Purification of, Generation of Monoclonal Antibodies to, and Mapping of Phosphoribosyl *N*-Formylglycinamide Amidotransferase†

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ABSTRACT: 5′-Phosphoribosyl *N*-formylglycinamide (FGAR) amidotransferase (EC 6.3.5.3) catalyzes the fourth reaction in the *de novo* synthesis of purines, that is, the conversion of FGAR to 5′-phosphoribosyl *N*-formylglycinamidine (FGAM). This is the only step of the pathway for which a vertebrate gene has not been cloned. FGAR amidotransferase has been highly purified from Chinese hamster ovary (CHO) cells, and this preparation has been used to generate monoclonal antibodies in mice. Two of these antibodies, designated BD4 and DD2, have been shown to recognize a 150-kDa protein in CHO-K1 cells that is of very low abundance in Ade⁻B cells, a CHO line in which FGAR amidotransferase activity is undetectable. Furthermore, the protein recognized by these antibodies is 5–10-fold more abundant in Az^r cells. The CHO Az^r cell line was made resistant to azaserine, a potent inhibitor of FGAR amidotransferase, and displays a 5–10-fold increase in FGAR amidotransferase activity over the parental K1 line. FGAR amidotransferase activity and the 150-kDa protein recognized by both monoclonal antibodies were found to immunoprecipitate concomitantly using antibody BD4. Monoclonal antibody DD2 cross-reacted with a human protein of identical molecular mass. A number of Ade⁻B/human hybrid cells were generated by somatic cell fusion and subsequent 5-bromo-2-deoxyuridine segregation. Analysis of these lines, together with two independently generated human/mouse hybrid cell lines, by both cytogenetics and immunoblotting with antibody DD2 revealed that the human FGAR amidotransferase gene is located on chromosome 17p.

Purines are of fundamental importance in the biology of all organisms. Purine nucleotide triphosphates are essential building blocks of both DNA and RNA. Adenosine triphosphate is the basic unit of energy metabolism and is also a prerequisite for the phosphorylation-dependent regulation of enzymes. Cyclic AMP and cGMP are both key messengers in signal transduction. Given such an integral role, it might be supposed that any defect in the metabolism of purines would be lethal and consequently of limited medical interest. However, a growing number of seemingly unrelated human disorders have been attributed to, or associated with, perturbations in purine metabolism, including hyperuricemia and gout (Palella & Fox, 1989), Lesch-Nyhan disease (Stout & Caskey, 1989), severe combined immune deficiency (Giblett *et al.*, 1972, 1975), severe mental retardation with autism (Jaeken & Van den Berghe, 1984; Jaeken *et al.*, 1988; Stone *et al.*, 1991), sensorineural deafness (Becker *et al.*, 1988), and certain aspects of Down's syndrome (Pant *et al.*, 1968). Purine biosynthesis is also the site of action for a number of anticancer, antiviral, and immunosuppressant drugs [for a review, see Elion (1988)].

The *de novo* synthesis of purines, as illustrated in Figure 1, appears to be invariant between species. However, the organization of the enzymes catalyzing the various reactions differs widely. For example, 5′-phosphoribosylglycinamide (GAR) transformylase (EC 2.1.2.2), GAR synthetase (EC 6.3.4.13), and 5′-phosphoribosylaminoimidazole (AIR) synthetase (EC 6.3.3.1) activities are catalyzed by three different proteins in *Escherichia coli* and *Bacillus subtilis*, by two proteins in yeast, and by a single multifunctional protein in *Drosophila melanogaster*, chickens, and humans. To complicate the issue further, the AIR synthetase domain is duplicated in the *D. melanogaster* multifunctional protein, and in *D. melanogaster*, chickens, and humans an additional protein is encoded that contains only the GAR synthetase domain (Zalkin & Dixon, 1992).

While regulation of the *de novo* purine biosynthetic pathway is well-documented for *E. coli*, and to a lesser extent for *B. subtilis*, little is known about regulation in higher animals [for a review, see Zalkin and Dixon (1992)]. The main reason for this had been that, until recently, none of the genes for purine biosynthesis in higher animals had been isolated. In the last 3 years, cDNA clones encoding 9 of the 10 enzymatic steps involved in the synthesis of inosine monophosphate (IMP[1]) and 3 of the 4 genes dedicated to the conversion of IMP to either AMP or GMP have been obtained from chickens and/or humans [Zalkin and Dixon (1992) and references therein]. This should greatly facilitate regulation studies in these animals.

The only enzyme in the *de novo* synthesis of IMP for which a gene has not been isolated from a vertebrate is 5′-phosphoribosyl *N*-formylglycinamide (FGAR) amidotransferase (EC 6.3.5.3, 5′-phosphoribosyl-*N*-formylglycinamidine (FGAM) synthetase). FGAR amidotransferase catalyzes the

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[1] Abbreviations: BrdU, 5-bromo-2-deoxyuridine; CHO, Chinese hamster ovary; FISH, fluorescence *in situ* hybridization; FGAM, 5′-phosphoribosyl *N*-formylglycinamidine; FGAR, 5′-phosphoribosyl-*N*-formylglycinamide; IMP, inosine monophosphate; PRPP, 5′-phosphoribosyl 1-pyrophosphate; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; TBS, Tris-buffered saline; TLC, thin-layer chromatography.

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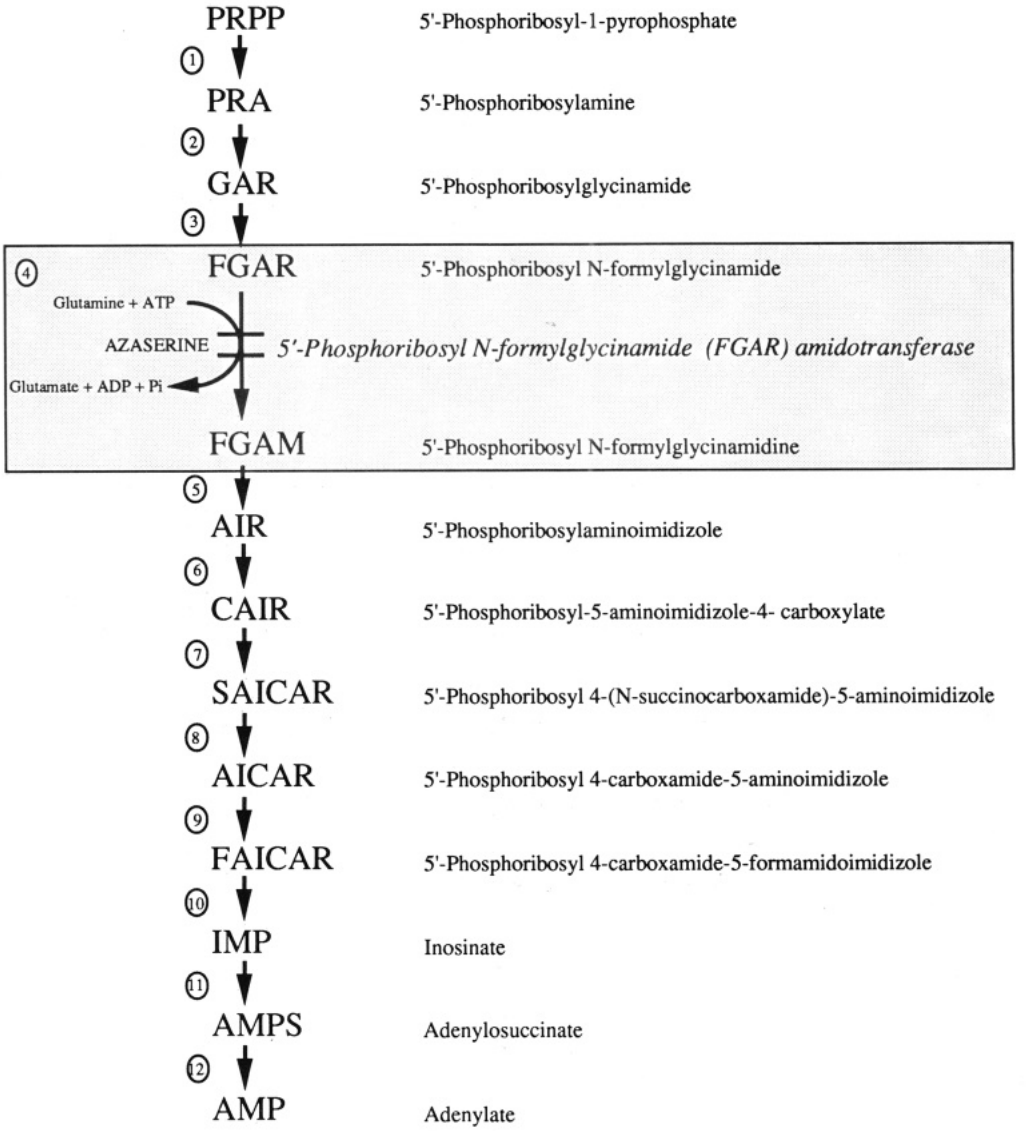

FIGURE 1: *De novo* biosynthesis of purines. The reaction catalyzed by 5′-phosphoribosyl *N*-formylglycinamide amidotransferase (FGAR amidotransferase) is shaded.

fourth reaction of *de novo* purine biosynthesis, that is, the irreversible conversion of FGAR to FGAM with the concomitant deamination of glutamine to glutamate and the hydrolysis of ATP to ADP and inorganic phosphate. Genes encoding FGAR amidotransferase have been isolated from *E. coli* (Schendel *et al.*, 1989), *B. subtilis* (Ebbole & Zalkin, 1987), *Saccharomyces cerevisiae* (Giani *et al.*, 1991), and *Lactobacillus casei* (Gu *et al.*, 1992). The amidotransferase protein has been highly purified from, in chronological order, *Salmonella typhimurium* (French *et al.*, 1963), chicken (Mizobuchi & Buchanan, 1968; Buchanan *et al.*, 1978; Schendel & Stubbe, 1986), Erlich ascites tumor cells (Chu & Henderson, 1972), and *E. coli* (Schendel *et al.*, 1989). In most cases the protein is reported to be a monomer with a molecular mass of approximately 135 kDa. The mechanism of action of chicken FGAR amidotransferase has been studied extensively by Buchanan and co-workers [for reviews, see Buchanan (1973, 1988)].

FGAR amidotransferase is a peculiar protein in the pathway for a number of reasons. It is one of only two single-function proteins in the pathway to IMP, the other being glutamine 5′-phosphoribosyl 1-pyrophosphate (PRPP) amidotransferase (EC 2.4.2.14). For a single-function polypeptide it has an unusually high molecular mass. The two steps immediately preceding and the step following FGAR amidotransferase are catalyzed by the same multifunctional protein in higher organisms. The existence of a CHO mutant, $Ade^{-}P_{AB}$ (Oates *et al.*, 1980), defective for both FGAR amidotransferase and glutamine PRPP amidotransferase activities, would suggest that the two amidotransferases are in some way linked physically. This is of particular interest since glutamine PRPP amidotransferase is the presumed regulatory protein of the pathway. We present here the purification of CHO FGAR amidotransferase, the generation of monoclonal antibodies to this protein, and the localization of the human FGAR amidotransferase gene to chromosome 17. The monoclonal antibodies will help to isolate both the CHO and human genes and to answer many of the questions regarding FGAR amidotransferase and the *de novo* purine biosynthesis pathway.

MATERIALS AND METHODS

Materials. Cell lines GM10498A and GM10479A were purchased from the Human Genetic Mutant Cell Repository at the Coriell Institute for Medical Research. Mouse myeloma line Ag8.653 was a generous gift from Dr. W. T. Melvin (University of Aberdeen, Aberdeen, Scotland). The biotinylated total human genomic and chromosome 17 "paint" probes were obtained from Oncor Inc. (Gaithersburg, MD).

Cells and Media. CHO-K1, Ade$^-$B, and Az^r cell lines were maintained on Ham's F12 medium supplemented with 5% fetal calf serum. All hybrid lines generated at this institute were maintained on Ham's F12D medium, also supplemented with 5% dialyzed fetal calf serum. GM10498A and GM10479A cells were maintained as directed by Coriell. Ag8.653 cells were maintained on Dulbecco's modified Eagle's medium supplemented with 10% horse serum.

Isolation of the Ade$^-$B and Azaserine-Resistant Cell Lines. The Ade$^-$B cell line is devoid of FGAR amidotransferase activity, and its isolation has been described previously (Kao & Puck, 1972a; Patterson, 1975; Patterson *et al.*, 1974).

Isolation of an azaserine-resistant cell line was accomplished by exposing CHO-K1 cells to increasing concentrations of azaserine in the absence of exogenous purines. Initially, the cells were plated in 0.045 μg/mL azaserine, a concentration that allowed 50% growth. After the azaserine concentration was slowly increased by 200-fold to 10 μg/mL, an azaserine clone (Az^r) was isolated. This clone exhibited a 5–10-fold increase in FGAR amidotransferase activity compared with CHO-K1.

Preparation of Cell Protein Extracts. Cells were grown to near-confluence and harvested using trypsin. After they were washed once with phosphate-buffered saline, the cells were lysed by resuspension in lysis buffer (20 mM Tris (pH 7.5), 1% Triton X-100, 50 mM NaCl, 5 mM $MgCl_2$, 0.7 μg/mL pepstatin, 0.5 μg/mL leupeptin, and 34 μg/mL phenylmethanesulfonyl fluoride) and incubation on ice for 15 min. Cell debris was removed by centrifugation at 100000*g* for 15 min. If used for immunoprecipitation, the supernatant was diluted to 10 mg/mL and used immediately. If used for SDS–PAGE, the supernatant was diluted to 7.5 mg/mL, mixed with 0.5 vol of boiling mix [150 mM Tris (pH 6.8), 6% (w/v) SDS, 15% (v/v) 2-mercaptoethanol, 30% (v/v) glycerol, and 0.05% (w/v) bromophenol blue], heated to 100 °C for 2 min, and stored at −20 °C.

Isolation of [^{14}C]FGAR. Ade$^-$B cells were plated at 10^6 cells per 100-mm plate and grown overnight in Ham's F12 supplemented with 5% fetal calf serum. After they were washed with PBS, the cells were incubated for 7 h in Ham's F12D supplemented with 5% dialyzed fetal calf serum and containing 50 μCi/mL [^{14}C]formic acid (Amersham, 52.5 mCi/mmol). After the plates were washed thoroughly with PBS, the cells were extracted with 80% ethanol. After the removal of any cell debris by centrifugation, the supernatant was spotted onto a cellulose thin-layer chromatography (TLC) plate (J. T. Baker), which was developed using a butanol/acetic acid/H_2O (2:1:1) liquid phase, dried, and exposed to X-ray film. The area of cellulose on the TLC plate corresponding to [^{14}C]FGAR was marked, scraped off, and resuspended in H_2O. After centrifugation to remove the cellulose, the supernatant was freeze-dried. The [^{14}C]FGAR was then brought to 10^5 cpm/μL in H_2O and stored at 4 °C.

FGAR Amidotransferase Assay. Cell protein extracts were prepared as described above. Protein extract (10 μL, 10 mg/mL) was mixed with 75 μL of H_2O and 10 μL of 10× Rxn mix [500 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH 7.0), 50 mM glutamine, 30 mM ATP, and 50 mM $MgCl_2$]. The reaction was started by the addition of 5 μL of [^{14}C]FGAR (0.23 μCi), allowed to continue at 37 °C for 15 min, and stopped by the addition of 100 μL of 95% ethanol. The mix was cooled on ice for 5 min and centrifuged at 12000*g* for 5 min. The supernatant (40 μL) was spotted onto a cellulose TLC plate (J. T. Baker), which was developed using a butanol/acetic acid/H_2O (2:1:1) liquid phase, dried, and exposed to X-ray film. For quantitation, spots corresponding to [^{14}C]-FGAR and [^{14}C]FGAM on the TLC plate were marked, cut out, and counted using a Beckman LS 1801 scintillation counter.

Purification of FGAR Amidotransferase. All purification steps were carried out at 4 °C unless otherwise stated. All dialyses were performed overnight with at least 500 vol and two changes of the required buffer. All concentrations were performed using an Amicon Centriprep 30 concentrator.

CHO-K1 cells (30 g) were resuspended in 25 mL of buffer J [20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH 7.2), 5% (v/v) glycerol, 5 mM $MgCl_2$, 5 mM glutamine, and 5 mM dithiothreitol] supplemented with 0.5 μg/mL leupeptin, 0.7 μg/mL pepstatin, and 34 μg/mL phenylmethanesulfonyl fluoride. The suspension was freeze–thawed twice and centrifuged at 100000*g* for 30 min. The supernatant was carefully decanted, brought to 30% $(NH_4)_2SO_4$, stirred on ice for 30 min, and centrifuged at 10000*g* for 10 min. The resulting supernatant was brought to 60% $(NH_4)_2SO_4$ and then stirred and centrifuged as before. This time the supernatant was discarded, and the pellet was resuspended in 18 mL of buffer J, dialyzed against buffer J, and applied to a DEAE–cellulose column previously equilibrated with buffer J. After the column was washed with 2 column vol of buffer J, bound protein was eluted using a 0–200 mM KCl gradient in buffer J. Fractions exhibiting FGAR amidotransferase activity (see below) were pooled, concentrated, and dialyzed against buffer H (buffer J without glutamine). This solution was applied to an azaserine affinity column preequilibrated with buffer H. The azaserine affinity column was prepared in advance by attaching the carboxyl group of azaserine to activated AH-Sepharose (as directed by the manufacturer). After the column was washed with buffer H, bound protein was eluted using a 25–100 mM KCl gradient in buffer H. The fractions containing the FGAR amidotransferase activity were pooled, concentrated, and dialyzed against buffer J. The protein solution was then applied to an Affigel-Blue (Bio-Rad) column preequilibrated with buffer J. FGAR amidotransferase activity, which was not retained by this column, was recovered by washing with 2 column vol of buffer J and collecting the eluant. After concentration to approximately 1 mg/mL, this highly purified FGAR amidotransferase preparation was routinely stored at 4 °C.

Generation of Hybridoma Lines. Monoclonal antibodies were generated essentially as described by Kohler and Milstein (1975), using the modifications described by Barnes *et al.* (1987). Briefly, female BALB/c mice, 8 weeks old, were immunized by intraperitoneal injection with 50 μg of the highly purified FGAR amidotransferase in 50% complete Freund's adjuvant in 0.9% NaCl. The mice were boosted on weeks 1, 2, and 25 as described above, but incomplete adjuvant was used. Three days after the final boost, spleen cells were fused with myeloma Ag8.653 cells. Colonies that were positive by immunoblotting were cloned twice by limiting dilution to ensure line monoclonality and stability. Monoclonal antibodies were prepared for use by 45% $(NH_4)_2SO_4$ precipitation of cell culture supernatant.

Immunoblotting. Proteins from crude cell extracts or purified preparations were resolved by discontinuous SDS–PAGE (Laemmli, 1970), using 7.5% (w/v) polyacrylamide resolving gels. Electroblotting of the resolved proteins onto nitrocellulose paper (Schleicher and Schuell, BA 85, 0.45-μm pore size) was performed as described by Towbin *et al.* (1979). The nitrocellulose paper was incubated successively in the following: 5% nonfat dried milk in Tris-buffered saline (TBS)

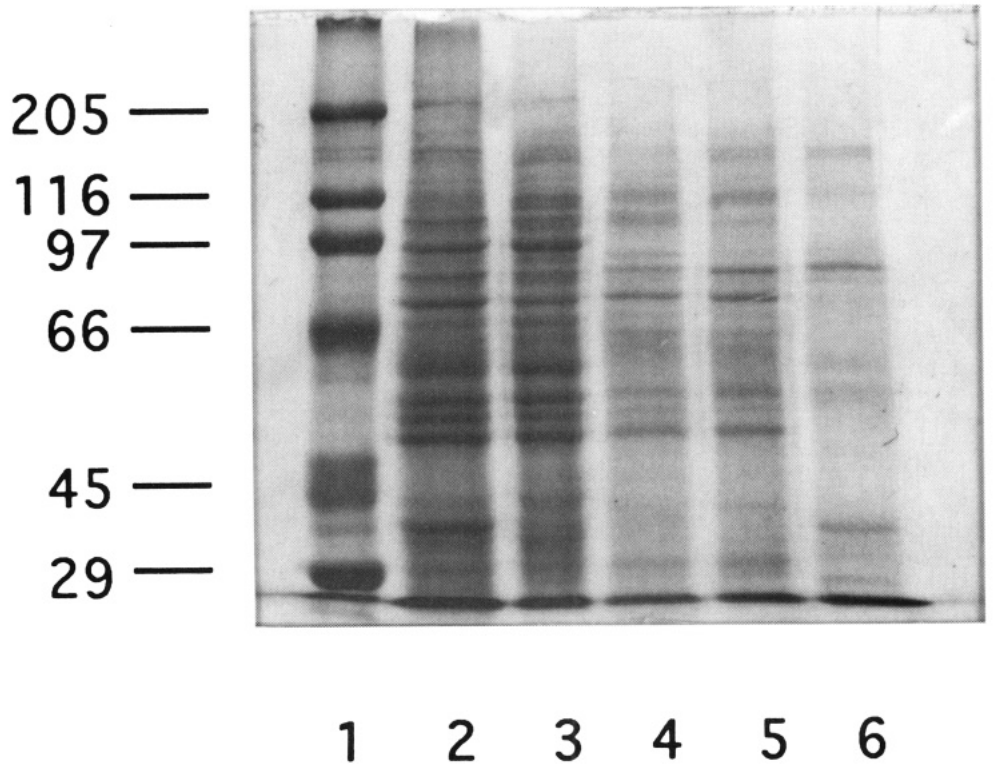

FIGURE 2: Purification of 5′-phosphoribosyl *N*-formylglycinamide amidotransferase from CHO-K1 cells. The proteins present in pertinent fractions of the purification procedure were resolved by 7.5% SDS–PAGE and visualized by Coomassie Blue staining: lane 1, molecular weight markers; lane 2, crude fraction of soluble K1 proteins (25 μg); lane 3, DEAE column load (25 μg); lane 4, azaserine affinity column load (5 μg); lane 5, Affigel-Blue column load (5 μg); lane 6, Affigel-Blue column eluent (5 μg).

for 30 min; "blot wash" [1% nonfat dried milk and 0.2% Triton X-100 in TBS] containing monoclonal antibody for 90 min; blot wash for 5 min, three times; blot wash containing rabbit anti-mouse IgG–horseradish peroxidase conjugate (Bio-Rad) for 60 min; blot wash for 5 min, twice; TBS for 5 min; and finally Amersham ECL reagents for 1 min. After the paper was sealed with plastic wrap, it was used to expose X-ray film for 15–120 s. To visualize the results, the film was developed as directed by the manufacturer.

Immunoprecipitation. CHO cell protein extract (100 μL at 10 mg/mL) was incubated with monoclonal antibody (500 μg) on ice for 30 min. Protein A–Sepharose CL-4B (Sigma) (300 μL of a 50% (v/v) solution) was pelleted, and the antibody mixture was used to resuspend the pellet. The suspension was incubated at 4 °C for 3 h with end-over-end rotation. After pelleting, the supernatant was removed for assay. The pellet was washed three times with 1 mL of lysis buffer (see the Preparation of Cell Protein Extracts section) and finally resuspended with 50 μL of lysis buffer for assay.

Somatic Cell Hybrid Production. CHO Ade$^-$B cells and human lymphocytes were fused using inactivated Sendai virus (Moore *et al.*, 1977). Hybrids in which the purine nutritional requirement of the Ade$^-$B cells had been complemented by the newly acquired human chromosomes were obtained by growth in selective medium (Ham's F12 without hypoxanthine, supplemented with 5% dialyzed fetal calf serum). Segregants, hybrids in which the human chromosome originally responsible for complementation of the Ade$^-$B locus had been lost, were selected by using 5-bromo-2-deoxyuridine (BrdU; Barton *et al.*, 1991).

Cytogenetic Analysis. The complement of human chromosomes contained by the various hybrid lines was determined by Giemsa–trypsin banding (GTG) followed by Giemsa-11 (G-11) staining on the same metaphase chromosome spreads (Barton *et al.*, 1991). The analyses and, in particular, the presence of human chromosome 17 were confirmed by fluorescence *in situ* hybridization (FISH) using total human genomic and chromosome 17 paint probes (Lichter *et al.*, 1990).

Table 1: Isolation of FGAR Amidotransferase from CHO-K1 Cells

fraction	fraction vol[a] (mL)	total protein (mg)	total activity (cpm)	specific activity (cpm/mg of protein)	fold purification
crude	40	924	300 300	325	1×
$(NH_4)_2SO_4$ pellet	20	318	222 600	700	2×
DEAE cellulose	72	40	600 000	1.5×10^4	46×
azaserine	10.5	4.8	480 000	1.0×10^5	314×
Affigel-Blue	7.5	0.3	159 000	5.3×10^5	1631×

[a] Fraction volume before concentration.

RESULTS

Purification of FGAR Amidotransferase. Purification of FGAR amidotransferase was carried out as described in the Materials and Methods section. Purification data are given in Table 1, while analysis of the pertinent pooled fractions by SDS–PAGE is illustrated in Figure 2. The final preparation exhibited a high level of FGAR amidotransferase activity and contained a protein of 150 kDa molecular mass. This mass is identical to that reported for chicken FGAR amidotransferase by SDS–PAGE (Frère *et al.*, 1971).

The high degree of purification accomplished, 1631-fold over that of the crude CHO-K1 extract, would suggest that the final FGAR amidotransferase preparation should be close to homogeneity. Although the SDS–PAGE analysis showed the end product to contain four prominent polypeptides in addition to the 150-kDa species (apparent molecular masses of 95, 65, 60, and 35 kDa), there are several reasons to believe that many of these polypeptides may be degradation products

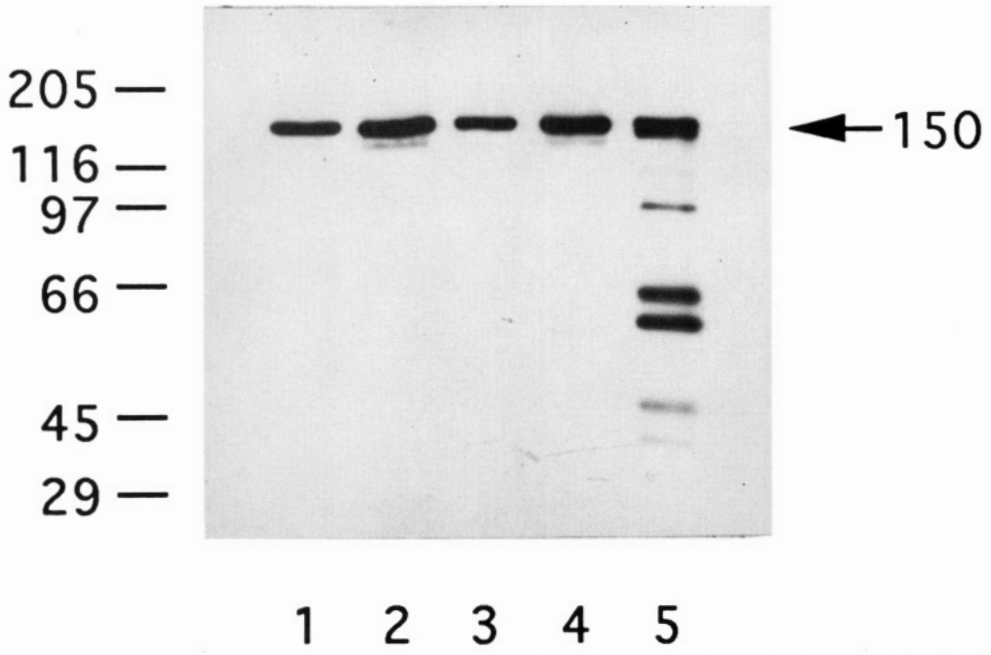

FIGURE 3: Immunoblot of purification fractions with monoclonal antibody BD4. The proteins present in pertinent fractions of the purification procedure were resolved by 7.5% SDS–PAGE and immunoblotted with monoclonal antibody BD4 as described in the text: lane 1, crude fraction of soluble K1 proteins (25 μg); lane 2, DEAE column load (25 μg); lane 3, azaserine affinity column load (5 μg); lane 4, Affigel-Blue column load (5 μg); lane 5, Affigel-Blue column eluent (5 μg).

of FGAR amidotransferase. Firstly, as mentioned above, there is the high "fold" of purification accomplished. Secondly, we were unable to further isolate the 150-kDa polypeptide. That is, even after application of the FGAR amidotransferase preparation to additional affinity or sizing columns, any fraction containing the 150-kDa polypeptide still contained additional lower molecular mass species. This would suggest that the various polypeptides are associated, either as members of a single polymer or as degradation products of a monomer. Finally, the 95-, 65-, 60-, and 35-kDa polypeptides present in the final fraction (Figure 2, lane 6) did not appear to be present in the pooled fraction loaded onto the final column (Figure 2, lane 5), suggesting that the lower molecular mass species are degradation products of the 150-kDa polypeptide and not subunits of the same protein, since such subunits should be present throughout the SDS–PAGE analysis. Thus, it was presumed that FGAR amidotransferase was, at the very least, a major component of the final preparation, and the preparation was used to immunize mice.

Generation and Characterization of the Monoclonal Antibodies. Mice were immunized with the highly purified CHO FGAR amidotransferase preparation, and antibody screening was carried out using a crude extract of CHO protein. Six hybridoma lines were isolated from a single fusion using this procedure. Two of these lines secreted antibodies, designated BD4 and DD2, that recognized a single protein of 150 kDa molecular mass when immunoblotted against the crude extract of CHO proteins. Since this molecular mass corresponds to the mass of the proposed FGAR amidotransferase protein, these two antibodies were chosen for further study.

Antibody BD4 was first immunoblotted against the same series of pooled protein fractions used for the SDS–PAGE analysis of the CHO-FGAR amidotransferase purification (Figure 3). With the exception of the final fraction, antibody BD4 recognizes a single protein of 150 kDa (lanes 1–4). In the final fraction, the eluent from the Affigel-Blue column (lane 5), antibody BD4 recognizes six protein bands. The molecular masses of five of these polypeptides closely correspond to the five prominent bands identified after SDS–PAGE analysis of this fraction. This result substantiates the opinion that the final fraction contains essentially intact FGAR amidotransferase and several degradation products.

In order to characterize the monoclonal antibodies BD4 and DD2 further, they were immunoblotted against crude protein extracts from a panel of different cell lines (Figure 4). The 150-kDa protein recognized by BD4 was not detected in Ade$^-$B, a CHO-K1-derived mutant cell line apparently devoid of FGAR amidotransferase activity (Figure 4A, lane 2). Furthermore, this protein was significantly more abundant in Az^r than K1 (Figure 4A, lane 3). Az^r is a CHO-K1 line that had been made resistant to azaserine, a potent inhibitor of FGAR amidotransferase, and exhibits a 5–10-fold increase in the activity of this enzyme over its parental line (see Materials and Methods). The antibody DD2 recognized a similar pattern of protein bands when immunoblotted against the same panel (Figure 4B). However, DD2 appeared to be somewhat more sensitive than BD4, since it did detect a small amount of the 150-kDa protein in Ade$^-$B. Furthermore, whenever DD2 was immunoblotted against an increased amount of the 150-kDa protein, as is present in Az^r for example, it also recognized additional bands of lower molecular mass, presumably degradation products.

Different cross-reactivities with proteins from other species were observed for the two monoclonal antibodies. When immunoblotted against proteins from the mouse cell line A9, neither BD4 nor DD2 recognized a protein (Figure 4A,B, lanes 5), a result that was anticipated since the antibodies were generated in mice. However, although BD4 did not recognize a protein in the human cell line HEP G2, DD2 did cross-react with an analogous protein of 150 kDa molecular mass (Figure 4A,B, lanes 4). This was an important result since it allowed the use of DD2 to chromosomally map the FGAR amidotransferase gene with the extensive panel of CHO-Ade$^-$B/human hybrid cell lines generated at this institute.

Immunoprecipitation of FGAR Amidotransferase. To prove definitively that BD4 and DD2 recognize the same 150-kDa protein and that this protein is FGAR amidotransferase,

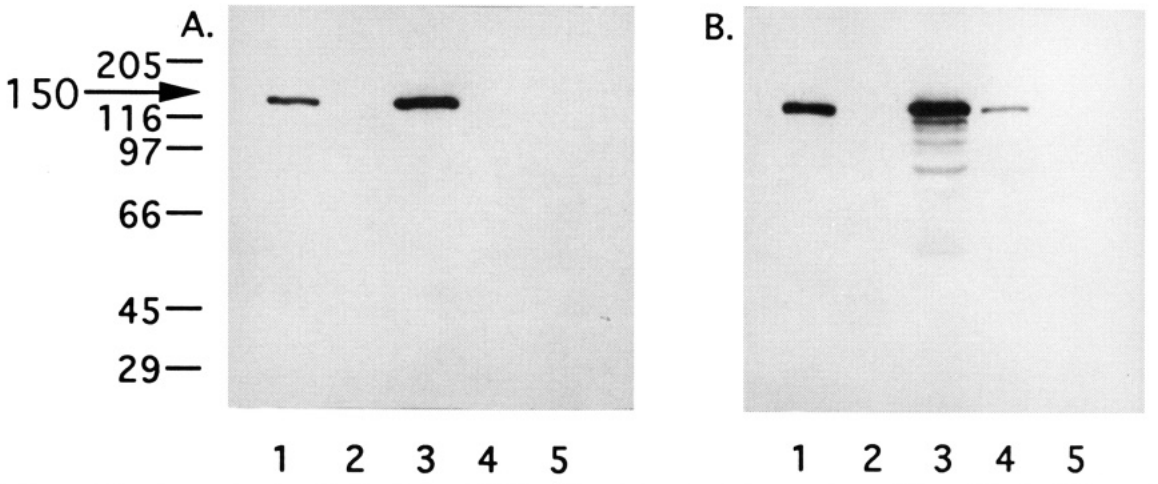

FIGURE 4: Immunoblot of monoclonal antibodies BD4 and DD2 with protein extracts from various cell lines. Total soluble proteins (12.5 μg) from K1 (lane 1), Ade⁻B (lane 2), Az^r (lane 3), human HEP G2 (lane 4), and mouse A9 cells (lane 5) were resolved by 7.5% SDS–PAGE and immunoblotted with either monoclonal antibody BD4 (A) or DD2 (B).

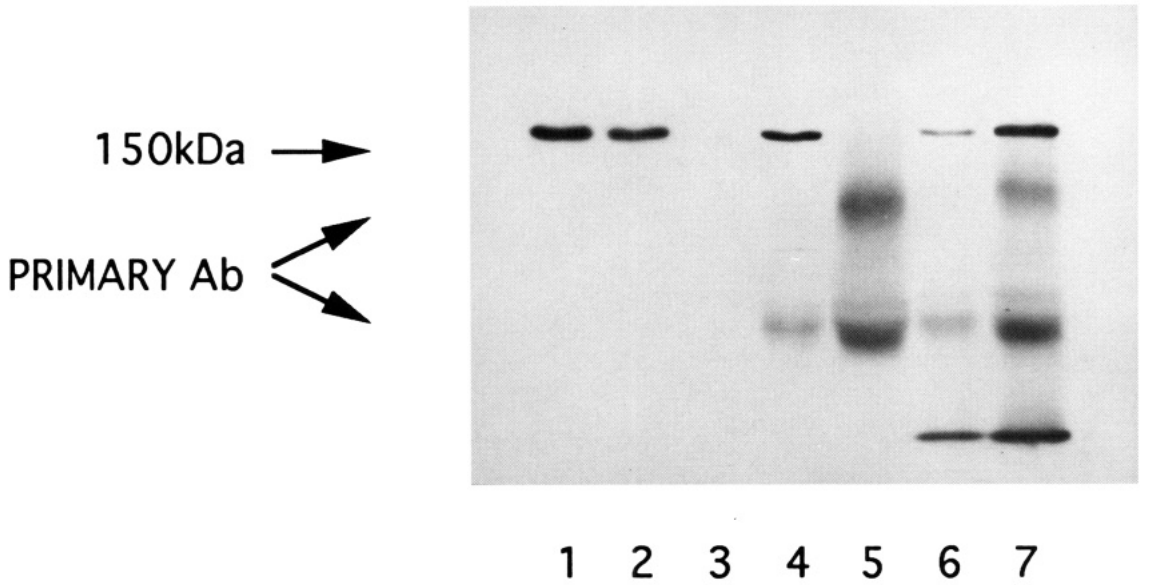

FIGURE 5: Immunoprecipitation of the 150-kDa protein recognized by monoclonal antibody BD4. Monoclonal antibody BD4 was used to precipitate its antigen under the conditions described in the text. Precipitated and unprecipitated protein fractions were resolved by 7.5% SDS–PAGE and immunoblotted with BD4: lane 1, CHO-K1 soluble proteins (reference); lanes 2 and 3, fractions not precipitated and precipitated, respectively, in the absence of a primary antibody; lanes 4 and 5, fractions not precipitated and precipitated, respectively, using a miscellaneous antibody; lanes 6 and 7, fractions not precipitated and precipitated, respectively, using BD4.

precipitation of the immunoreactive protein was attempted using the antibodies (Figures 5 and 6). Under the conditions described in the Materials and Methods section, BD4 immunoprecipitated a large proportion of the available 150-kDa protein from a crude K1 protein extract (Figure 5, lanes 6 and 7), while no immunoreactive protein was precipitated either in the absence of primary antibody (lanes 2 and 3) or in the presence of a miscellaneous primary antibody (lanes 4 and 5). The precipitated and unprecipitated fractions were also assayed for FGAR amidotransferase activity by TLC, as described in Materials and Methods. The presence of FGAR amidotransferase activity was found to correspond closely with the distribution of the 150-kDa protein (Figure 6, lane designations are the same as for Figure 5). Quantitation of the radioactivity present in the FGAR and FGAM spots after TLC revealed that BD4 precipitated 85% of the available amidotransferase activity, while less than 6% of the available activity was precipitated either in the absence of primary antibody or in the presence of the miscellaneous antibody. Thus, monoclonal antibody BD4 must recognize and precipitate FGAR amidotransferase.

Conditions could not be found under which DD2 would precipitate sufficient protein to give a positive activity assay. However, DD2 did recognize the 150-kDa protein precipitated by BD4 and did not recognize anything precipitated without primary antibody or with the miscellaneous primary antibody (results not shown). Thus, DD2 also recognizes FGAR amidotransferase, but does not precipitate it.

Production of Somatic Cell Hybrids and Assignment of the FGAR Amidotransferase Gene to Chromosome 17. CHO Ade⁻B cells and human lymphocytes were fused and plated in medium without hypoxanthine. Under these conditions, only Ade⁻B cells in which the mutated FGAR amidotransferase locus has been complemented by a gene present on a human chromosome will form viable colonies. Eighteen CHO/human hybrids were produced in this manner (NLB-1-26). The human chromosomes retained by these hybrids were determined by sequential GTG/G-11 banding/staining and

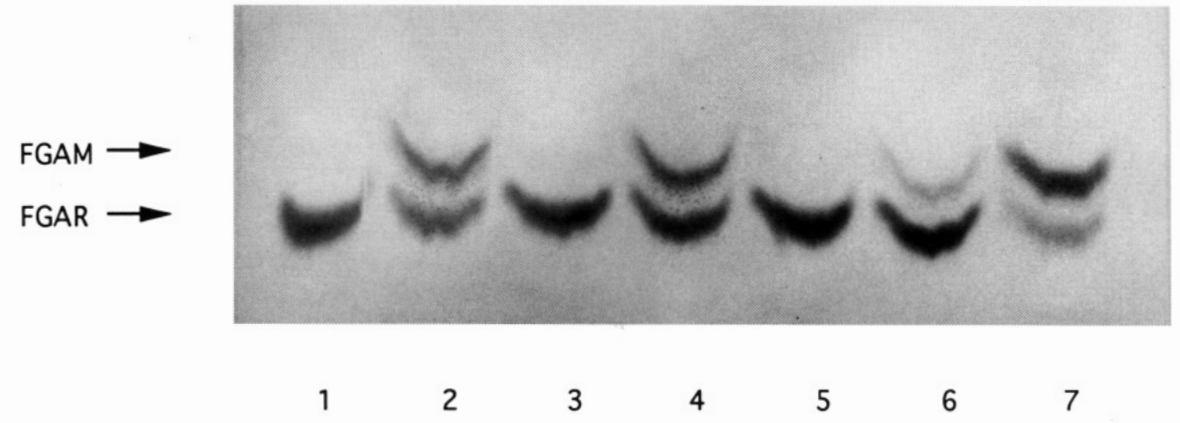

FIGURE 6: Immunoprecipitation of FGAR amidotransferase activity by monoclonal antibody BD4. FGAR amidotransferase activity was assayed by measuring the conversion of [^{14}C]FGAR to [^{14}C]FGAM as described in Materials and Methods. Protein samples assayed, and their lane designations, were identical with those described in Figure 5. CHO-K1 soluble proteins in lane 1 act as the "zero" time point.

Table 2: Results of Cytogenetic Analysis of Fusion Hybrids

	human chromosomes																							
clone	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	X	Y
NLB-1	+		+	+	+	+	+	+	+	+	+	+	+	+			+		+		+	+	+	
NLB-3	+	+	+	+	+	+	+	+	+	+	+	+		+	+		+		+	+		+	+	
NLB-4	+	+	+	+	+		+	+	+		+	+		+	+		+		+	+		+	+	
NLB-5	+		+	+	+		+		+		+	+	+	+	+		+				+	+	+	
NLB-6	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+		+		+	+	+	+	+	
NLB-8	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+		+	+	+	
NLB-9	+		+														+						+	
NLB-11	+		+		+		+	+			+						+		+	+	+	+	+	
NLB-13A			+	+			+	+			+						+					+	+	
NLB-13B			+	+			+	+			+			+			+			+	+	+	+	
NLB-15	+		+	+	+	+			+				+				+	+	+		+	+	+	
NLB-17	+			+	+		+	+			+	+		+	+		+		+	+	+	+		
NLB-20	+		+	+	+	+	+				+	+	+	+	+		+		+	+	+	+	+	
NLB-21	+		+						+			+					+					+	+	
NLB-22						+		+				+		+	+		+		+		+			
NLB-23																	+[a]							
NLB-25	+		+	+	+	+	+				+	+	+	+	+		+		+			+		
NLB-26	+		+		+	+		+			+	+					+		+			+	+	
total	14	4	15	12	12	9	12	11	8	4	13	12	7	11	9	1	18	2	12	7	10	15	14	0
%	78	22	83	67	67	50	67	61	44	22	72	67	39	61	50	6	100	11	67	39	56	83	78	0

[a] Chromosome 17 is divided and translocated onto hamster material.

are listed in Table 2. While the complement of human chromosomes varies significantly between the hybrids, only chromosome 17 is retained by all of the hybrids. In particular, attention is drawn to hybrid NLB-23, in which the only human genetic material is derived from chromosome 17. The analysis of NLB-23 was confirmed by FISH using both a total human genomic DNA probe (Figure 7A) and a human chromosome 17 specific paint probe (Figure 7B). Clone NLB-23 retains the human 17q and 17p chromosome arms translocated onto two separate CHO chromosomes. Thus, human chromosome 17 showed 100% concordance with the Ade$^-$B locus, while no other human chromosome exhibited a concordance greater than 83%.

To verify that human chromosome 17 encodes the Ade$^-$B locus, five NLB hybrid clones were selected for BrdU segregation analysis. Essentially, this technique selects for lines that have lost their purine prototrophy and have therefore lost the human chromosome responsible for complementing the Ade$^-$B locus. Of the 34 BrdU segregants isolated (Table 3), 18 had lost the complete human chromosome 17 and 15 had lost the 17p arm (the 17q arm was translocated onto a hamster chromosome). One segregant, NLB-26 S7, apparently contained an intact human chromosome 17. Thus, by both somatic cell fusion and segregation analysis, the 17p arm is the only human chromosomal material to show almost complete concordance with purine prototrophy and should, therefore, encode the Ade$^-$B locus.

Immunoblot Analysis of Chromosome 17 Hybrids and Segregants. In order to confirm the placement of the Ade$^-$B locus on the p arm of human chromosome 17, a number of the fusion and segregant lines were immunoblotted with monoclonal antibody DD2 (Figure 8). As mentioned above, DD2 cross-reacts with human FGAR amidotransferase, but does not recognize the enzyme in mice. Since the fusion and segregant lines are all derived from CHO-Ade$^-$B, only a small amount of CHO FGAR amidotransferase is detected. The immunoblot clearly demonstrates that the 150-kDa human amidotransferase protein is almost always present in cell lines that contain a complete chromosome 17 (lanes 2–4), the one exception being segregant NLB-26 S7 (lane 7). Thus, even though NLB-26 S7 appears to contain an intact chromosome 17, it does not express human FGAR amidotransferase. Furthermore, the human protein is absent from all lines tested containing no chromosome 17 material (lane 5) or containing only the q arm of 17 (lane 6). Once again, attention is drawn to the hybrid NLB-23, in which the only human genetic material originates from chromosome 17 (lane 4). That this line contains an elevated amount of the 150-kDa protein recognized by antibody DD2 is very strong evidence that human FGAR amidotransferase must be encoded by chro-

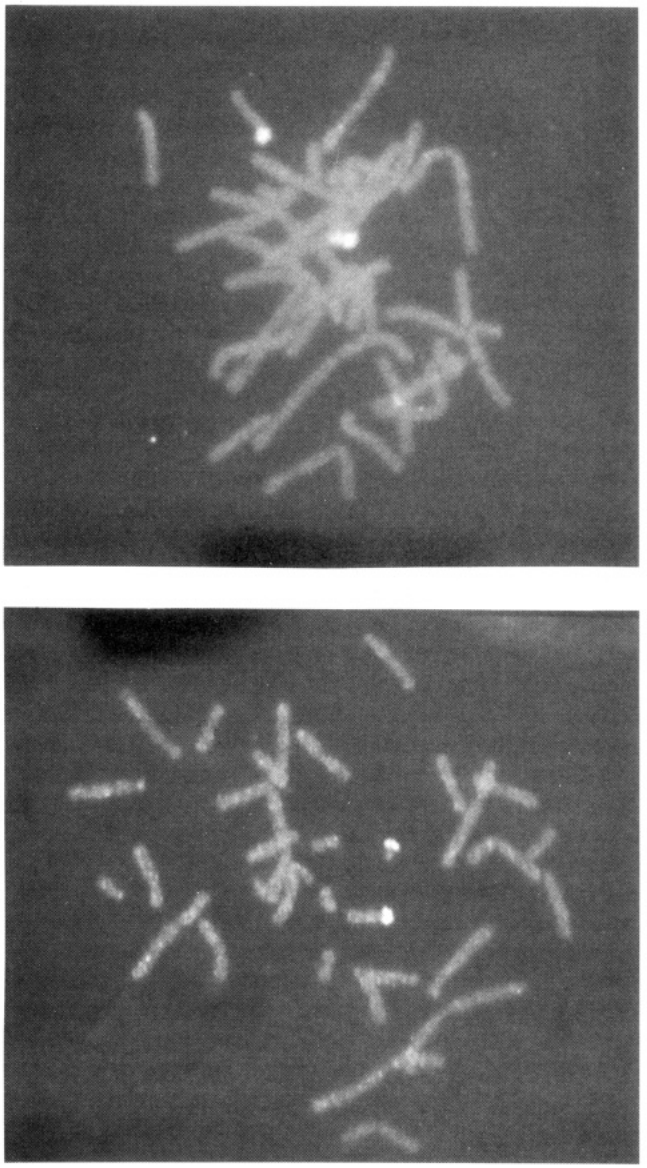

FIGURE 7: Fluorescence *in situ* hybridization of hybrid NLB-23. Metaphase spreads of hybrid NLB-23 were prepared, and FISH analysis was preformed as described in the text. FISH probes were directed against total human genomic DNA (A, top) and human chromosomal 17 DNA (B, bottom).

mosome 17. This was substantiated by immunoblotting DD2 against protein extracts from GM10498A and GM10479A, human/mouse hybrids purchased from the Human Genetic Mutant Cell Repository at the Coriell Institute for Medical Research. The 150-kDa protein was exhibited by GM10498A (lane 9), which contains only human chromosome 17, but was completely absent from GM10479A (lane 8), which contains only human chromosome 14. These results are in complete agreement with those obtained by somatic cell fusion and segregation analysis; that is, FGAR amidotransferase is encoded by the Ade$^-$B locus, and this locus maps to the p arm of chromosome 17 in humans.

DISCUSSION

FGAR amidotransferase has been purified 1631-fold from CHO-K1 cells, based on the end product's ability to convert ^{14}C-labeled FGAR to FGAM. The specific activity for the purified protein given here cannot be compared directly with specific activities reported elsewhere, since the [^{14}C]FGAR obtained in the manner described above remains mixed with an unknown quantity of unlabeled FGAR. However, the fold purification of 1631× compares very favorably with the values obtained for the purification of chicken liver FGAR amidotransferase by Buchanan *et al.* (1978) and by Schendel and Stubbe (1986). It should be noted that since both of these groups reported a homogeneous end product, the much higher degree of purification reported here is probably a simple reflection of the lower abundance of the amidotransferase in our CHO starting material. Although the highly purified CHO FGAR amidotransferase fraction contains five prominent polypeptide bands by SDS–PAGE analysis, several reasons were presented in the results to suppose that many of the bands are degradation products of a 150-kDa FGAR amidotransferase. This conclusion was borne out by the fact that, while both of the FGAR amidotransferase monoclonal antibodies recognize a single protein of 150 kDa in crude CHO cell extracts and in all initial fractions of the purification procedure, they recognize multiple bands in the end fraction. However, if the amidotransferase is indeed degraded and only a small proportion remains intact, how could such a high fold purification be obtained? One explanation might be that degradation was caused by the addition of SDS–PAGE boiling mix, thereby affecting analysis of molecular mass but not activity. However, such an effect would have to be dependent on enzyme purity since degradation is not seen in the cruder preparations. Another explanation might be that, since FGAR amidotransferase is likely to be constructed of two or more active domains, the intact protein may be breaking into fragments that remain active. If purification is the cause of the degradation, then it occurs on the final Affigel-Blue column. Consequently, we are currently seeking conditions to accomplish the final purification step of the enzyme without its partial degradation. In brief, this is the first report of a highly purified, essentially homogeneous mammalian FGAR amidotransferase.

Using the CHO FGAR amidotransferase to immunize mice, six independent monoclonal antibodies have been generated. Two of these monoclonal antibodies, designated BD4 and DD2, recognize CHO FGAR amidotransferase. Both antibodies recognize a protein of 150 kDa molecular mass (by SDS–PAGE) that is present in the amidotransferase preparation and in CHO-K1 cells, is practically absent from Ade$^-$B cells, and is elevated in Az^r cells. Moreover, this protein can be immunoprecipitated concomitantly with CHO FGAR amidotransferase activity by antibody BD4. While DD2 itself did not precipitate the amidotransferase, it did recognize the immunoreactive protein precipitated by BD4. Although DD2 was isotyped as IgG$_1\kappa$ and protein A does not react efficiently with mouse antibodies of this isotype, immunoblotting indicates that DD2 is precipitated by an excess of protein A, just as is seen for BD4 in Figure 5. That DD2 should recognize FGAR amidotransferase but not precipitate it is not unusual since any monoclonal antibody requires a much higher affinity to precipitate a protein than to recognize it by immunoblot.

The molecular mass of FGAR amidotransferase appears to be highly conserved between species. Antibody DD2 reveals that, by SDS–PAGE, the molecular mass of human FGAR amidotransferase is 150 kDa, which is identical to that of the CHO amidotransferase. This value is also identical to that reported by Buchanan's group (Frère *et al.*, 1971) for chicken by SDS–PAGE. Since this paper also gives a molecular mass of 133 kDa for the chicken amidotransferase by analytical ultracentrifugation, the molecular mass of CHO and human FGAR amidotransferase may also be close to the value of 135 kDa reported for *S. typhimurium* and *E. coli* (see the

Table 3: Results of BrdU Segregation Analysis

	human chromosomes[a]																						
clone/SEG[b]	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	X	Y
NLB-1	+		+	+	+	+	+	+	+	+	+	+	+	+			+		+		+	+	+	
NLB-1/S2	+			+	+	+					+	+	+	+					+			+	+	
NLB-1/S4	+			+	+	+					+	+	+	+					+		+	+	+	
NLB-1/S5						+			+													+	+	
NLB-1/S6a	+			+	+				+	+	+	+	+	+					+		+	+		
NLB-9	+		+														+						+	
NLB-9/S1	p		+														q						+	
NLB-9/S2	p		+														q						+	
NLB-9/S4a																	q							
NLB-9/S5	p		+														q						+	
NLB-9/S6b	p		+														q						+	
NLB-21	+		+						+			+					+					+	+	
NLB-21/S1	p		q									+										+	+	
NLB-21/S3	p		+									+										+	+	
NLB-21/S5	+		+									+										+		
NLB-21/S7	p		q									+										+		
NLB-21/S9	p		q									+										+		
NLB-21/S11	p		q									+										+		
NLB-21/S13												+										+	+	
NLB-21/S15	p		q									+										+		
NLB-22						+		+				+		+	+		+		+		+			
NLB-22/S2						+		+				+		q	+		q		+		+			
NLB-22/S4						+		+				+		q	+		q		+		+			
NLB-22/S6						+		+				+		q	+		q		+		+			
NLB-22/S7						+		+				+		q			q		+		+			
NLB-22/S9						+		+				+		q	+		q				+			
NLB-22/S12						+		+				+		q	+		q				+			
NLB-22/S14						+		+				+		q	+		q		+		+			
NLB-26	+		+		+	+		+			+	+					+		+			+	+	
NLB-26/S1	+		+		+	+													+			+	+	
NLB-26/S2	+		+		+	+					+	+							+			+	+	
NLB-26/S3	+		+		+	+					+								+			+	+	
NLB-26/S4	+		+		+	+					+	+							+			+	+	
NLB-26/S5	+		+		+	+					+	+							+			+	+	
NLB-26/S6	+		+		+	+					+	+					q		+			+	+	
NLB-26/S7	+		+		+	+					+	+					+		+			+	+	
NLB-26/S8	+		+		+	+					+	+					q		+			+	+	
NLB-26/S9	+		+		+	+					+	+					q		+			+	+	
NLB-26/S10	+		+		+	+					+	+					q		+			+		

[a] Borders outline chromosomes present in fusion hybrids but missing from most BrdU segregants. [b] Clone in bold indicates parental fusion hybrid.

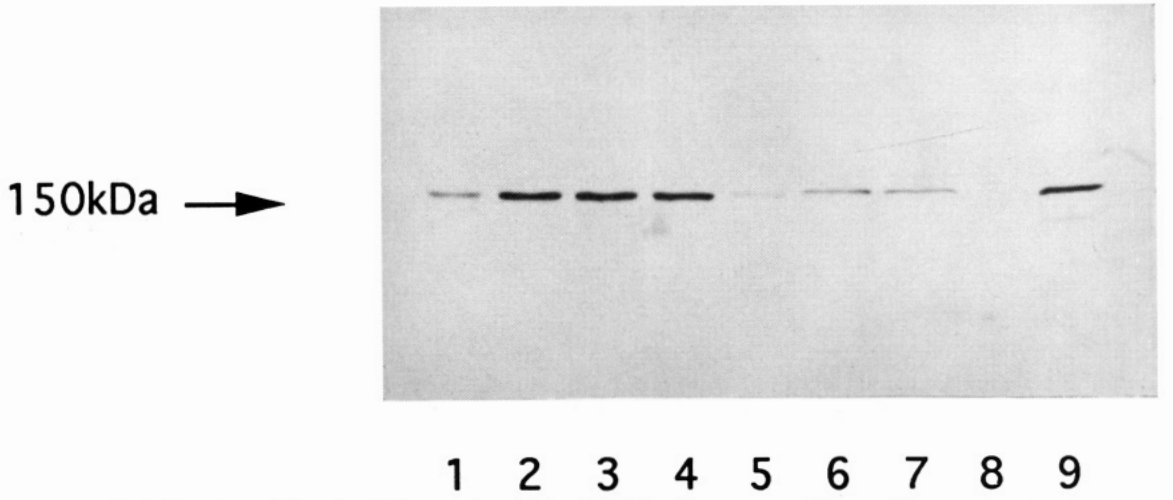

FIGURE 8: Immunoblot of crude protein extracts from various fusion hybrid and segregant cell lines with monoclonal antibody DD2. Total soluble proteins (12.5 μg) from Ade⁻B (lane 1), NLB-21 (lane 2), NLB-17 (lane 3), NLB-23 (lane 4), NLB-21 S5 (lane 5), NLB-9 S4a (lane 6), NLB-26 S7 (lane 7), GM10479A (lane 8), and GM10498A (lane 9) were resolved by 7.5% SDS–PAGE and immunoblotted with monoclonal antibody DD2 as described in the text.

introduction for references). Why the high molecular mass of FGAR amidotransferase should be so similar between a wide variety of organisms remains an important question.

The human FGAR amidotransferase gene has been provisionally assigned to chromosome 4 or 5 (Kao & Puck, 1972b) and to chromosome 14 (Kao, 1980). In both cases, CHO-Ade⁻B cells were fused with human cells, and the human chromosome exhibiting concordance with purine prototrophy was identified. However, these studies were performed several years ago, and in both cases isozyme analysis was used to

determine the human chromosomal content of the hybrids. Because isozyme analysis identifies only one or two points on a chromosome, the method is only accurate when dealing with intact chromosomes. With the assistance of more modern cytogenetic techniques, it is now possible to determine a more accurate picture of hybrid chromosomal contents. After analysis of 18 fusion hybrids and 34 BrdU segregants, we find 98% concordance between the p arm of human chromosome 17 and the FGAR amidotransferase locus. Of the 52 lines examined, only one did not show concordance, segregant NLB-26 S7.

The placement of the human FGAR amidotransferase gene on 17p was confirmed by immunoblotting monoclonal antibody DD2 against several of the fusion clones and segregants generated as a part of this study and also against two mouse/human hybrids generated independent of this institute. Both of the mouse hybrids contained only a single chromosome of human origin. One hybrid contained chromosome 17 and the other chromosome 14, one of the chromosomes to which the FGAR amidotransferase locus had been assigned previously. With the exception of segregant NLB-26 S7, antibody DD2 always recognized an abundant 150-kDa protein in lines containing 17p, but never in lines not containing 17p. Immunoblotting DD2 with NLB-26 S7 revealed the absence of human FGAR amidotransferase in this segregant. Thus, the only cell line in the cytogenetic study to exhibit discordance between the human FGAR amidotransferase locus and chromosome 17p does not, in fact, express the amidotransferase protein. While this segregant still contains an apparently intact chromosome 17 after BrdU treatment, it is possible that a small piece of the chromosome, cytogenetically invisible and containing the FGAR amidotransferase locus, has been lost. The fact that the chromosome 17 contained by this line is sometimes divided and translocated onto different CHO chromosomes lends additional weight to this idea. Either way, this segregant will be worth further study. With NLB-26 S7 essentially accounted for, we report incontrovertible evidence that the Ade$^-$B locus, and therefore the FGAR amidotransferase gene, is located on the p arm of human chromosome 17.

We have previously reported the isolation of a CHO purine auxotroph, Ade$^-P_{AB}$, which is lacking both FGAR amidotransferase and glutamine PRPP amidotransferase activities (Oates *et al.*, 1980). Revertants of these mutants can be isolated at a frequency that suggests that the Ade$^-P_{AB}$ mutation is a single genetic lesion. Previously, we and others assigned a gene necessary for glutamine PRPP amidotransferase activity to human chromosome 4 (Stanley & Chu, 1978). The demonstration that a gene required for FGAR amidotransferase activity is located on human chromosome 17 adds further complexity to this observation. Since both Ade$^-$A (glutamine PRPP amidotransferase deficient) and Ade$^-$B (FGAR amidotransferase deficient) mutations are recessive, the assignment of these genes to different chromosomes strongly implies the existence of a third locus essential for both activities. The existence of the antibodies described here should aid in understanding these findings.

In genomic dimensions, the p arm of human chromosome 17 is small, only about 30 megabases. Several human disorders have been mapped to 17p, including plasmin inhibitor deficiency, Bernard–Soulier syndrome, Miller–Dieker lissencephaly syndrome, colorectal cancer, Li–Fraumeni syndrome, astrocytoma, Charcot–Marie–Tooth neuropathy, Smith–Magenis syndrome, and possibly a form of breast cancer [for references, see Cuticchia *et al.* (1993)]. While it is unlikely that any one of these diseases will be associated with FGAR amidotransferase, it is possible that mapping of this gene will provide a useful tool for the isolation of the affected genes.

The purification of CHO FGAR amidotransferase and the generation of monoclonal antibodies to this protein should allow the isolation of the corresponding gene. Having both the protein and the gene should allow a more complete analysis of how FGAR amidotransferase is regulated, how it is affected in the Ade$^-P_{AB}$ mutant, and what its role is in the *de novo* synthesis of purines.

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