >95% purity. Expression yields are given in Table 1.

With the exception of the myeloma-derived S107, the yields are in the range of 1–3 mg/liter for four antibodies and 0.1–0.3 mg/liter for the remaining three, which compares well with reported values (11). No obvious correlation between sequence homology and expression level was observed. The Fab fragments retained their hapten-binding and catalytic activity where tested (Table 1). Importantly, in all antibodies tested, the majority of the protein was produced in soluble form, whereas only a small fraction of Fab was soluble in the systems that we had examined previously. Even the small amounts of S107 Fab fragments were soluble, and only for the 2E11 clone was >10% of total antibody associated with the cytoplasmic fraction (data not shown). Large quantities of recombinant protein could be produced in high-density (70–100 OD_{580} units) fermentations in a 2.5-liter Bioflow III fermentor by using a modification of the protocol described by Carter et al. (12). Again, the antibodies were produced in soluble form, associated with the periplasm, and yields (10–150 mg/liter) were sufficient for crystallization and extensive kinetic studies (Table 1).

On Western blots of crude periplasmic fractions of AZ-28, an additional band below the functional Fab fragment was detected. Deleting the heavy chain sequences from the plasmid and comparing the pattern of expression indicated that the additional band was a disulfide-linked light chain dimer. Deletion of the 48G7 heavy chain sequences resulted in light chains that migrated as monomers on a nonreducing gel (data not shown). The expression levels of these light chains were strongly reduced compared to the complete Fab fragment, indicating that isolated light chains are fairly unstable in the E.

A

	FR1	CDR1	FR2	CDR2	FR3	CDR3	FR4
1	DIVMTQSPTFLAVTASKKVTISC	TASESLYSSKHKVHYLA	WYQKKPEQSPKLLIY	GASNRYI	GVPDRFTGSGSGTDFTLTISSVQVEDLTHYYC	AQFYSYPLT	FGAGTKVEIK
2	DIVMTQTPLSLPVSFGDQVSISC	RSSQSLANSYGNTYLS	WYLHKPGQSPQLLIY	GISNRFS	GVPDRFSGSGSGTDFTLKISTVKPEDLGMYYC	LQGTRQPPMYT	FGGGTKVEIK
3	ELVMTQTPLSLPVSLGDQASISC	RFSQSIVHSNGNTYLE	WYLQKSGQSPKLLIY	KVSNRFS	GVPDRFSGSGSGTDFTLKISRVEAEDLGVYYC	FQGSHVPRT	FGGGTKLEIK
4	ELVMTQTPLSLPVSLGDQASISC	RSSQSLLHSNGNTYLH	WYLQKPGQSPKLLIY	KVSNRFS	GVPDRFSGSGSGTDFTLKISRVEAEDLGVYFC	SQVTHVPPT	FGGGTKLEIK
5	DIQMTQSPSSLSASLGERVSLTC	RASQEINGYLG	WLQQKPDGTIKRLIY	AASTLHS	GVPKRFSGSRSGSDYSLTISSLESEDFADYYC	LQYASYPRT	FGGGTKVEIK
6	ELVMTQSPSSLSASLGERVSLTC	RASQDISNYLI	WVQQKPDGTIKRLIY	STSTLDS	GVPKRFSGSRSGSDYSLTISSLESEDFADYYC	LQYASSPFT	FGSGTKLEIK
7	ELVLTQSPSSMYASLGERVTITC	KASQDINSYLN	WFQQKPGKSPKTLIY	RTNRLVD	GVPSRFSGSGSGQDYSLTISSLEYEDMGIYYC	LQYDEFPYT	FGSGTKLEIK
8	ELVMTQTPKFMSTTVGDRVSITC	KASQNVGTPVA	WYQQKPGQSPKLLIY	SASNRYT	GVPDRFTGSGSGTDFTLTISNMQSEDLADYFC	QQYSSYPLT	FGGGTKVEIK

B

	FR1	CDR1	FR2	CDR2	FR3	CDR3
1	QVQLLESGAEIVRPGASVKLSCKASGYIFT	DYWMN	WVKQRPGQGLEWIG	GINPSDSSTNYNQKFKD	KVTLTVDTSSNSAYIQLSSLTSEDSAVYFCAT	SGRFRGGY
2	QVQLLESGAELVKPGASVKLSCKASGYTFT	SYWMH	WVKQRPGRGLEWIG	MIDPNSGGTKYNEKFKS	KATLTVDKPSNTAYMQLSSLTSEDSAVYYCTR	RDMDY
3	QVQLLESGAELMKPGASVKISCKATGYTFS	SFWIE	WVKQRPGHGLEWIG	EILPGSGGTHYNEKFKG	KATFTADKSSNTAYMQLSSLTSEDSAVYYCAR	GHSYYFYDGDY
4	QVQLQQSGAELVKPGASVKLSCTASGFNIK	DTYMH	WVKQRPKQGLEWIG	RIDPANVDTKYDPKFQD	KATITADTSSKTTYLQLSGLTSEDTAVYYCAS	YYGIY
5	QVQLLESGGGLGQPGGSLRLSCATSGFTFT	DYYFN	WARQPPGKALEWLG	FIRNKAGYTTEYSASVKG	RFTISRDNSQGILYLQMNTLRAEDSATYYCAR	WGSYAMDY
6	QVKLLESGGGLVQPGGSLRLSCATSGFTFS	DFYME	WVRQPPGKRLEWIA	ASRNKANDYTTEYSASVKG	RFIVSRDTSQSILYLQMNALRAEDTAIYYCAR	DYYGSSYWYFDV
7	QVQLLESGPELKKPGETVKISCKASGYTPT	NYGMN	WVKQAPGKGLKWMG	WINTYTGEPTYADDFKG	RFAFSLETSASTAYLQINNLKNEDTATYFCVQ	AERLRRTFDY
8	QVQLLESGPGIFQPSQTLSLTCSFSGFSLS	TDGIGVG	WIRQPSGKGLEWLA	HIWWNDNKYYNIVLKS	RLTISKDTSNNQVFLKIASVDTADTGTYYCVR	IRDYYGYAMDY

	FR4
1	WGAGTTVTVSS
2	WGAGTTVTVSS
3	WGQGTSVTVSS
4	WGQGTTLTVSS
5	WGQGTSVTVSS
6	WGAGTTVTVSS
7	WGAGTTVTVSS
8	WGQGTSVTVSS

FIG. 2. Amino acid sequences of the cloned V regions. Subgroup numbers are indicated in parentheses. (A) Light chains. Sequences: 1, S107 (I); 2, 2E11 (II); 3, 28B4.2 (II); 4, 39, A11.1 (II); 5, 48G7 (V); 6, 18R.136.1 (V); 7, AZ-28 (V); 8, 7G12-A10 (V). (B) Heavy chains. Sequences: 1, 18R.136.1 (IIA); 2, 7G12-A10 (IIA); 3, AZ-28 (IIB); 4, 48G7 (IIC); 5, 28B4.2 (IIIA); 6, S107 (IIIA); 7, 39,A11.1 (misc.); 8, 2E11 (misc.). FR1–4, framework regions.
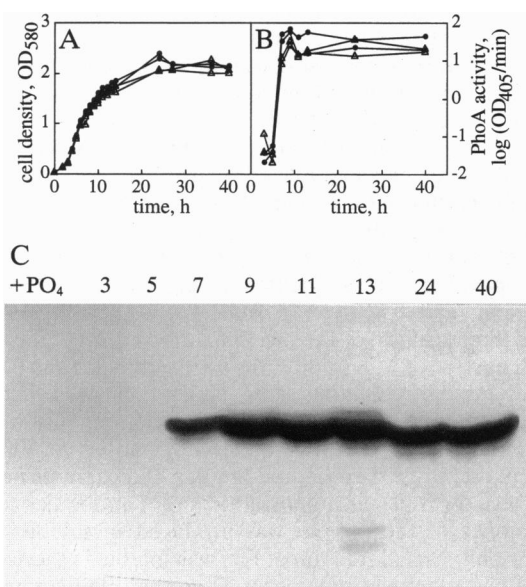
FIG. 3. Time course of antibody expression in shake flasks. Δ, 48G7; ●, 18R.136.1. (*A*) OD_{580} of the cultures after inoculation at a 1:200 dilution of an overnight culture. (*B*) Induction of the PhoA promoter, as measured by specific PhoA activity of the periplasmic lysates prepared at the indicated times. The activity [log(OD_{405} units/min)] of a lysate prepared from a culture grown in the presence of 2.0 mM sodium phosphate was −1.64. (*C*) Western blot of 18R.136.1 in the periplasmic fractions at the indicated times (20 μl per lane). +PO_4, lysate from a culture grown in the presence of 2.0 mM sodium phosphate.

coli periplasm. Since the dimers do not bind to the protein G affinity column, they do not present a problem for purification.

Characterization of Chain Combinations. Part of the diversity of the immune response is generated by the random combination of heavy and light chains during B-cell development. Due to this combinatorial diversity, antibody chains are well adapted to associate randomly with each other to form stable pairs. The association derives mainly from the interaction between the C regions (37), but the V regions are promiscuous enough to allow for random pairings. This property is exploited in phage and *in vivo* expression libraries.

To test whether the chimeric expression system would accommodate pairs of heavy and light chains that were not selected to associate with each other during an immunization process, chain combinations between 48G7 and the other clones were constructed and expressed. In no case was the expression of a hybrid clone worse than that of the poor partner, indicating that the yield depends to a large degree on

Table 1. Yields and activities of the cloned Fab fragments

Antibody	Yield, mg/liter		Activity present	
	Shake flask	Fermentor	ELISA	Catalysis
48G7	2.7 ± 29%	156	+	+
AZ-28	1.6 ± 22%	54	+	+
18R.136.1	0.34 ± 20%	20	+	+
2E11	0.25 ± 41%	13	+	ND
28B4.2	0.11 ± 17%	ND	+	+
S107	<0.02	ND	ND	ND
7G12-A10*	1.6	56	+	+
39,A11.1*	1.3	ND	+	ND

For shake flasks, the yield after purification is shown, based on four cultures grown under identical conditions (mean ± SD as a percentage of the mean). For the fermentor, the yield after purification is shown, based on one experiment. ND, not determined.
*Preliminary results based on one culture obtained independently (F.E.R., H.D.U., P.G.S., unpublished results).

the expression level of the individual chains and not their association. The combinations also revealed which of the chains was responsible for the varying expression levels observed. For example, the combination of the AZ-28 heavy chain and the 48G7 light chain expressed like AZ-28 itself, whereas the reverse combination showed levels comparable to 48G7, and light chain dimers were no longer observed, indicating that for AZ-28, the heavy chain limits expression. In contrast, the poor expression of S107 could not be attributed to one of the chains in particular, since neither combination with 48G7 resulted in any appreciable increase in expression compared to S107 itself. In all other cases, the combinations showed expression levels somewhere in between those of the two partners. Based on these results, it is likely that the chimeric expression system will be suitable for the generation of expression libraries by cloning random combinations of V regions from the spleen of an immunized mouse without significant losses in diversity resulting from insufficient expression levels.

Effects of Point Mutations on the Expression Level. Protein produced in this system has been used to solve the crystal structure of the recombinant Fab fragment of 48G7 to 2.0 Å (P.A.P. and P.G.S., unpublished results). To better understand the mechanism of this antibody a series of amino acid substitutions around the active site was generated. Interestingly, although the use of rare codons was avoided in all cases, some of the mutations had large effects on the protein yield (Fig. 4). According to their expression levels, the mutants fell into three classes. The Tyr33^{H}His, His35^{H}Ala, His35^{H}Gln, and His35^{H}Glu mutants, Tyr → Phe mutants at positions 99^H and 100^H, and the V$_\kappa$ triple mutation G^{L}48G7^H (Asn30^{L}Ser/Gly34^{L}Ser/His55^{L}Asp) all behaved like the wild-type 48G7. The Tyr91^{L}Lys, Tyr94^{L}Ala, Arg96^{L}Ala, Arg96^{L}Lys, and Tyr100^{H}His mutants showed 2- to 10-fold reductions in expression yields. In contrast to the Tyr99^{H}Phe mutant, expression of the other three Tyr99^H mutants was estimated to be at least 100-fold lower than that of wild-type 48G7 and comparable to the level of S107.

All mutations lie in the complementarity-determining region (CDR) loops or the β-strands immediately behind a loop, and none of them affect buried residues. Changes from a Tyr to a hydrophilic or charged amino acid like His, Arg, or Lys occur in all three classes. Most notably, Tyr99^H seems to accommodate no other change than the conservative replacement with Phe, even though it resides in a highly variable solvent-exposed position at the tip of the heavy chain CDR3 loop. Similar behavior is observed for the Phe and His mutants of the adjacent residue, Tyr100^H. Tyr91^L, Tyr94^L, and Arg96^L lie in the light chain CDR3 loop, and in the latter case, even the conservative change of Arg (which makes a hydrogen bond
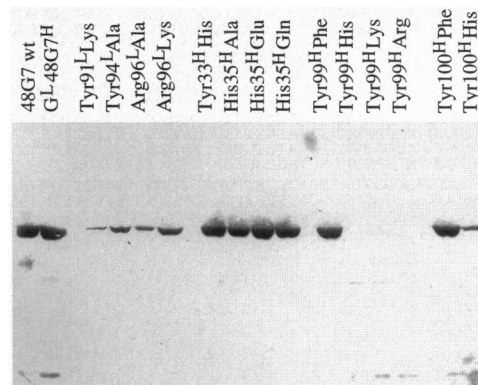

FIG. 4. Effects of point mutations on the expression level. Western blots were prepared from 25-ml cultures of 48G7 and the indicated mutants in pDEI440 (20 μl per lane) grown in parallel under identical conditions. wt, Wild type; G^{L}48G7^H = Asn30^{L}Ser/Gly34^{L}Ser/His55^{L}Asp.

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to the hapten) to Lys leads to a significant reduction in expression level. In contrast, significant changes can be made to Tyr-33^H and His-35^H in CDR1 of the heavy chain without significant effects (both residues contact the hapten). One possibility is that the *E. coli* translation machinery might be affected by small alterations in the mRNA, as observed by Dueñas *et al.* (38). Alternatively, amino acid substitutions in the loops could make the protein thermodynamically unstable, thereby rendering it susceptible to proteolytic attack. This phenomenon has also been reported by other groups (39, 40). Alfthan *et al.* (41) have observed similar correlations of expression levels and protease sensitivity with temperature stability of Fab fragments that use different murine C_H1 regions. On the other hand, Knappik and Plückthun (9) found positions in the heavy chain loops of the single-chain antibody McPC603 that influenced expression efficiency mainly through solubility effects without affecting the *in vitro* stability of the protein. The mutations in 48G7 seem to be of a different nature, however, since increased amounts of insoluble aggregates, as found by Knappik and Plückthun (9), were not observed in our poorly expressing clones. It is possible that the human C regions, which are absent in Plückthun's system, keep even unstable or partly unfolded V regions in solution, thus preventing aggregation and allowing proteases to remove the unfolded chains. It should be interesting to investigate whether the changes in expression titer in our system can indeed be correlated with protein stability.

Conclusion. We have demonstrated that a range of different hybridoma-derived antibodies and combinations of heavy and light chains not selected as pairs *in vivo* can be expressed as murine–human chimeric Fab fragments in *E. coli*. This expression system provides good yields of seven of eight antibodies assayed, and in each case the protein remained in soluble form. This chimeric system seems to be a viable compromise between the high expression levels of humanized antibodies and the low levels observed for murine Fab fragments, while avoiding the risk of losing catalytic activity by changing the V regions. Crystallography should be facilitated due to the greater homogeneity of the recombinant antibody when compared with murine Fab fragments generated by papain digestion, and site-directed mutagenesis becomes much less complicated, with the caveat of mutation effects on expression levels. Most importantly, however, attempts to improve the catalytic efficiency of existing antibodies by genetic approaches or the selection of catalytic clones from *in vivo* cDNA libraries (3, 4) should now become feasible.

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