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Isolation of a rat liver δ-aminolevulinate dehydrase (ALAD) cDNA clone: Evidence for unequal ALAD gene dosage among inbred mouse strains

(heme biosynthetic enzymes/polysome immunopurification/monoclonal antibody/enzyme regulation)

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ABSTRACT We have isolated several cDNA clones encoding δ-aminolevulinate dehydrase [ALAD; porphobilinogen synthase; 5-aminolevulinate hydro-lyase (adding 5-aminolevulinate and cyclizing), EC 4.2.1.24], the second enzyme in the heme biosynthetic pathway. We used a rabbit polyclonal antibody developed against the purified 35-kDa subunit of rat liver ALAD to screen a λgt11 cDNA expression library constructed from rat liver mRNA. A prototype clone (ALAD-1) contained a 680-base-pair insert and expressed a 140-kDa β-galactosidase gene cDNA insert fusion protein immunoreactive with both polyclonal and monoclonal anti-ALAD. Identity of ALAD-1 was verified by hydridization to ALAD mRNA that had been enriched via immunopurification of rat liver polysomes with anti-ALAD. Intensity of such hybridization to a 1500-nucleotide-long mRNA was ≈200-fold greater than that realized with whole liver mRNA, a result consistent with the degree of immunoenrichment of ALAD mRNA found independently by analysis of cell-free translation products. A second ALAD cDNA clone (ALAD-3) was isolated when the rat liver cDNA expression library was rescreened with ALAD-1. The identity of both ALAD cDNA clones was established by correspondence between their nucleotide sequence and the reported amino-terminal protein sequence of bovine ALAD. Hybridization of ALAD cDNA to mouse genomic DNA indicates that the previously unexplained incremental differences in ALAD enzymatic activity among inbred mouse strains has arisen through alterations in ALAD gene dose.

δ-Aminolevulinate dehydrase [ALAD; porphobilinogen synthase; 5-aminolevulinate hydro-lyase (adding 5-aminolevulinate and cyclizing), EC 4.2.1.24], is the second enzyme in the heme biosynthetic pathway (1). In this report, we describe the isolation of recombinant cDNA clones encoding rat liver ALAD. We have used one of these clones to examine the source of the roughly 3:2:1 incremental differences in ALAD enzyme level observed among inbred mouse strains (2, 3). We find that different strains exhibit different doses of ALAD gene DNA in a way that parallels, and accounts for, the reported differences in ALAD enzyme activity.

MATERIALS AND METHODS

Materials. Radiochemicals were obtained from New England Nuclear or Amersham, endonucleases were from Bethesda Research Laboratories, and reverse transcriptase was from Life Sciences (St. Petersburg, FL). DNA from individual mouse strains was purchased, as such, from The Jackson Laboratory. A plasmid bearing mouse adult β-globin cDNA (pCR1βM9) (4) and a plasmid bearing mouse carbonic anhydrase II cDNA (p6-69) (5) were gifts from M. Edgell (University of North Carolina) and P. Curtis (Wistar Institute), respectively.

ALAD Purification. Liver cytosol from adult Wistar rats was prepared and fractionated with protamine sulfate as described by Nakakuki *et al.* (6). ALAD, as judged by enzyme assay (7), was precipitated by 50% saturation with $(NH_4)_2SO_4$ from the fraction remaining soluble after 35% saturation. Thereafter, ALAD was resuspended in 6.8DP buffer (0.1 mM dithiothreitol/7 mM potassium phosphate, pH 6.8) and applied to a DE52 (Whatman) column equilibrated in 6.8DP. After washing with a large volume of 6.8DP containing 0.1 M KCl, ALAD was eluted at 25°C by a linear KCl gradient (0.125–0.35 M) in 6.8DP. ALAD active fractions were precipitated by $(NH_4)_2SO_4$, resuspended in 6.8DP buffer containing 0.5 M KCl, and applied to a Sephacryl S200 (Pharmacia) column (5 × 86 cm) equilibrated with the same solution at 4°C. The ALAD-bearing peak corresponded to a protein of ≈273 kDa. ALAD active S200 fractions were precipitated by $(NH_4)_2SO_4$, resuspended in 6.5DP buffer (3 mM dithiothreitol/10 mM sodium phosphate, pH 6.5), and serially passaged three times through an IEX-545 DEAE (Beckman) HPLC column. The column and sample were equilibrated each time with 6.5DP buffer. Columns were all developed with a linear salt gradient, which contained 0.45 M NaCl end buffer, but ranged from 0.1 M to 0.25 M NaCl in starting buffers and from 12 ml to 30 ml in total volume. Fractions were examined by $NaDodSO_4$/polyacrylamide gel electrophoresis and then by silver staining. When purified to homogeneity after the third IEX-545 passage, rat ALAD consisted of a single subunit of ≈35 kDa (35-kDa ALAD)—i.e., like the ALAD subunit size found in other species (8–10).

To ensure that ALAD to be used as immunogen was free of trace contaminants, the 35-kDa ALAD was isolated by electroelution (11, 12). Starting materials for this purpose consisted of ALAD active S200 or IEX-545 fractions. Applied ALAD varied in purity from 15% to 50% and exhibited >6.5 enzyme units (7) per mg of protein. To aid later assays, portions of applied materials were labeled (0.12 μCi/μg; 1 Ci = 37 GBq) by reductive alkylation (13) with NaB^3H_4. After $NaDodSO_4$/polyacrylamide gel electrophoresis, ALAD subunits were identified by gel immersion in 4 M sodium acetate and electroeluted from gel slices with 25% to 60%

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Abbreviations: ALAD, δ-aminolevulinate dehydrase; IPTG, isopropyl β-D-thiogalactoside.

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recovery and, as judged by later electrophoresis, >99% purity.

Preparation and Characterization of Antibodies to 35-kDa ALAD. One microliter of rabbit (R574) anti-ALAD, developed after three injections of electroeluted 35-kDa ALAD (400 μg), removed 50% of enzymatically active ALAD after reaction with 2.7 milliunits of enzyme and later with particulate *Staphylococcus aureus* protein A (Calbiochem). Under equivalent conditions, 1 μl of R574 bound 24% (9 ng) ^{3}H-labeled 35-kDa ALAD. Affinity-purified R574, obtained by adsorption–desorption (14) from a $NaDodSO_4$-treated S200 eluate of ALAD bonded to Sepharose 4B (Pharmacia), bound 50% of ^{3}H-labeled 35-kDa ALAD after reaction at an IgG/antigen molar ratio of 20:1.

Monoclonal antibodies were developed from a BALB/c mouse given 11 intraperitoneal injections (1–10 μg) of electroeluted 35-kDa ALAD overa 20-week period. Immunized mouse spleen cells were fused with SP2/0-Ag14 mouse myeloma cells previously treated with 50 ng of demecolcine (Sigma) per ml (15). Two weeks after seeding in the presence of peritoneal macrophages and after beginning selection with hypoxanthine/aminopterin/thymidine (16), 190 hybridomas were assayed (17) for reactivity with 35-kDa ALAD bound to nitrocellulose. Twelve anti-ALAD clones were detected by ELISA reactions. Like R574 antisera, ascites fluids derived from subclones of one of these hybridomas (33-4A) bound selectively to 35-kDa bands in immunoblots (18) prepared from less than pure ALAD preparations, including a 4% pure fraction from rat liver, a DE-52 eluate (9) of reticulocyte protein from anemic rats, and a DE-52 eluate from human liver.

Isolation of cDNA Clones Immunoreactive with Anti-35-kDa ALAD. A λgt11 cDNA expression library, constructed by Schwarzbauer *et al.* (19) from adult rat liver poly(A)$^+$ RNA, was kindly provided by Richard Hynes (Massachusetts Institute of Technology). In the immunological screening of this library, the methods of Young and Davis (20) were adopted. Affinity-purified R574 anti-35-kDa ALAD (1 μg of IgG per ml) was used; blocking solution contained 3% bovine serum albumin, 0.5% gelatin in 0.5 M NaCl, 20 mM Tris·HCl (pH 7.4); after incubation with R574, all solutions included 0.05% Tween 20 (Bio-Rad). Immunoreactive clones, gauged by parallel immunoreactivity of 1.5 ng of 35-kDa ALAD, were detected by secondary incubation with ^{125}I-labeled protein A.

Generation of Fusion Proteins. Lysogens were prepared (21) in *Escherichia coli* Y1089 (Vector Cloning Systems, San Diego, CA) (20). Upon attainment of appropriate growth at 30°C, phage *cI857* repressor was inactivated by shifting cultures to 45°C. Thereafter, a portion of each culture was incubated at 37°C in the presence, and a portion in the absence, of the *lac* operon inducer, isopropyl β-D-thiogalactoside (IPTG). Cell lysates were harvested 2 hr later.

Immunological Enrichment of Rat Liver ALAD mRNA Polysomes. Liver polysomes were prepared by the method of Taylor and Schimke (22). One thousand A_{260} of polysomes reacted with 6.5 mg of an RNase-free IgG fraction derived from R574 anti-35-kDa ALAD. Reaction conditions, harvest of ALAD polysome immunocomplexes with *S. aureus* protein A, methods of later poly(A)$^+$ RNA isolation, cell-free translation of mRNA in the presence of tritiated amino acids, electrophoresis, and immunoassay closely followed those previously used for the immunopurification and analysis of rabbit carbonic anhydrase I mRNA (23). Concentrations of mRNA used to generate radiolabeled cell-free translation products were estimated after the fact by (*i*) assay of trichloroacetic acid-precipitable radioactivity in an aliquot of translates and (*ii*) calibration via parallel translation of known quantities of rabbit reticulocyte mRNA (23).

Nucleotide Sequence Analysis. Nucleotide sequences of cDNA inserts subcloned in M13 mp8 (24) were determined by the dideoxy-chain termination method of Sanger *et al.* (25) using deoxyadenosine 5′-[[α-^{35}S]thio]triphosphate and either the Klenow fragment of DNA polymerase I (Bethesda Research Laboratories) or reverse transcriptase.

RESULTS AND DISCUSSION

Immunologic Detection and Characterization of ALAD cDNA Clones. Four clones out of 105,000 recombinant phage screened in an amplified rat liver λgt11 expression library persistently reacted with rabbit anti-rat liver 35-kDa ALAD during two rounds of plaque purification. After isolation of phage DNA by plate lysis (26), all four clones were found to contain 680-base-pair (bp) inserts as determined by *Eco*RI digestion of recombinant phage DNA and subsequent electrophoresis. When examined for nucleotide sequence, each insert proved indistinguishable from the others. A radiolabeled *Eco*RI insert from one of these immunologically detected clones (λALAD-1) was used to rescreen the rat liver library via nucleic acid hybridization (26). Thereby, an additional clone, λALAD-3, was isolated and found to contain a 450-bp *Eco*RI insert. In what follows, derivatives containing *Eco*RI inserts are designated according to vector used—e.g., λALAD-1 insert in pUC8 (27) is termed pALAD-1 and, in M13 mp8, M13ALAD-1. Where no prefix is used, λ vector is to be assumed.

Characterization of the protein produced by ALAD-1 is illustrated in Fig. 1*A*, where IPTG-mediated induction led to the emergence of 116-kDa β-galactosidase and to a 140-kDa ALAD-1 fusion protein. In Fig. 1*D*, each sample is reactive with anti-β-galactosidase. However, only the fusion protein

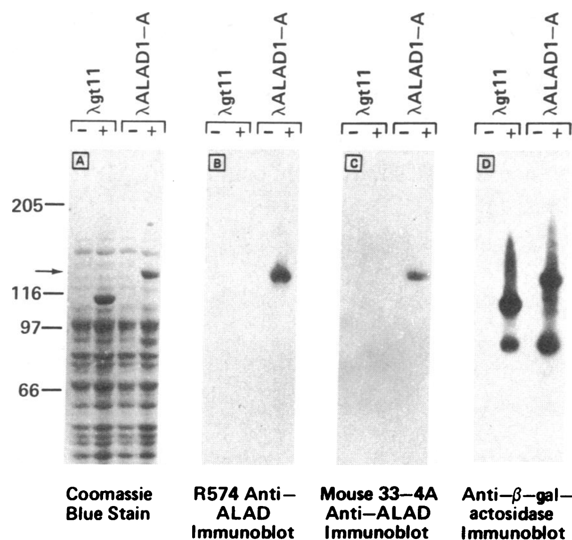

FIG. 1. Immunoblot analysis (18) of fusion proteins. Bacterial lysogens of λgt11 and pALAD-1A (a subclone of ALAD-1) were prepared with (+) and without (−) IPTG-mediated induction of β-galactosidase. After electrophoresis in $NaDodSO_4$/6% polyacrylamide gels, samples were analyzed four ways. *A* was developed with Coomassie blue. (*B–D*) Gel contents were electrophoretically transferred to nitrocellulose (24). Thereafter, samples in *B* reacted with affinity-purified rabbit anti-35-kDa ALAD (1 μg/ml), those in *C* reacted with mouse monoclonal anti-35-kDa ALAD (ascites fluid, 1:500), and those in *D* reacted with rabbit anti-*E. coli* β-galactosidase (Cooper Biomedical). Immunoreactivity was then detected in *B* and *D* by incubation with ^{125}I-labeled protein A and in *C* by incubation with ^{125}I-labeled sheep anti-mouse IgG. Procedures were otherwise similar to those used for cDNA library screening. Molecular sizes (kDa) derive from electrophoresis of protein standards. Arrow denotes migration of 140-kDa fusion protein.

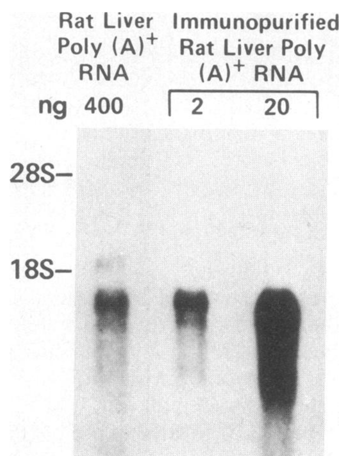

FIG. 2. Comparison of pALAD-1 hybridization to unfractionated rat liver polysome mRNA with hybridization to immunologically enriched ALAD mRNA. After electrophoresis in a formaldehyde/1.3% agarose gel, RNA was transferred to nitrocellulose paper and hybridized with ^{32}P-labeled nick-translated (26) pALAD-1. Conditions for hybridization followed those described by Wahl *et al.* (28) except that dextran sulfate was omitted. Indicated nanograms of poly(A)$^+$ mRNA present before and after R574 anti-35-kDa ALAD-mediated polysome purification were estimated by calibrated cell-free translation as described. Molecular sizes at left are based on ribosomal RNA standards. The minor slow-moving component seen in whole mRNA was presumably lost during polysome immunopurification.

reacted with rabbit anti-35-kDa ALAD (Fig. 1*B*). The fusion protein also reacted with a mouse monoclonal antibody (33-4A) to the ALAD subunit (Fig. 1*C*). The fusion peptide thus binds antibodies raised in two different species against highly purified 35-kDa ALAD, a result that minimized the chance that the ALAD-1 insert encodes anything but the ALAD subunit.

Hybridization of Cloned cDNA with ALAD mRNA from Unfractionated and Enriched Preparations. Two further lines of evidence that ALAD-1 indeed contains ALAD sequences

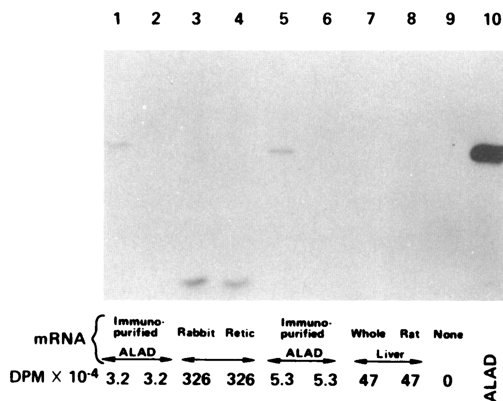

FIG. 3. NaDodSO$_4$/polyacrylamide gel electrophoresis of immunoprecipitates developed from tritiated cell-free translation products of indicated mRNA (see text). In each instance, ^{3}H-labeled amino acid incorporated during translation had been previously quantified by precipitation with trichloroacetic acid (23). Untreated aliquots containing the denoted quantities of incorporated radiolabel reacted with either 5 μg of R574 anti-ALAD (odd-numbered lanes) or 5 μg of normal rabbit IgG (even-numbered lanes). Thereafter, immunoprecipitates were formed by overnight reaction with goat anti-rabbit IgG (Cooper Biomedical). Washed immunoprecipitates were boiled and applied as shown. After electrophoresis, En^{3}Hance (New England Nuclear)-treated gels were exposed to x-ray film at $-85°$C for 300 hr and later, as shown here, for 2700 hr.

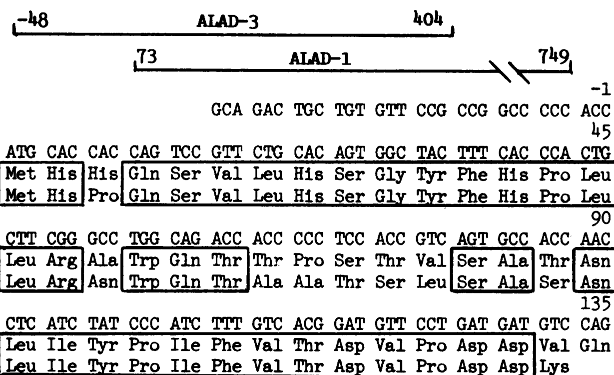

FIG. 4. Nucleotide sequences obtained for portions of rat liver ALAD M13 ALAD-1 and M13 ALAD-3 clones compared with rat liver ALAD protein sequence deduced from nucleotide sequence (upper rows of amino acids) and reported bovine liver ALAD protein sequence (29) (lower rows of amino acids). Boxed residues denote between-species homology. Clone geometries, with position of first and last nucleotide, are shown at top of figure. Overlaps between clones were located by nucleotide sequence analysis. All nucleotide assignments here derive from bidirectional analysis.

were deduced by hybridization with rat liver polysomal poly(A)$^+$ RNA. First, as shown in Fig. 2, ^{32}P-labeled pALAD-1 hybridizes with RNA whose 1500-nucleotide length is appropriate for encoding 35-kDa ALAD protein. Second, the intensity of hybridization with 400 ng of mRNA from unfractionated polysomes (lane 1) is similar to that realized with 2 ng of mRNA from immunopurified ALAD-bearing polysomes (lane 2). The several hundred-fold enrichment of ALAD mRNA thus estimated is much like that independently adduced (Fig. 3) after electrophoresis of washed immunoprecipitates formed from reactions of rabbit anti-ALAD and anti-antibody with known quantities of tritiated cell-free translates. Specifically, immunoprecipitates of cell-free translates developed from the "immunopurified" mRNA used in Fig. 2 contain in the Fig. 3 autoradiograph a 35-kDa element (lanes 1 and 5). This element is indistinguishable from authentic ALAD (lane 10). No such element is seen in Fig. 3 when anti-ALAD is replaced with nonimmune rabbit IgG (lanes 2 and 6) and none was seen when unfractionated polysomal poly(A)$^+$ rat liver mRNA (lanes 7 and 8) or rabbit reticulocyte mRNA (lanes 3 and 4) served as a template for cell-free translation. Since the quantity of radiolabeled protein subject to immunoprecipitation was 15-fold greater for lane 7 than for lane 1, since nothing is seen in lane 7, and since results equivalent to those

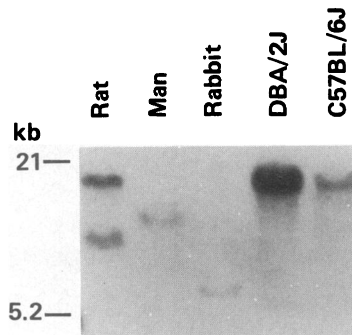

FIG. 5. Southern blot analysis (30) of ALAD genomic DNA. *Hin*dIII digests of ≈5 μg of whole genome DNA were separated, together with radiolabeled markers (not shown), by 0.8% agarose gel electrophoresis; transferred to nitrocellulose; and hybridized with ^{32}P-labeled pALAD-1 DNA (10^6 dpm/ml) using conditions described in Fig. 2. kb, Kilobases.

Table 1. DBA/2/C57BL/6 ratios of autoradiographic intensities realized after hybridization of several ^{32}P-labeled probes to Southern blots of genomic DNA

Condition	Analysis no.	Probe			ALAD/β-globin or ALAD/CA II ratio
		ALAD	β-Globin	CA II	
*Hind*III digests assessed in					
Separate lanes	1	2.98*		0.90†	3.31
Same lane	2	3.20†		1.43†	2.24
*Eco*RI digests assessed in					
Separate lanes	3	3.11†	1.24*		2.51
Same lane	4	4.06†		2.10†	1.93

Individual digests of DNA from each mouse strain were prepared for each analysis. Thereafter, a digest was either applied to one lane (analyses 2 and 4) or equal volumes were applied to multiple lanes (analyses 1 and 3). Data in analyses 1 and 3 derive from single probing of each lane. In analyses 2 and 4, each lane was probed first with ALAD and then, as described in Fig. 6 legend, with carbonic anhydrase II (CA II). Probes consisted of pALAD-1, mouse adult β-globin cDNA, and mouse CA II cDNA. In each analysis, autoradiographic intensities of the entirety of each reactive band were assessed in a Corning model 760 densitometer. Band densities were then summed for each lane and the denoted between-strain ratios were calculated by reference to a digest of DNA from the other mouse strain assayed in an adjacent lane.

*Mean of two parallel determinations.

†One determination.

in Fig. 3 were also seen in a shorter autoradiographic exposure (1/9th the time), it follows that the estimated level of ALAD mRNA in the enriched preparation (lane 1) is at least 135-fold greater than in the unfractionated one (lane 7). The fact that this minimal estimate of ALAD mRNA enrichment is like that realized by nucleic hybridization in Fig. 2 supports our contention that ALAD-1 encodes ALAD sequences.

Comparison of ALAD Amino Acid Sequences and M13 ALAD Nucleotide Sequences. Conclusive evidence for the identity of the overlapping M13 ALAD-1 and M13 ALAD-3 cDNA clones appears in Fig. 4. As shown there, amino acid residues deduced from rat liver M13 ALAD-1 and M13 ALAD-3 nucleotide sequences exhibit 80% homology with the 44 amino acid residues of bovine liver ALAD described by Lingner and Kleinschmidt (29).

ALAD Genomic DNA Sequences and Gene Dose Differences in Mice. As illustrated in Fig. 5, rat liver ALAD cDNA readily reacts with genomic DNA from several species. The relative number of ALAD copies, however, differs between mouse strains. Four sets of densitometric analyses of autoradiographic intensities (Table 1) suggest that ALAD gene dose, when normalized by reference to either β-globin or carbonic anhydrase II dose, is 2 to 3 times greater in DBA/2 mice than in C57BL/6 mice. Such findings are extended in Fig. 6 to *Rsa* I digests of DNA from six kinds of mice including five inbred strains and the B6D2 F_1 hybrid from a C57BL/6 × DBA/2 cross. The ALAD gene dose differences between mouse samples in Fig. 6*A* are validated in Fig. 6*B* where the intensities in all samples became similar when the radiolabeled ALAD probe was dissociated and the samples were reprobed with ^{32}P-labeled mouse carbonic anhydrase II cDNA. We interpret these results, like those in Table 1, to mean that between-strain differences in ALAD gene dose are genuine, assuming that carbonic anhydrase II gene dose is constant among mice sampled. Further support for the authenticity of ALAD gene dose differences is evident in Fig. 6*C*. Here the normalized ratios for ALAD/carbonic anhydrase II autoradiographic intensities closely correspond to the average levels of hepatic ALAD enzyme activity calculated from the reports of Hutton and Coleman (2) and Doyle and Schimke (3). As shown in Fig. 6*C*, mice are roughly divisible, whichever criterion is used, into three groups: AKR and DBA/2, C58 and the B6D2 F_1, and C57BL/6 and SM. The bulk of the known differences in enzymatic levels between inbred mouse strains is therefore explicable by increments in ALAD gene dose such that the groups just listed have three active sets of ALAD genes, two

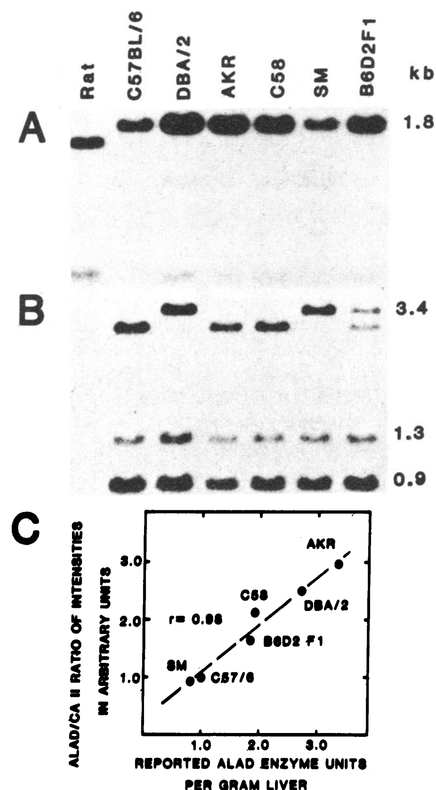

FIG. 6. Southern blot analysis of *Rsa* I digests of genomic DNA from rat (2 μg) and denoted inbred mouse strains (5 μg). (*A*) Samples were probed, as described in Figs. 2 and 5, with ^{32}P-labeled pALAD-1 DNA. (*B*) ALAD probe was dissociated by two 15-min washes at 85°C in 10 mM Tris·HCl/1 mM EDTA, and the filter was rehybridized with ^{32}P-labeled carbonic anhydrase II cDNA prepared by the oligolabeling method of Feinberg and Vogelstein (31). (*C*) Ratios of ALAD/carbonic anhydrase II (CA II) autoradiographic intensities (see Table 1) plotted versus average levels of hepatic ALAD enzyme activity calculated from prior reports (2, 3). For convenience, enzyme levels have been normalized by taking the C57BL/6 value as unity. kb, Kilobases.

active sets, or only one active set, respectively. In fact, the regression of the ordinal values in Fig. 6*C* upon these 3, 2, and 1 doses of ALAD genes has a correlation of 0.97 and a nearly 0 intercept. Thus, while we cannot exclude the possibility that ALAD pseudogenes contribute to the ordinal values in Fig. 6*C*, it seems unlikely that they do so out of proportion to the number of active ALAD genes present in each mouse strain.

As it turns out, we can eliminate one class of pseudogenes from further consideration. The class in question is the processed pseudogenes, which are thought to arise by the random reinsertion into the genome of cDNA copies of mRNA. The usual hallmark of processed pseudogenes is that they lack intervening sequences and are thereby foreshortened relative to genomic sequences from which they arose (32). Consequently, if this process had figured in generating the ALAD gene dose differences evident in Figs. 5 and 6 and in Table 1, we would expect to have found differences in restriction patterns between strains. The fact is that all mouse samples in Fig. 6*A* have identical patterns. The same is true for *Hin*dIII and *Eco*RI digests. It is also true when DNA from DBA/2 and C57BL/6 are digested with any of 10 other restriction endonucleases (data not shown). While the pattern for each digestion is characteristic, the restriction pattern in the two strains is indistinguishable. These results not only exclude processed pseudogenes from the outcome shown in Fig. 6*C* but also raise a new question: Why are restriction patterns among strains all alike with respect to ALAD yet so different with respect to β-globin (4) and carbonic anhydrase II (e.g., Fig. 6*B*)? We must suppose either that ALAD gene regions have been held invariate, except for dose, or that ALAD gene homogenization and generation of dose differences has occurred comparatively recently. The inference, in either case, is that mouse ALAD genes have been subject to recent intense selection.

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