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Molecular cloning of a cDNA sequence complementary to porphobilinogen deaminase mRNA from rat

(heme synthesis/mRNA purification/acute intermittent porphyria)

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ABSTRACT **A cDNA clone containing sequences complementary to the mRNA coding for anemic rat spleen porphobilinogen deaminase (EC 4.3.1.8) has been isolated. A cDNA library was prepared from partially purified mRNA (1% purity). This library was then screened by colony hybridization, using a cDNA probe derived from porphobilinogen deaminase mRNA further enriched (10–20% purity) by gel electrophoresis in the presence of methylmercury hydroxide. Colonies hybridizing with the probe were analyzed by hybrid-selected translation using anemic rat spleen mRNA. Four recombinant plasmids containing porphobilinogen deaminase cDNA sequences were identified by specific immunoprecipitation of the translational product from hybrid-selected mRNA. Porphobilinogen deaminase mRNA was shown to contain 1800 bases by blot hybridization analysis. The cloned cDNA sequence consists of 1500 bases. Hybridization analysis of poly(A)$^+$ RNA from uninduced and induced mouse erythroleukemic cells indicated that induction to erythroid differentiation by dimethyl sulfoxide results in a 10-fold increase of porphobilinogen deaminase mRNA. The rat cDNA clones hybridize to the corresponding sequences encoding human porphobilinogen deaminase. This property will be useful for isolation of human gene(s) and further characterization of the molecular lesion(s) responsible for acute intermittent porphyria.**

Porphobilinogen deaminase [PBG-D; porphobilinogen ammonia-lyase (polymerizing), EC 4.3.1.8] is the third enzyme of the biosynthetic pathway leading to the production of heme. The structural gene encoding this enzyme has been assigned to the distal part of the long arm of chromosome 11 in man (11q23→11qter) (1, 2). Inherited deficiency of this enzyme is associated with the autosomal dominant disease acute intermittent porphyria, which is the most frequently encountered acute porphyria (3). Studies using antibodies specifically directed against PBG-D failed to detect any inactive enzyme in all but few patients for whom the presence of noncatalytic immunoreactive protein (cross-reacting immunological material, CRIM) was established (4); therefore, acute intermittent porphyria appears to be heterogeneous at the molecular level. In the vast majority of patients whose PBG-D defect can be classified as CRIM-negative, it is not known whether the enzymatic deficiency is due to an abnormality of the structural gene or to a regulatory defect.

Other interesting features of PBG-D relate to its expression during erythropoiesis: a dramatic increase in the enzymatic activity of PBG-D takes place before hemoglobin accumulation, when virus-transformed (5) as well as normal erythroid precursor cells (6) are induced to differentiate *in vitro*. An induced activity of the other enzymes of the heme biosynthetic pathway has also been documented in these cells (5, 7) after dimethyl sulfoxide (Me_2SO) treatment. However, the molecular events leading to their coordinated expression are still unknown.

Further characterization of the mutation(s) that cause acute intermittent porphyria and of the mechanisms controlling PBG-D expression during erythroid differentiation clearly requires investigations at the mRNA and gene levels. In this paper, we report the molecular cloning of rat PBG-D cDNA sequences.

MATERIAL AND METHODS

Material. L-[^{35}S]methionine (1200 Ci/mmol; 1 Ci = 37 GBq) and α-^{32}P-labeled deoxynucleotides (410 Ci/mmol) were obtained from Amersham. Enzymes were purchased from New England Biolabs, Bethesda Research Laboratories, or Boehringer Mannheim. Glutaraldehyde-activated Ultrogel was from Industrie Biologique Française (Villeneuve-La-Garenne, France). Oligo(dT)-cellulose type 3 was from Collaborative Research (Waltham, MA). Dextran T 40 and Sepharose 4B were from Pharmacia Fine Chemicals. Nitrocellulose was purchased from Sartorius or Millipore. Ampholine polyacrylamide gel plates (pH 4.0–6.5) were purchased from LKB.

PBG-D Purification and Antibodies. PBG-D was purified to homogeneity from human erythrocytes according to Anderson and Desnick (8) except that an additional chromatography on hydroxyapatite was introduced after the phenyl-Sepharose step. Fractions from phenyl-Sepharose were loaded onto a column of hydroxyapatite (diameter 2 cm, length 10 cm) equilibrated with 20 mM potassium phosphate buffer (pH 6.4). The enzyme was eluted by a gradient from 20 mM potassium phosphate buffer (pH 6.4) to 300 mM potassium phosphate buffer (pH 6.8).

An antiserum was raised in rabbits as described (4). Specific antibodies were prepared by affinity chromatography of the IgG fraction using human PBG-D immobilized on glutaraldehyde-activated Ultrogel (9). Cross-reactivity of the anti-human PBG-D antibodies with rat PBG-D was established by their ability to immunoprecipitate the rat enzyme activity (unpublished results). Pure human PBG-D was labeled with ^{14}C according to Dottavio-Martin and Ravel (10).

***In Vitro* Translation and Immunoprecipitation.** Cell-free translation was carried out in micrococcal nuclease-treated rabbit reticulocyte lysate prepared as described by Pelham and Jackson (11). The biological activity of mRNA preparations was measured by determining the amount of radioactivity precipitable with hot trichloroacetic acid above back-

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Abbreviations: PBG-D, porphobilinogen deaminase; CRIM, cross-reacting immunological material; Me_2SO, dimethyl sulfoxide; bp, base pair(s); kb, kilobase(s).

ground. ^{35}S-labeled peptides were separated by NaDodSO$_4$/polyacrylamide gel electrophoresis (12) and detected by fluorography. Immunoprecipitations were carried out according to Opperman *et al.* (13). Pure ^{14}C-labeled human PBG-D was used as a molecular weight marker.

RNA Isolation and Partial Purification of PBG-D mRNA. Sprague–Dawley rats were treated with *N*-acetylphenylhydrazine to induce anemia. Total RNA was isolated from tissues (rat liver, rat spleen, and human anemic spleen) by the LiCl method (14) and poly(A)$^+$ RNA was prepared by chromatography on oligo(dT)-cellulose (15).

Rat poly(A)$^+$ RNA (300 μg) was fractionated by preparative gel electrophoresis with continuous elution (preparative gel electrophoresis system 100 PB, Bethesda Research Laboratories), as described by Brûlet and Roskam (16), except that 0.15% NaDodSO$_4$ was used in the electrophoresis and elution buffers and that dextran T 40 was added as a carrier for ethanol precipitation instead of tRNA.

An additional enrichment was obtained by electrophoresis through a methylmercury hydroxide agarose gel, using the same preparative gel electrophoresis system and a sodium borate buffer (17).

The relative amount of PBG-D message in each fraction was estimated from its specific translational activity in the cell-free system.

Cloning of Double-Stranded cDNA. After the first electrophoretic separation, an aliquot from the fractions most enriched for PBG-D mRNA (corresponding to 0.5 μg of mRNA) was used to direct the synthesis of double-stranded cDNA as described by Wickens *et al.* (18). After treatment with S1 nuclease, double-stranded cDNA was size fractionated by Sepharose 4B chromatography. cDNA molecules longer than 600 base pairs (bp) were inserted in the *Pst* I site of pBR322 by using the homopolymeric tailing and hybridization method according to Michelson and Orkin (19). The recombinant plasmids were used to transform *Escherichia coli* MC 1061 as described by Hanahan (20). Bacteria were plated on a single nitrocellulose filter (diameter 14 cm) laid on agar containing L broth and tetracycline (10 μg/ml).

Replicas of the filter, obtained as described by Hanahan and Meselson (21), were used for colony hybridization or frozen for long-term storage of the library.

Screening of the cDNA Library by Colony Hybridization. After the second electrophoretic step, carried out on methylmercury hydroxide agarose gel, the most enriched fractions were pooled and used for the synthesis of a single-stranded cDNA probe by reverse transcription in the presence of [α-^{32}P]dATP (22).

A replica filter of the library was laid on a plate containing chloramphenicol (150 μg/ml) and incubated overnight. The colonies were lysed and hybridized with 1.5×10^6 cpm of the cDNA probe (23). Clones giving a positive signal were individually picked and transferred to L plates for a secondary screening with the same cDNA probe.

RNA Rescue. Individual clones selected in the secondary screening were analyzed by positive selection. Hybrid-selected translation analysis of plasmid DNA was carried out according to Parnes *et al.* (24) with several modifications: DNA was isolated from 5-ml overnight cultures as described by Holmes and Quigley (25). Plasmid DNA (2 μg in 20 μl) was heated to 100°C for 10 min and quickly chilled in ice. Then an equal volume of 1 M NaOH was added and the mixture was incubated at room temperature for 20 min. After neutralization with 1 M HCl, the cDNA was pipetted onto nitrocellulose disks, which were air dried and baked for 2 hr at 80°C in a vacuum oven. Ten micrograms of anemic rat spleen mRNA was used for each hybridization. Translation was carried out in a 25-μl volume, 12 μl was applied directly to a NaDodSO$_4$/10% polyacrylamide gel, and the remainder was immunoprecipitated with anti-PBG-D IgG.

Restriction Maps of the cDNA Clones. Plasmid DNA was purified by the acid/phenol method (26). All restriction enzyme analyses were performed using the reaction conditions recommended by the supplier. Restriction fragments were analyzed either by 1.5% (wt/vol) agarose, or 12% (wt/vol) polyacrylamide gel electrophoresis in a 90 mM Tris/90 mM borate/1 mM EDTA buffer and stained with ethidium bromide.

Cell Culture and mRNA Isolation from MEL Cells. MEL cells, clone 745, were grown in RPMI medium supplemented with 10% fetal calf serum and induced by treatment with 1.8% (vol/vol) Me_2SO as described (27); mRNA was prepared according to Curtis (28).

RNA Blot Analysis. Size fractionation of RNA preparations on denaturing methylmercury hydroxide agarose gel was carried out according to Chandler *et al.* (29) except that samples were made up in 10 mM methylmercury hydroxide and applied on gel containing 1.3% agarose and 10 mM methylmercury hydroxide. After electrophoresis, the gel was washed before blotting sequentially in 100 ml of 10 mM phosphate buffer containing 1.1 M formaldehyde and 5 mM 2-mercaptoethanol for 40 min, then in the same buffer with 7 mM iodoacetate instead of 2-mercaptoethanol for 30 min, and finally in phosphate buffer plus formaldehyde alone for 20 min (30). The RNA was then transferred to a nitrocellulose filter (31) and hybridized to a nick-translated [^{32}P]cDNA probe (32). The washed filters were autoradiographed with an intensifying screen at −80°C against a Kodak X-Omat x-ray film.

DNA Blot Analysis. DNAs were digested with restriction endonucleases, electrophoresed in 0.8% agarose gels, and transferred to nitrocellulose filters as described (22).

RESULTS

Construction of a cDNA Library from Rat Spleen mRNA Enriched in PBG-D-Specific Sequences. Preliminary experiments were performed to assess the relative abundance of PBG-D mRNA in poly(A)$^+$ RNA from rat liver and anemic spleen by cell-free translation. Antibodies raised in rabbits against human PBG-D were used to immunoprecipitate the newly synthesized rat enzyme. Analysis of the immunoprecipitate by NaDodSO$_4$/polyacrylamide gel electrophoresis revealed a single radioactive band with the same apparent molecular mass as the human enzyme [42 kilodaltons (kDa)]. This value is in good agreement with the values previously reported (8, 33). Anemic rat spleen contained the highest proportion of specific mRNA. From measuring the radioactivity of the band after gel electrophoresis of the immunoprecipitate, we estimated that 0.1% of the [^{35}S]methionine incorporated into proteins was associated with newly synthesized PBG-D.

PBG-D message was enriched to 1% by preparative polyacrylamide gel electrophoresis. Then 0.5 μg of the mRNA from enriched fractions was used for cloning. From 20 ng of the hybrid plasmid, 6000 clones were obtained and plated onto a single nitrocellulose filter.

Identification of Plasmids Containing PBG-D mRNA Sequences. To get a specific probe, 20 μg of the mRNA enriched for PBG-D message was further fractionated under highly denaturing conditions, using methylmercury hydroxide agarose gel electrophoresis. This procedure resulted in a final 150-fold purification of PBG-D mRNA, with a translational activity representing 10–20% of the translatable RNAs in the selected fractions (Fig. 1). Four fractions (93–96, Fig. 1) were pooled and their mRNA was used to derive a ^{32}P-labeled single-stranded cDNA. We screened 6000 bacterial transformants with this probe, and 40 individual colonies giving a positive signal were selected and characterized by hybridization selection using rat spleen mRNA.

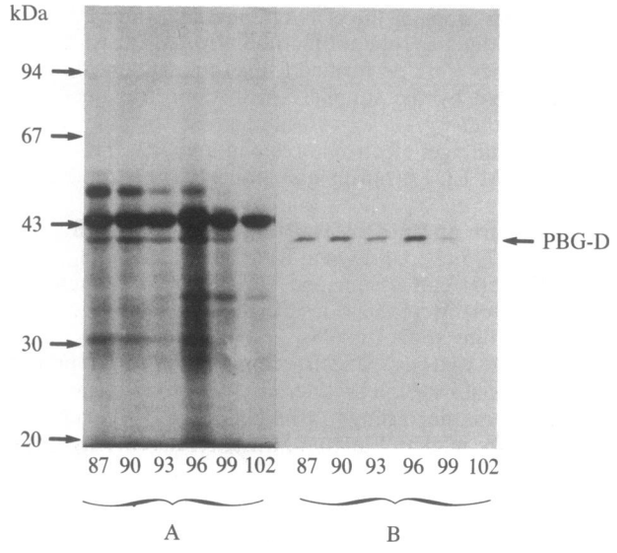

FIG. 1. Cell-free translation of rat spleen mRNA fractions after electrophoresis through methylmercury/agarose gel. Enriched mRNA (10 μg) from the first purification step was submitted to electrophoresis through 10 mM methylmercury/1.3% agarose gel. One hundred and fifty 600-μl fractions were collected, mRNA was precipitated with ethanol and resuspended in 10 μl of water, and 2 μl from each fraction was translated *in vitro* in 25 μl of translation mixture. (*A*) Analysis of the total newly synthesized products by Na-DodSO$_4$ polyacrylamide gel electrophoresis (10 μl per slot from the translation mixture, corresponding to 10,000–20,000 cpm of protein labeled with ^{35}S *in vitro*). (*B*) Ten microliters from the same translation mixtures as in *A* was immunoprecipitated with PBG-D antibodies before electrophoresis.

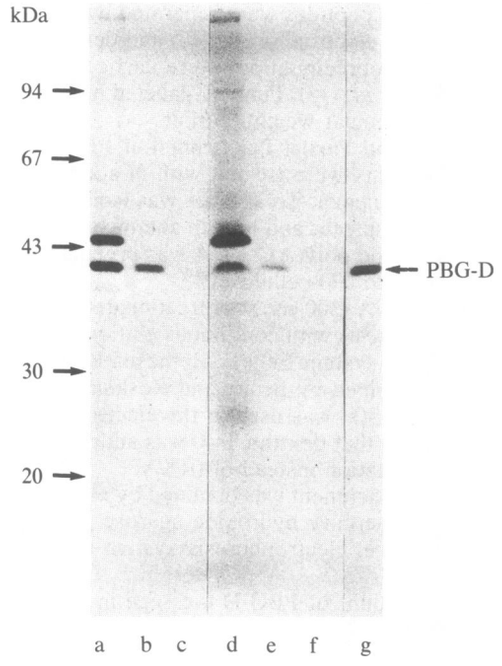

FIG. 2. Identification of cDNA sequences corresponding to rat PBG-D by positive hybridization selection. Lane a, translation products of plasmid pPBG-D29-selected rat spleen mRNA. Lane b, immunoprecipitation with anti-PBG-D IgG of an equal aliquot of cell-free translation products from selected rat mRNA. Lane c, same as in lane b but in presence of 10 μg of unlabeled pure human PBG-D. Lanes d, e, and f, same as in lanes a, b, and c, but with human mRNA. Lane g, ^{14}C-labeled PBG-D purified from human erythrocytes. The 45-kDa band represents the [^{35}S]methionine-protein adduct and is also observed in the absence of added mRNA.

Four recombinant plasmids reproducibly selected mRNA species, the translation product of which was identified as being PBG-D according to its molecular weight and immunoreactivity with anti PBG-D antibodies. Specificity of the immunoprecipitation was checked by competition with pure human PBG-D (Fig. 2).

We took advantage of a peculiar property of PBG-D to confirm the identity of the recombinant plasmid pPBG-D29: when submitted to nondenaturing electrophoresis, PBG-D migrates as a set of discrete bands (4, 8, 33). Anderson and Desnick (8) presented evidence that the resulting pattern reflects the presence in the preparation of stable enzyme–substrate intermediates at various stages of the sequential condensation of four molecules of porphobilinogen. Incubation with porphobilinogen was shown to elicit the interconversion of these different forms of PBG-D (8). The translational product from the rat spleen mRNA selected by pPBG-D29 was analyzed by isoelectric focusing and fluorography of the gel. The newly synthesized protein migrated as a single major radioactive band; however, incubation with porphobilinogen prior to isoelectric focusing resulted in the appearance of several additional bands that focused at more acidic pH values (not shown). These data support the idea that PBG-D was synthesized *in vitro* as a free enzyme and that the incubation with porphobilinogen generated the observed more acidic forms.

When tested with human anemic spleen mRNA, the rat PBG-D recombinant plasmid pPBG-D29 was able to select the human PBG-D message (Fig. 2).

A screening of the library was carried out by colony hybridization with a probe obtained by nick-translation of the largest *Pst* I fragments from PBG-D recombinant plasmid pPBG-D11 (see restriction map in Fig. 3). Four clones hybridized with this probe and corresponded to those already

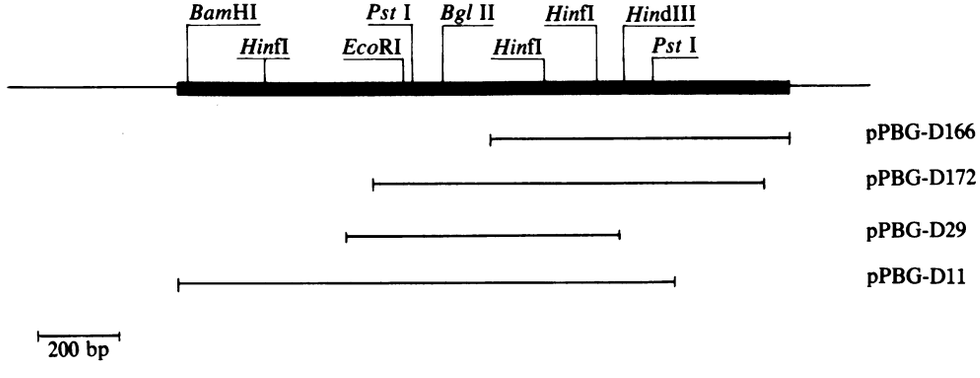

FIG. 3. Restriction maps of four pPBG-D cDNA inserts. Plasmid DNA was isolated from the pPBG-D11, pPBG-D29, pPBG-D166, and pPBG-D172 clones and digested with the restriction endonucleases shown. These plasmids were not cut by *Hin*cII.

identified by positive hybridization selection.

Restriction Maps of the cDNA Clones. Recombinant plasmids (pPBG-D11, pPBG-D29, pPBG-D166, and pPBG-D172) were analyzed and compared by restriction mapping of their inserted sequences (Fig. 3). pPBG-D11, the largest cDNA insert (1280 bp) displays two internal *Pst* I sites. Its sequence partially overlaps that of pPBG-D166 and pPBG-D172 inserts and entirely contains the sequence of the pPBG-D29 insert. This was determined by alignment of restriction sites and establishment of identity between restriction fragments by Southern analysis with appropriate probes.

Blot Hybridization Analysis of PBG-D mRNA. An electrophoretic analysis of poly(A)$^+$ RNA from rat liver and rat anemic spleen as well as from human anemic spleen was performed under denaturing conditions (Fig. 4, lanes 1, 2, and 5).

After transfer to nitrocellulose filter and hybridization with pPBG-D29 nick-translated cDNA insert, a single prominent band was observed with all preparations, corresponding to a mRNA 1800 nucleotides in length.

The intensity of the hybridization signal obtained with liver mRNA was lower than that of the signal obtained with anemic spleen mRNA, although the amount of poly(A)$^+$ RNA submitted to electrophoresis was 4 times higher. These data well correlate with differences observed in specific enzymatic activities measured in homogenates from these two tissues (data not shown).

Analysis of human anemic spleen RNA by using the rat cDNA probe indicates that human PBG-D mRNA has the same length as the homologous rat sequence.

Changes in PBG-D mRNA in MEL Cells Measured by cDNA Hybridization. The rat cDNA probe was also used to detect PBG-D mRNA changes in MEL cells after treatment with Me_2SO (Fig. 4, lanes 3 and 4). An approximate 10-fold increase in PBG-D message was noticed upon induction. This value corresponds to the increase observed for the protein measured either as an antigen (unpublished results) or by its enzymatic activity (5).

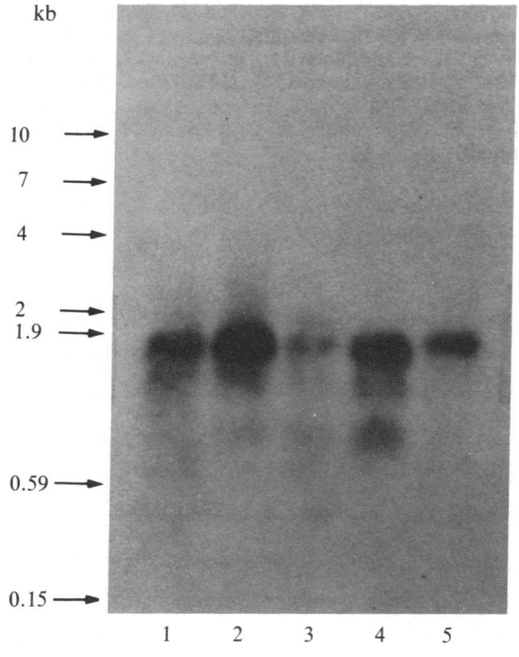

FIG. 4. Blot hybridization analysis of PBG-D mRNA. Lane 1, 10 μg of mRNA from rat liver; lane 2, 2.5 μg of mRNA from anemic rat spleen; lane 3, 6 μg of mRNA from uninduced murine erythroleukemia (MEL) cells; lane 4, 6 μg of mRNA from MEL cells induced with Me_2SO for 3 days; lane 5, 5 μg of mRNA from human anemic spleen. The human spleen was from a thalassemic patient. The standards are λ phage DNA restriction fragments, digested with *Hin*dIII. kb, Kilobase(s).

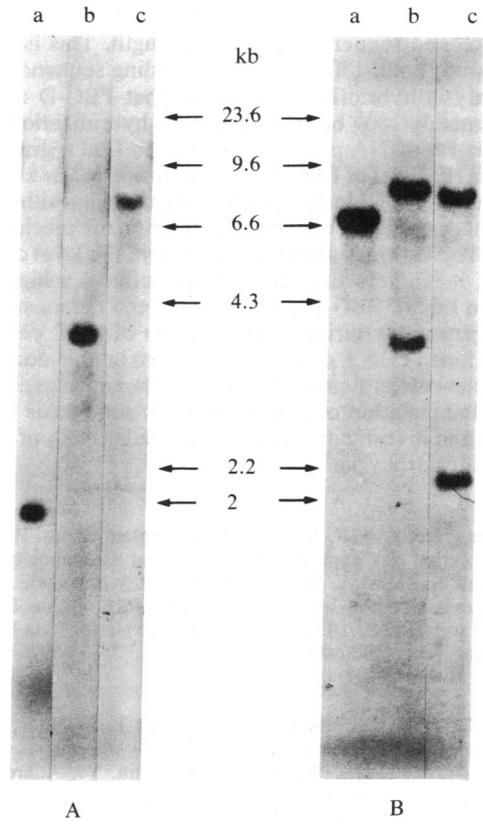

FIG. 5. Identification of PBG-D sequences in human and rat DNAs by blot hybridization. Total cellular DNAs were digested with the indicated restriction enzymes, electrophoresed in agarose, blotted to nitrocellulose, and hybridized with the rat cDNA probe. Size markers consisted of phage λ DNA digested with *Hin*dIII. (*A*) Rat DNA; (*B*) human DNA. Lane a, *Eco*RI; lane b, *Bgl* II; lane c, *Bam*HI.

Southern Blot Analysis of PBG-D Sequences in Rat and Human DNA. As a first step towards the study of human acute intermittent porphyria we used the nick-translated cDNA insert pPBG-D29 as a probe against both rat and human DNAs in Southern blot analyses. Fig. 5 shows the results of these experiments. The simple restriction pattern obtained suggests that genomic sequences encoding PBG-D do not belong to a multigenic family. Additional experiments are necessary to determine the genomic map of human PBG-D gene, and there may be restriction fragment length polymorphisms that would be useful for tracing mutant genes among family members.

DISCUSSION

In this paper, we report the molecular cloning of cDNA sequences complementary to PBG-D mRNA. A cDNA library was prepared from partially purified mRNA from rat anemic spleen. This library was then screened with a cDNA probe derived from a mRNA fraction highly enriched for PBG-D sequences by two successive steps of gel electrophoresis under various conditions of denaturation. Colonies giving a positive signal were further analyzed by hybridization selection, and four clones containing PBG-D cDNA sequences were isolated. The recombinant plasmids from these clones were identified by their ability to select mRNA species encoding a protein that displayed typical immunological and electrophoretic properties of PBG-D. Restriction mapping of the PBG-D plasmids established that two overlapping cDNA

inserts (from pPBG-D166 and pPBG-D172) represent an overall cloned sequence 1500 bp in length. This is likely to correspond to most if not all of the coding sequence since a blot analysis hybridization revealed that PBG-D mRNA is approximately 1800 bp in length. Blot hybridization studies using the rat cDNA probe allowed us to find a dramatic increase in the level of PBG-D mRNA during *in vitro* erythroid differentiation of Me_2SO-treated MEL cells. Although experiments are clearly needed to determine the mechanism by which Me_2SO brings about an increase in the level of PBG-D mRNA, it is likely that this finding reflects a higher transcription rate of PBG-D gene, as has been demonstrated for globin genes (34) during differentiation of MEL cells.

The cloned cDNA cross-hybridizes to human corresponding sequences as demonstrated by blot analyses. This will permit the isolation of human genomic sequences encoding PBG-D and investigations of the molecular basis of acute intermittent porphyria.

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