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Biochemistry

Generation of antibody activity from immunoglobulin polypeptide chains produced in *Escherichia coli*

(carcinoembryonic antigen/monoclonal antibody/Fab fragment/chain recombination/modified immunoglobulin)

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ABSTRACT **Plasmids have been constructed that direct the synthesis in *Escherichia coli* of heavy chains and/or light chains of an anti-carcinoembryonic antigen (CEA) antibody. Another plasmid was constructed for expression of a truncated form of heavy chain (Fd′ fragment) in *E. coli*. Functional CEA-binding activity was obtained by *in vitro* reconstitution in *E. coli* extracts of heavy chain or Fd′ fragment mixed with extracts containing light chain.**

Hybridomas have an intrinsic limitation; they can only produce the natural sequence antibodies found in the B-cell population. Production of antibodies by recombinant DNA techniques, on the other hand, makes possible the construction of immunoglobulin derivatives designed for specific purposes. For this and other reasons, such as stability problems of hybridomas during large-scale growth, we sought to clone, characterize, and express antibody genes derived from cDNA.

Carcinoembryonic antigen (CEA) is a well-characterized human tumor marker for colon cancer (1, 2), and hybridoma lines producing anti-CEA monoclonal antibodies of high affinity have been obtained (3–8). As a model system to test the feasibility of antibody production based on recombinant techniques, we used an anti-CEA mouse hybridoma as a source of mRNA for cDNA preparation, and plasmids were constructed that direct the expression of either heavy (H; γ_1) or light (L; κ) chains, or both H and L chains in *Escherichia coli*. A plasmid was also constructed that allows expression of a truncated fragment of H chain (Fd′) in *E. coli*. *In vitro* reconstitution experiments with bacterially produced immunoglobulin polypeptide chains have yielded molecules with CEA-binding specificity.

EXPERIMENTAL

Cloning of Anti-CEA H- and L-Chain Genes. Hybridoma CEA 66-E3, which originated as a fusion of a SP2/0-Ag14 lymphocytoma cell with a mouse spleen cell (7), secretes only an anti-CEA IgG antibody with a κ L chain and γ_1 H chain. Total RNA from CEA 66-E3 was extracted as reported by Lynch *et al.* (9) and enriched for mRNA by oligo(dT) cellulose chromatography. Five micrograms of unfractionated poly(A) mRNA was used as a template for oligo(dT)-primed preparation of double-stranded cDNA as described (10, 11). The cDNA was fractionated according to size by electrophoresis on a 6% polyacrylamide gel, and DNA fragments >600 base pairs long were cloned into the *Pst* I site of pBR322 using the G·C tailing method (12). DNA hybridization probes were prepared (13) complementary to coding sequences in the constant region of L chain (5′ T-C-C-A-T-C-T-T-C-C-C-A-C-C) (14) or H chain (5′ C-T-G-G-G-A-T-G-C-C-T-G-G-T-C) (15). Approximately 8500 *E. coli* strain 294 (ATCC 31446) transformants were screened (16), of which 200 colonies contained L-chain and 40 colonies contained H-chain cDNA inserts. Further analysis of these clones revealed that the CEA 66-E3 cell line produces at least three different L-chain mRNAs and at least two different H-chain mRNAs (unpublished data).

Determination of Anti-CEA H-Chain Sequences. Two plasmids (pγ298 and pγ11) were found in which the combined nucleotide sequences did account for the entire coding region, and these were used in the construction of vectors for expression of H chain and Fd′ fragment.

The nucleotide sequence that we determined (17, 18) for the variable regions of the mouse anti-CEA H-chain mRNA, as well as the corresponding amino acids, is shown in Fig. 1*A*. Sequence data for the constant region are not shown but are in agreement with the sequence determined by Honjo *et al.* (15). The deduced amino acid sequence of the NH_2-terminal region of mature γ_1 chain corresponds perfectly with the sequence of the first 23 NH_2-terminal amino acids of hybridoma-derived anti-CEA γ_1 chain, which we determined by protein microsequence analysis (19). The CEA γ-chain cDNA codes for a putative 19 amino acid signal sequence and 447 amino acids of mature H chain. The mature unglycosylated H chain (M_r, 49,200) has a variable region of 123 amino acids, including an assumed diversity region of 12 amino acids, a J4 joining region of 13 amino acids, and a constant region of 324 amino acids. H-chain Fab fragment or Fd′ fragment to be used in the construction of the Fab portion of IgG consists of the first 226 amino acids of mature H chain with a M_r of 24,156.

Determination of Anti-CEA L-Chain Sequences. Several plasmids reacting with the κ-chain probe were found to contain cDNA inserts large enough to encode full-length κ chain. The complete nucleotide sequence of the L-chain gene was determined by the dideoxynucleotide chain-termination method (17), after subcloning restriction endonuclease cleavage fragments into M13 mp8 or mp9 vectors (18).

Fig. 1*B* shows the nucleotide sequence for the variable region of the mouse anti-CEA L-chain mRNA. The cDNA insert codes for a tentative 24 amino acid signal sequence and 214 amino acids of mature L chain. The deduced amino acid sequence of the NH_2-terminal region of mature κ chain corresponds perfectly with the first 23 NH_2-terminal amino acids of the hybridoma-derived mouse anti-CEA κ chain as determined by our amino acid sequence analysis of the purified protein (19). The mature L chain (M_r 23,598) has a variable region of 107 amino acids, including a J1 joining region of 12 amino acids, and a constant region of 107 amino acids.

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Abbreviations: CEA, carcinoembryonic antigen; H chain, heavy chain of immunoglobulin; L chain, light chain of immunoglobulin.

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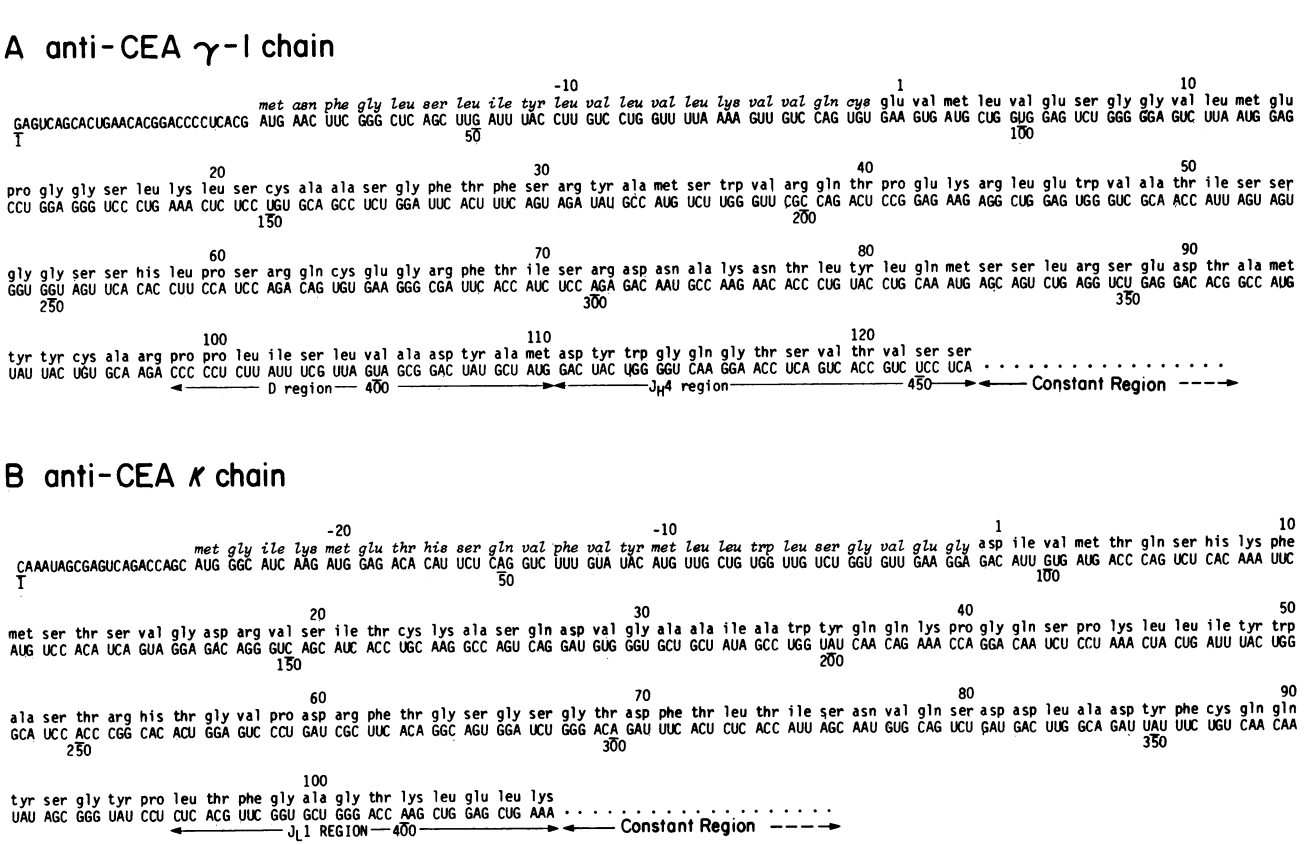

FIG. 1. Nucleotide sequence and deduced amino acid sequence of the variable region of anti-CEA γ_1-chain mRNA (*A*) and anti-CEA κ-chain mRNA (*B*). Putative presequences of 19 amino acids and 24 amino acids have been assigned to H and L chain, respectively. Diversity region (D) and joining regions (JH4 and JL1) are underlined. The numbers above each line refer to amino acid position of mature H or L chain. The numbers below each line refer to nucleotide position.

Construction of Plasmids for Expression of Antibody Chains in *E. coli*. Details of the constructions are outlined in the legend to Fig. 2. It was our strategy to replace the human growth hormone (*HGH*) gene in plasmid pHGH207-1* with either the gene for H or L chain of Fd' fragment. The plasmid pHGH207-1* resembles in all aspects the expression vector pHGH207-1 (21), except that the *Eco*RI restriction site 5' from the *trp* promoter was eliminated. The nucleotide sequence between the *trp* leader Shine–Dalgarno sequence and the initiation codon for the immunoglobulin chains is as follows:

S–D initiation

5'...*trp* promoter...A-A-G-G-G-T-A-T-C-T-A-G-A-A-T-T-A-T-G...antibody chain...

Xba I

Construction of this efficient ribosome binding site was based on observations that (*i*) an AT-rich region between the Shine–Dalgarno sequence (S–D) and ATG results in efficient expression of *HGH* (22) and bovine growth hormone (*BGH*) (23); (*ii*) efficiency of β-interferon synthesis is optimal when a distance of 7–11 bases is maintained between the Shine–Dalgarno sequence and ATG (24).

Construction of Vector pγCEAtrp207-1* for the Direct Expression of H Chain. Since neither of the cDNA plasmids included a full-length H-chain gene, it was necessary to construct the entire gene from two plasmids: pγ298, which contains the 5' end of the H-chain gene; and pγ11, which completes its 3' end. Two intermediate plasmids were required for the construction of the final expression vector. The first intermediate plasmid (pγCEAIntI) encodes the COOH-terminal region of H chain. In the construction of the second intermediate plasmid (pγCEAInt2), the gene for mature full-length H chain was reconstructed and the *E. coli trp* promoter/operator sequences were placed upstream from the structural gene. The most significant feature of this construction was the use of a synthetic deoxyoligonucleotide primer to position an ATG initiation codon directly in front of the DNA sequence coding for mature H chain (20). In the final construction, the tetracycline resistance (*Tet*^{R}) gene from pBR322 was introduced to yield pγCEAtrp207-1*.

Construction of Vector pγCEAFd'trp207-1* for the Direct Expression of Fd' Fragment. The gene for Fd' was constructed by introducing a stop codon in the hinge region of the H-chain gene. The TGT triplet coding for cysteine (nucleotide position 764–766; see figure 5 of ref. 15) was converted to a TGA stop codon by primer repair (20). The cysteine at nucleotide position 758–760 (see figure 5 of ref. 15) was retained to allow disulfide-bond formation with κ chain on *in vitro* reassociation, as is found in Fab antibody fragments generated by papain treatment (25).

Construction of Vector pκCEAtrp207-1* for Direct Expression of L Chain. Details of the assembly are not presented in Fig. 2, but techniques used were similar to those described for the construction of the H-chain expression vector, including primer repair to position an initiation codon in front of the mature L-chain gene.

RESULTS

Production of Immunoglobulin Polypeptide Chains. *E. coli* strain W3110 (ATCC 27325) was transformed with either pγCEAtrp207-1*, pκCEAtrp207-1*, or pγCEAFd'trp207-

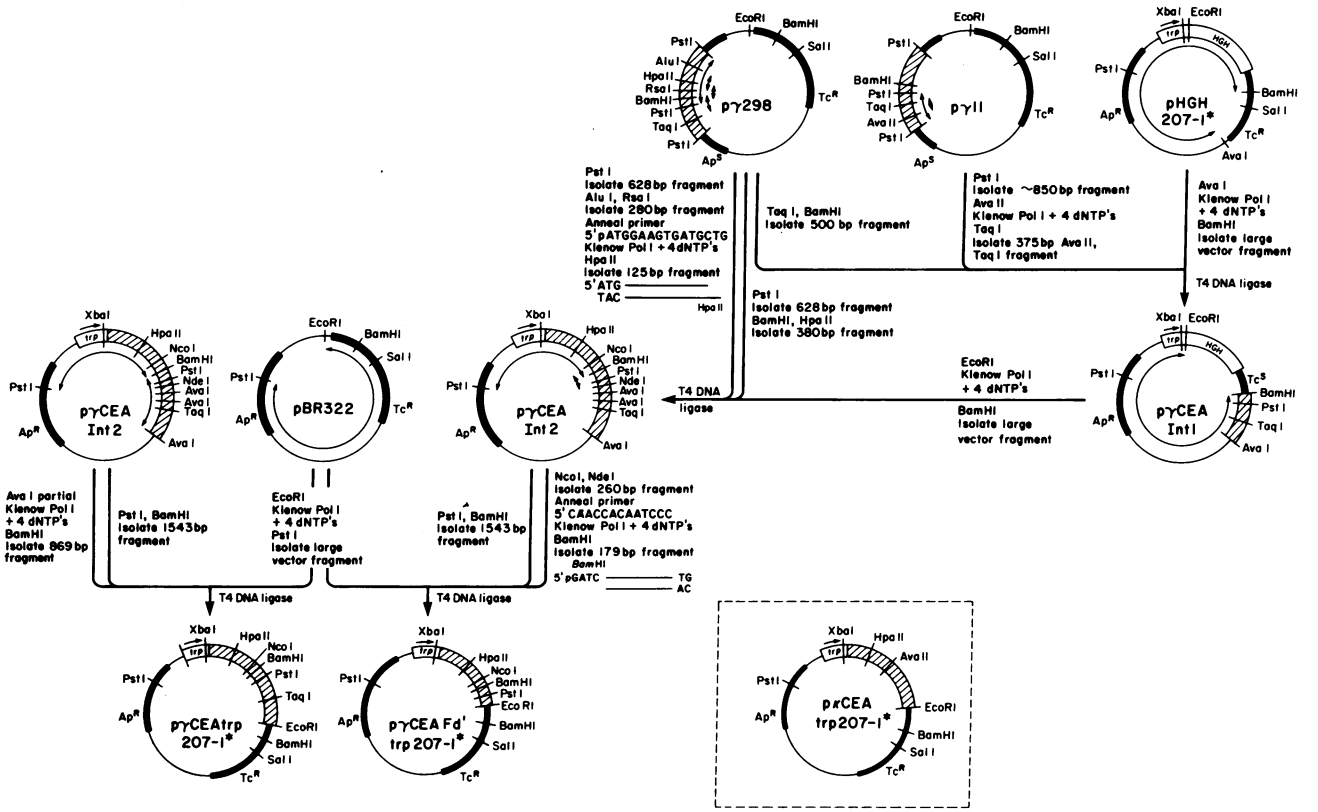

FIG. 2. Construction of vectors for expression in *E. coli* of immunoglobulin chains. An account of the enzymatic steps involved in the generation of new plasmids is presented in chronological order. Purification of DNA and ligation reactions were done as described (20). DNA restriction nuclease fragments used in these constructions are indicated by arrows inside the plasmid circles. Primer repair (20) was used to create initiation and termination codons. The primer 5′ A-T-G-G-A-A-G-T-G-A-T-G-C-T-G, which hybridizes to positions 87–99 of the anti-coding strand of cDNA (Fig. 1*A*), was used to insert an ATG 5′ to position 87. The primer 5′ C-A-A-C-C-A-C-A-A-T-C-C-C, which hybridizes to the coding strand of γ_1-chain cDNA from position 752–765 (see figure 5 of ref. 15), was used to facilitate conversion of the TGT cysteine codon at position 227 to the stop codon TGA.

1*. Transformants were grown in L broth containing 5 μg of tetracycline per ml to an A_{550} value of 5, and they were then induced with 50 μg of indoleacrylic acid per ml. Total cellular protein of induced cells was analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis. Cells transformed with either pγCEAtrp207-1* or pγCEAFd′trp207-1* had prominent new protein bands at the sizes expected for H chain or Fd′ fragment (data not shown). Cells transformed with pκCEAtrp107-1* had a band of increased intensity in the expected region, but other *E. coli* proteins of similar size precluded positive identification by protein staining. It was confirmed by immunoblotting (26) that immunoglobulin L-chain, H-chain, and Fd′ fragment were made in good yield (Fig. 3). Rough quantitation was done by comparing the amounts made in *E. coli* with different amounts of authentic CEA H and L chains. Estimated yield (percent of total cellular protein) of immunoglobulin polypeptides was 3% H chain and 0.5% L chain. Yield of Fd′ fragment could only be determined from Coomassie-blue stained gels and was ≈4%.

Expression of H and L Chains in the Same Cell. To obtain transformants producing both chains in the same cell, *E. coli* strain W3110 was first transformed with pκCEAtrp207-1*Δ, which is an ampicillin sensitive (Ap^S) derivative of pκCEAtrp207-1* made by deleting the *Pst* I/*Pvu* I fragment from the β-lactamase gene. Cells containing pκCEAtrp207-1*Δ were used as recipients for a second transformation using pγCEAInt2 (Fig. 2). Successful double transformants were identified by their Ap^R Tet^R phenotype. Double-transformed cells showed protein bands for both H and L chains when induced with indoleacrylic acid and analyzed by immunoblotting as described above (Fig. 3).

Reconstitution of Antibody Activity. *E. coli* extracts were prepared by suspension of 1 g of frozen cells in 9 ml of lysis buffer (7.6 M guanidine hydrochloride/50 mM Tris·HCl, pH 8/1 mM EDTA). 2-Mercaptoethanol was added to 0.1 M, and the suspension was incubated at 37°C for 1 hr, then centrifuged at 15,000 rpm (Sorvall SS-34 rotor) for 30 min. The clarified supernatant was stored at 4°C. Simple mixing of cell lysates containing γ_1 and κ chains does not generate detectable antibody activity, nor is activity observed in a lysate of cells coproducing the two chains (Table 1). Our reconstitution procedure was designed to completely reduce and solubilize the γ_1 and κ chains in mixtures of crude extracts, followed by a gradual return to native conditions in the continued presence of a mild redox buffer (29) that promotes thiol–disulfide interchange. Hybridoma anti-CEA was converted to denatured disulfide-free chains in the form of the protein S-sulfonates (30). This was accomplished by reacting hybridoma anti-CEA (0.5 mg/ml) in 7 M guanidine hydrochloride/1 mM EDTA/50 mM Tris·HCl, pH 8.5, for 6–16 hr at room temperature with sodium sulfite (20 mg/ml) and sodium tetrathionate (10 mg/ml), followed by dialysis (4°C) against 25 mM Tris·HCl (pH 8.5) in 9 M urea. A nonreducing $NaDodSO_4$/polyacrylamide gel (not shown) indicated that chain separation was complete within the limit of detection, and no anti-CEA activity was detectable (Table 1). For reconstitution, the above extracts were diluted to give a potential IgG content of 25 μg/ml in 8 M guanidine hydrochloride/50 mM Tris·HCl, pH 8/1 mM EDTA, and dialyzed 1–2 hr at 4°C against 20 vol of 8 M urea (deionized)/50 mM sodium glycinate, pH 10.8/10 mM glycine ethyl ester/1 mM EDTA/1 mM reduced glutathione/0.1 mM oxidized glutathione. Dialysis

Table 1. Anti-CEA activities of chain combination reactions

Preparation	Goat anti-mouse IgG (H and L chains)			Goat anti-mouse IgG F(ab')$_2$ fragment		
	Anti-CEA activity, ng/ml			Anti-CEA activity, ng/ml		
	With CEA	Without CEA	Yield, %	With CEA	Without CEA	Yield, %
No treatment						
Hybridoma γ- and κ-chain S-sulfonates	4	0	0.02	0	0	0
E. coli, γ_1- and κ-chain extract	4	0	0.02	0	0	0
Denaturation/renaturation						
Hybridoma γ_1- and κ-chain S-sulfonates	199	14	0.7	119	2	0.5
Hybridoma γ_1- and κ-chain S-sulfonates and *E. coli*/IFN-αA extract	163	8	0.6	143	3	0.6
E. coli, γ_1-chain extract and *E. coli*, κ-chain extract	454	28	1.7	717	20	2.8
E. coli, γ_1- and κ-chain extract double-transformed	880	11	3.5	1412	10	5.6
E. coli, Fd' fragment extract and *E. coli*, κ-chain extract	164	0	0.6	360	5	1.4
E. coli, γ_1-chain extract	17	10	0.03	1	0	0.004
E. coli, κ-chain extract	7	0	0.03	10	0	0.04
E. coli, Fd' fragment extract	6	0	0.02	0	0	0
E. coli, IFN-αA extract	10	0	0.04	0	0	0

Reactions were analyzed in an ELISA using polyvinyl chloride microtiter plates coated with CEA antigen by incubation for 16 hr at room temperature with a solution of 5 μg/ml. Nonspecific sites were sealed with 5% gelatin, and then samples of hybridoma anti-CEA (for the standard curve) and folding reactions were incubated in the wells for 90 min at 37°C. After washing with phosphate-buffered saline, wells were incubated with goat anti-mouse IgG (H and L chains) or goat anti-mouse IgG F(ab')$_2$ conjugated with alkaline phosphatase (both from Zymed Laboratories, South San Francisco), then with phosphatase substrate (Sigma). Samples were read according to a standard curve (with CEA). Nonspecific background was assessed on wells treated only with 5% gelatin (without CEA). Association yields were calculated as follows. Based on estimates of levels of immunoglobulin chains in *E. coli*, the theoretical yield of IgG in experiments involving κ and γ chains was 25 μg/ml; yield of Fab was 17 μg/ml. In experiments containing only κ chain, γ chain, or interferon (IFN) extracts, a hypothetical yield of 25 μg/ml was assigned; for Fd' alone it was 17 μg/ml. For IgG percent yield, the amount of specific binding (anti-CEA activity with CEA minus anti-CEA activity without CEA) was multiplied by 100 and divided by 25,000 ng/ml. For Fab, the apparent specific binding (ng/ml) from the standard curve was multiplied by 0.67 to reflect that two-thirds of the molecular weight of the CEA-associated ligand in the standard curve was Fab fragment sensitive to goat anti-mouse IgG F(ab')$_2$. This number was multiplied by 100 and divided by 17,000 ng/ml. The results shown are the averages of two dilutions of each of two independently conducted folding reactions.

bags were placed in 20 vol of fresh buffer in a graduated cylinder at 4°C, and N_2-saturated native buffer (the above buffer minus urea) was delivered over a 15-hr period to give a final urea concentration of 1 M. At this time, serum albumin was added to each dialysis bag to a concentration of 0.5 mg/ml, and the bags were transferred to phosphate-buffered saline/0.1 mM phenylmethylsulfonyl fluoride, not degassed, and dialyzed at 4°C for another 2 hr.

The final dialysate was assayed for CEA-specific binding activity using an ELISA procedure. Table 1 shows that no significant anti-CEA activity is obtained for *E. coli* extracts producing γ_1 chain, κ chain, Fd' fragment, or subtype A of human α-interferon (31) alone. On the other hand, significant binding is obtained when κ-chain extract is mixed with either γ_1 chain or Fd' fragment extract. Extract from *E. coli* expressing both γ_1 and κ chains generates comparable activity. Since we had observed that H- and L-chain specific anti-mouse IgG antibodies were directed primarily toward Fc determinants (see legend to Fig. 3), we independently assayed the extracts with a goat anti-mouse IgG conjugate specific for F(ab')$_2$ (Table 1).

DISCUSSION

The expression of cloned genomic κ-chain immunoglobulin genes in mouse lymphoid cell lines has been reported (32–34), but the expression of complete H- and L-chain genes derived from cDNA has not previously been obtained. Our results demonstrate that complete antibody chains can be produced efficiently in *E. coli*. There is, however, no detectable antibody activity in extracts of *E. coli* coproducing substantial levels of IgG H and L chains (Table 1). This may be due to the highly reducing intracellular environment (35, 36), which inhibits disulfide-bond formation, and to the accumulation of gene products in insoluble "refractile bodies" in the cell, a phenomenon noted in many cases of exogenous gene expression in *E. coli* (37). It is also possible that *E. coli* is lacking a protein that might be required for *in vivo* formation of IgG (28).

Most of the work described in the literature on *in vitro* antibody reconstitution from reduced denatured H and L chains was done with polyclonal antibodies. While formation of insoluble aggregates is a significant side reaction in such studies, reconstitution of at least several percent was obtained for some immunoglobulin derivatives (38). Better yields (17%) have been reported for reconstitution of reduced denatured Fab fragment rabbit anti-ribonuclease (39). Since it was necessary to solubilize *E. coli*-produced immunoglobulin polypeptides using denaturant, and since antibody activity has been regenerated in modified immunoglobulins from completely reduced and denatured molecules, we devised a method of IgG reconstitution starting from denaturing conditions. Under these conditions, the yield of anti-CEA activity from *E. coli* extracts was 3–5% when H and L chain were coexpressed in *E. coli*. For reasons not yet understood, reconstitution has been consistently better with *E. coli*-derived antibody chains than with hybridoma-derived material. It is possible that S-sulfonation inhibits reconstitution. The yield of activity with *E. coli*-produced Fab fragment was 1.4% when assayed in the ELISA using as antibody conjugate an anti-mouse IgG Fab antibody (Table 1).

The approach we have taken to express immunoglobulin chains from cDNA opens up new avenues toward production of recombinant antibodies. For example, this approach makes possible the construction at the DNA level of modified immunoglobulins with unique properties such as the Fab fragment described here. The construction of chimeric anti-CEA antibody chains by recombining the variable antigen-binding region from mouse hybridoma cDNA with the constant region from human IgG cDNA could produce antibodies with the advantage over mouse hybridomas of being less immunogenic when used for human *in vivo* diagnostic or

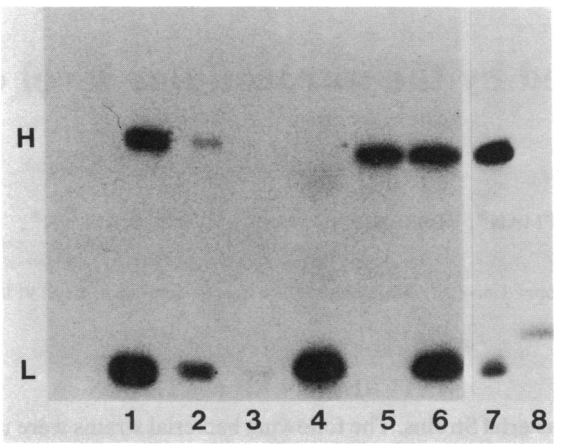

FIG. 3. Immunoblots of bacterially produced immunoglobulin peptide chains. Frozen *E. coli* cells were dispersed in 10 vol of 10 mM Tris·HCl, pH 7.5/1 mm EDTA/0.1 M NaCl/0.1 mM phenylmethylsulfonyl fluoride, disrupted by sonication, and partially clarified by centrifugation at 3000 rpm for 20 min in an SS-34 rotor. Aliquots of these extracts and of hybridoma-derived anti-CEA antibody were boiled for 5 min in 10 mM Tris·HCl/1 mM EDTA/0.1 M 2-mercaptoethanol/2.0% $NaDodSO_4$. The proteins were precipitated with 10 vol of acetone for 10 min, centrifuged for 5 min at 12,000 rpm, boiled for 5 min in O'Farrell's buffer (27), and size-fractionated on a 10% polyacrylamide gel in the presence of $NaDodSO_4$. Using the immunoblot technique (26), the proteins were transferred to nitrocellulose and the immunoglobulin chains were identified radioimmunochemically. Lanes 1–6, rabbit anti-mouse IgG and then ^{125}I-labeled protein A were used. Lanes 7 and 8, ^{125}I-labeled sheep anti-mouse IgG was used, because we found that pooled rabbit anti-mouse IgG did not react with Fd'. Even sheep anti-mouse IgG reacts less well with Fd' than with γ chain, probably because the major antigenic determinants are in the Fc region, which has been removed. Lanes 1–3 contain κ (H) and γ (L) chains from hybridoma-derived anti-CEA; the amounts are 1.0, 0.1, and 0.01 μg, respectively. Lane 4, *E. coli* κ chain; lane 5, *E. coli* γ chain; lane 6, *E. coli* κ and γ chains produced in the same cell; lane 7, *E. coli* κ and γ chains; lane 8, *E. coli* Fd' fragment.

therapeutic purposes. In addition, the expression of pre-heavy chain (or pre-Fd' fragment) and pre-light chain in *E. coli*, yeast, and mammalian cells could be pursued to establish conditions for *in vivo* assembly of secreted antibodies. These approaches could lead to alternative ways of preparing stable human monoclonal antibodies, as well as antibodies having properties custom-designed for particular research and therapeutic applications.

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1. Gold, P. & Freedman, S. O. (1965) *J. Exp. Med.* **121,** 439–448.
2. Shively, J. E. & Todd, C. W. (1981) in *Handbook of Cancer Immunology. Tumor Antigens: Structure and Function*, ed. Waters, H. (Garland, New York), Vol. 8, pp. 321–354.
3. Acolla, R. S., Carrel, S. & Much, J.-P. (1980) *Proc. Natl. Acad. Sci. USA* **77,** 563–567.
4. Kupchik, H. Z., Zurawski, V. R., Jr., Hurrell, J. G. R., Zamchek, M. & Black, P. H. (1981) *Cancer Res.* **41,** 3306–3310.
5. Hedin, A., Hammarstrom, S. & Larsson, A. (1982) *Mol. Immunol.* **19,** 1641–1648.
6. Primus, F. J., Newell, K. D., Blue, A. & Goldenberg, D. M. (1983) *Cancer Res.* **43,** 686–692.
7. Wagener, C., Yang, Y. H. J., Crawford, F. G. & Shively, J. E. (1983) *J. Immunol.* **130,** 2308–2315.
8. Wagener, C., Clark, B. R., Rickard, K. J. & Shively, J. E. (1983) *J. Immunol.* **130,** 2302–2307.
9. Lynch, K. R., Pennica, D., Ennis, H. L. & Cohen, P. S. (1979) *Virology* **89,** 251–254.
10. Goeddel, D. V., Heyneker, H. L., Hozumi, T., Arentzen, R., Itakura, K., Yansura, D. G., Ross, M. J., Miozzari, G., Crea, R. & Seeburg, P. H. (1979) *Nature (London)* **281,** 544–548.
11. Wickens, M. P., Buell, G. N. & Schimke, R. T. (1978) *J. Biol. Chem.* **253,** 2483–2495.
12. Chang, A. C. Y., Nunberg, J. H., Kaufman, R. J., Erlich, H. A., Schimke, R. T. & Cohen, S. N. (1978) *Nature (London)* **275,** 617–624.
13. Crea, R. & Horn, T. (1980) *Nucleic Acids Res.* **8,** 7331–7348.
14. Hamlyn, P. H., Gait, M. J. & Milstein, C. (1981) *Nucleic Acids Res.* **9,** 4485–4494.
15. Honjo, T., Obata, M., Yamawaki-Kataoka, Y., Kataoka, T., Kawakami, T., Takahashi, N. & Mano, Y. (1979) *Cell* **18,** 559–568.
16. Wallace, R. B., Johnson, M. J., Hirose, T., Miyake, T., Kawashima, E. H. & Itakura, K. (1981) *Nucleic Acids Res.* **9,** 879–894.
17. Smith, A. J. H. (1980) *Methods Enzymol.* **65,** 560–580.
18. Messing, J., Crea, R. & Seeburg, P. H. (1981) *Nucleic Acids Res.* **9,** 309–321.
19. Shively, J. E. (1981) *Methods Enzymol.* **79,** 31–48.
20. Goeddel, D. V., Shepard, H. M., Yelverton, E., Leung, D., Crea, R., Sloma, A. & Pestka, S. (1980) *Nucleic Acids Res.* **8,** 4057–4074.
21. de Boer, H. A., Comstock, L. J., Yansura, D. & Heyneker, H. L. (1982) in *Promoters: Structure and Function*, eds. Rodriguez, R. L. & Chamberlin, M. (Praeger, New York), pp. 462–481.
22. de Boer, H. A., Comstock, L. J., Hui, A., Wong, E. & Vasser, M. (1983) *Biochem. Soc. Symp.* **48,** 233–244.
23. Matteucci, M. & Heyneker, H. L. (1983) *Nucleic Acids Res.* **11,** 3113–3121.
24. Shepard, H. M., Yelverton, E. & Goeddel, D. V. (1982) *DNA* **1,** 125–131.
25. Anzel, L. M. & Poljak, R. J. (1979) *Annu. Rev. Biochem.* **48,** 961–997.
26. Burnette, W. N. (1981) *Anal. Biochem.* **112,** 195–203.
27. O'Farrell, P. H. (1975) *J. Biol. Chem.* **250,** 4007–4021.
28. Wabl, M. & Steinberg, C. (1982) *Proc. Natl. Acad. Sci. USA* **79,** 6976–6978.
29. Saxena, V. P. & Wetlaufer, D. B. (1970) *Biochemistry* **9,** 5015–5023.
30. Means, G. E. & Feeney, R. E. (1971) *Chemical Modification of Proteins* (Holden-Day, San Francisco), pp. 152–154.
31. Goeddel, D. V., Yelverton, E., Ullrich, A., Heyneker, H. L., Miozzari, G., Holmes, W., Seeburg, P. H., Dull, T., May, L., Stebbing, N., Crea, R., Maeda, S., McCandliss, R., Sloma, A., Tabor, J. M., Gross, M., Familletti, P. C. & Pestka, S. (1980) *Nature (London)* **287,** 411–416.
32. Rice, D. & Baltimore, D. (1982) *Proc. Natl. Acad. Sci. USA* **79,** 7862–7865.
33. Ochi, A., Hawley, R. G., Shulman, M. J. & Hozumi, T. (1983) *Nature (London)* **302,** 340–342.
34. Oi, V. T., Morrison, S. L., Herzenberg, L. A. & Berg, P. (1983) *Proc. Natl. Acad. Sci. USA* **80,** 825–829.
35. Freedman, R. B. & Hillson, D. A. (1981) in *Enzymology of Post-Translational Modification of Proteins*, eds. Freedman, R. B. & Hawkins, H. C. (Academic, New York), pp. 157–212.
36. Pollitt, S. & Zalkin, H. (1983) *J. Bacteriol.* **153,** 27–32.
37. Wetzel, R. & Goeddel, D. V. (1983) in *The Peptides: Analysis, Synthesis, Biology*, eds. Gross, E. & Meienhofer, J. (Academic, New York), Vol. 5, pp. 1–64.
38. Freedman, M. H. & Sela, M. (1966) *J. Biol. Chem.* **241,** 5225–5232.
39. Haber, E. (1964) *Proc. Natl. Acad. Sci. USA* **52,** 1099–1106.