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Identification of the Human and Bovine ATP:Cob(I)alamin Adenosyltransferase cDNAs Based on Complementation of a Bacterial Mutant*

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In humans, deficiencies in coenzyme B12-dependent methylmalonyl-CoA mutase (MCM) lead to methylmalonyl aciduria, a rare disease that is often fatal in newborns. Such deficiencies can result from inborn errors in the MCM structural gene or from mutations that impair the assimilation of dietary cobalamins into coenzyme B12 (Ado-B12), the required cofactor for MCM. ATP:cob(I)alamin adenosyltransferase (ATR) catalyzes the terminal step in the conversion of cobalamins into Ado-B12. Substantial evidence indicates that inherited defects in this enzyme lead to methylmalonyl aciduria, but the corresponding ATR gene has not been identified. Here we report the identification of the bovine and human ATR cDNAs as well as the corresponding human gene. A bovine liver cDNA expression library was screened for clones that complemented an ATR-deficient bacterial strain for color formation on aldehyde indicator medium, and four positive clones were isolated. The DNA sequences of two clones were determined and found to be identical. Sequence similarity searching was then used to identify a homologous human cDNA (89% identity) and its corresponding gene that is located on chromosome XII. The bovine and human cDNAs were independently cloned and expressed in *Escherichia coli*. Enzyme assays showed that expression strains produced 87 and 98 nmol/min/mg ATR activity, respectively. These specific activities are in line with values reported previously for bacterial ATR enzymes. Subsequent studies showed that the human cDNA clone complemented an ATR-deficient bacterial mutant for Ado-B12-dependent growth on 1,2-propanediol. This demonstrated that the human ATR is active under physiological conditions albeit in a heterologous host. In addition, Western blots were used to show that ATR expression is altered in cell lines derived from *cblB* methylmalonyl aciduria patients compared with cell lines from normal individuals. We propose that inborn errors in the human ATR gene identified here result in methylmalonyl aciduria. The identification of genes involved in this disorder will allow improvements in the diagnosis and treatment of this serious disease.

Enzymes dependent on the vitamin B12 coenzymes, adenosyl-B12 (Ado-B12)[1] and methyl-B12 (CH_3-B12), have a broad but uneven distribution among living forms (1–3). In higher animals, two B12-dependent enzymes are known. CH_3-B12-dependent methionine synthase is needed for the methylation of homocysteine to methionine (4, 5), and Ado-B12-dependent methylmalonyl-CoA mutase (MCM) plays an essential role in the conversion of propionyl-CoA to the trichloroacetic acid cycle intermediate, succinyl-CoA (6, 7). This latter process occurs in three steps: propionyl-CoA is carboxylated to (2*S*)-methylmalonyl-CoA, isomerized to (2*R*)-methylmalonyl-CoA, and finally rearranged to succinyl-CoA in a reaction catalyzed by Ado-B12-dependent MCM (Fig. 1). In higher animals, propionyl-CoA is produced from the breakdown of the amino acids valine, isoleucine, methionine, and threonine, as well as thymine, cholesterol, and odd-chain fatty acids; hence, MCM is essential for the complete catabolism of each of these compounds (6).

In humans, inherited defects that impair the activity of Ado-B12-dependent MCM lead to methylmalonyl aciduria, a rare but severe disease that is often fatal in the 1st year of life (8–10). Such inherited deficiencies can result from mutations in the MCM structural gene (*mut*) or from mutations that impair the acquisition of the required cofactor, Ado-B12 (8, 9). Higher animals are incapable of *de novo* synthesis and, hence, must obtain Ado-B12 by synthesis from complex precursors taken up from the diet (1). Suitable precursors include vitamin B12 (CN-B12) and other cobalamins with various β-ligands (X-B12s) (1). The pathway by which X-B12s are converted to Ado-B12 has been studied in several organisms and is thought to be similar in both prokaryotes and eukaryotes (Fig. 1); X-B12 is converted to glutathionyl-B12 (GS-B12), reduced to cob(II)alamin, further reduced to cob(I)alamin, and finally adenosylated to Ado-B12 (11–15). In humans, four complementation groups (*cblABCD*), associated with methylmalonyl aciduria, are thought to correspond to genes involved in the conversion of X-B12s to Ado-B12. Enzymatic assays of fibroblast extracts have indicated that the *cblC* and *cblD* complementation groups encode cytoplasmic enzyme(s) needed for the conversion of X-B12s to cob(II)alamin (16). Similar studies have indicated that the *cblA* and *cblB* complementation groups correspond to a mitochondrial cob(II)alamin reductase and an ATP:cob(I)alamin adenosyltransferase (ATR) enzymes, respectively (17, 18). To date, the human genes that correspond to the *cblABCD* complementation groups have not been identified. Progress in this area has been slow due the difficulties in

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[1] The abbreviations used are: Ado-B12, adenosyl-B12; CH_3-B12, methyl-B12; MCM, methylmalonyl-CoA mutase; ATR, ATP:cob(I)alamin adenosyltransferase; IPTG, isopropyl-β-D-thiogalactopyranoside; MTS, mitochondrial targeting sequence; DDH, ado-B12-dependent diol dehydratase.

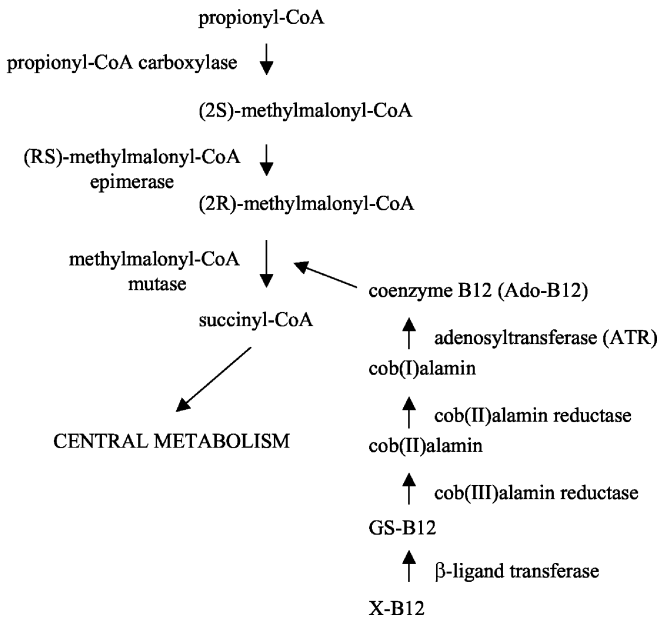

FIG. 1. **Pathways of propionyl-CoA catabolism and the intracellular metabolism of coenzyme B12.** The abbreviations used are: *X-B12*, inactive B12 precursors with one of several β-ligands; *GS-B12*, B12 with glutathione as the β-ligand.

purifying the relevant proteins (19).

We recently identified the PduO ATR of *Salmonella,* and showed that this enzyme has partial functional redundancy with the CobA enzyme (20). In these studies, it was also shown that a *Salmonella pduO cobA* double mutant lacked Ado-B12-dependent diol dehydratase (DDH) activity due to the ATR deficiency. Furthermore, it was found that a plasmid-encoded source of ATR enzyme restored DDH activity to a *cobA pduO* double mutant (20). Because this enzymatic activity can be readily detected on aldehyde indicator medium, we expected that the ATR-deficient *Salmonella* strain could be used to screen expression libraries for cDNAs that encode ATR enzymes. Such an approach would circumvent the difficulties associated with ATR purification.

Here, we report the isolation of an ATR cDNA, from a bovine liver library, by complementation of an ATR-deficient *Salmonella* mutant for color formation on aldehyde indicator medium. Subsequently, the homologous human cDNA and its corresponding gene were identified by sequence similarity searching. Both the human and bovine cDNAs are shown to express ATR activity and complement an ATR-deficient bacterial mutant. In addition, Western blots were used to show that expression of the human ATR was altered in cell lines derived from three *cblB* patients compared with a cell line derived from a normal individual. We propose that the human gene identified here coincides with the *cblB* complementation group, defects in which lead to methylmalonyl aciduria. The identification of genes involved in methylmalonyl aciduria is important for the development of improved methods for the diagnoses and treatment of this devastating disorder.

MATERIALS AND METHODS

Chemicals and Reagents—Vitamin B12 (CN-B12) and hydroxy-B12 (HO-B12) were purchased from Sigma. Titanium(III) citrate was prepared as described previously (21). 5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) and isopropyl-β-D-thiogalactopyranoside (IPTG) were from Diagnostic Chemicals Ltd., Charlottetown, Prince Edward Island, Canada. Restriction enzymes and T4 DNA ligase were from New England Biolabs, Beverly, MA. Other chemicals were from Fisher.

Bacterial Strains and Growth Media—Bacterial strains used in this study are listed in Table I. The minimal media used was NCE (22, 23) supplemented with 0.4% 1,2-propanediol, 200 ng/ml CN-B12, 1 mM $MgSO_4$, 0.3 mM each of valine, isoleucine, leucine, and threonine. Luria-Bertani (LB) was the rich medium used (Difco) (24). MacConkey and aldehyde indicator media were supplemented with 1,2-propanediol and CN-B12 and prepared as described previously (20).

General Protein and Molecular Methods and ATR Assays—Bacterial transformation, PCR, restriction enzyme digests, and other standard molecular and protein methods were performed as described previously (20, 25). ATR assays were also performed as reported previously (20).

P22 Transductions—Transductional crosses were performed as described previously (26). In preparing lysates of *galE* mutant strains, overnight cultures were grown on LB medium supplemented with 0.2% galactose and 0.2% glucose with the addition of appropriate antibiotics. A P22 HT105/1 *int-201* phage was used for transductional crosses at a concentration of 2×10^8 phage/ml (27).

Screening the Bovine and Human Liver cDNA Libraries—Uni-ZAP XR bovine and human liver cDNA libraries were from Stratagene (La Jolla, CA). Titering, amplification, and mass excision of the cDNA carried on pBlueScript SK(+/−) were done according to the manufacturer's protocol except that the titering procedure used F-top agar with 0.2 mM thymine, but no NaCl (24), instead of NZY top agar. Following mass excision, the cDNA expression plasmids were purified using a QIAprep spin mini prep kit (Qiagen, Chatsworth, CA). Then a portion of the resulting expression library was used to transform *Salmonella enterica* strain BE253 by electroporation. Strain BE253 is ATR-deficient due to *pduO* and *cobA* mutations and carries pSJS1240 which provides rare tRNAs that enhance expression of heterologous genes in *S. enterica.* Following electroporation, transformation mixtures were suspended in molten aldehyde indicator medium that had been supplemented with 1,2-propanediol, HO-B12, and 100 μg/ml ampicillin and cooled to 45 °C. The molten medium was poured into 100 × 15-mm sterile Petri dishes, allowed to solidify, and incubated at 37 °C in the dark for ~12 h. Resultant colonies were screened for red/brown color formation. This procedure allowed screening of about 5,000 transformants per plate.

Cloning of the Bovine ATR Coding Sequence for High Level Expression—PCR was used to amplify the bovine ATR coding sequence. Plasmid pNL121, isolated in this study from a bovine library cDNA library, provided the template DNA. The primers used for amplification of the full-length coding sequence were 5′-GCCGCCGGTACCGATGACGACGACAAGTTCGGCACGAGCCCGGGAGGT-3′ (forward) and 5′-GCCGCCAAGCTTGCTTGGTTCCTCGATGAAGCA-3′ (reverse). To eliminate the predicted mitochondrial targeting sequence (MTS), forward primer 5′-GCCGCCGGTACCGATGACGACGACAAGCCCCAGGGCGTGGAAGACGGG-3′ was used in conjunction with the reverse primer described above. These primers introduced *Kpn*I and *Hin*dIII restriction sites into the PCR products and were designed such that following cloning the bovine ATR coding sequence would be fused to both N-terminal glutathione *S*-transferase and His_6 tags. PCR products obtained using the primers described above were restricted with *Kpn*I and *Hin*dIII and ligated to the pET-41a expression vector (Novagen, Cambridge, MA) that had been similarly digested (25). Ligation mixtures were used to transform *Escherichia coli* DH5α by electroporation, and transformants were selected by plating on LB-Kan medium. Pure cultures were prepared from selected transformants, and plasmid DNA isolated from these strains was analyzed by restriction digestion and DNA sequencing. Clones having the expected DNA sequences were transformed into *E. coli* strain BL21DE3 RIL (Stratagene) for high level expression.

Cloning of the Human ATR Coding Sequence for High Level Expression—The human ATR coding sequence was cloned via PCR using a strategy similar to that described above for the bovine enzyme. IMAGE cDNA clone 2822202 provided the template DNA (Incyte Genomics, Palo Alto, CA). For amplification of the full-length coding sequence, the following primers were used: forward 5′-GCCGCCAGATCTGGATGACGACGACAAGATGGCTGTGTGCGGCCTGG-3′ and reverse 5′-GCCGCCAAGCTTTCAGAGTCCCTCAGACTCGGCCG-3′. To eliminate the putative MTS, primer 5′-GCCGCCAGATCTGGATGACGACGACAAGCCTCAGGGCGTGGAAGACGGG-3′ was used as the forward primer. The primers described above introduced *Bgl*II and *Hin*dIII restriction sites that were used for cloning into the pET-41a expression vector. The *Bgl*II site was positioned such that the resulting clones would express the human ATRs as fusion proteins with N-terminal glutathione *S*-transferase and His_6 tags. Ligation, transformation, and analysis of clones was performed as described above for the bovine expression clones. Clones with the expected DNA sequence were transformed into *E. coli* strain BL21DE3 RIL for high level expression.

Cloning of the Human ATR Coding Sequence for Complementation Studies—For complementation studies, the human ATR coding se-

TABLE I
Bacterial strains

Species	Strain	Genotype
E. coli	BL21 (DE3) RIL	(*E. coli* B) F$^-$*ompT hsdS* ($r_B^-m_B^-$) *dcm*$^+$ Ter^r *gal* λ (DE3) *endA* Hte (*argU ileY leuW* Cam^r)
	SOLR™ (Stratagene)	e14$^-$(McrA$^-$) Δ(*mcrCB-hsdSMR-mrr*)171 *sbcC recB recJ uvrC umuC*::Tn5 (Kan^r) *lac gyrA96 relA1 thi-1 endA1* λ^R [F′ *proAB lacI*^{q}*ZΔM15*] ^{c} Su$^-$ (nonsuppressing)
	XL1-Blue MRF′ (Stratagene)	D(*mcrA*)*183* Δ(*mcrCB-hsdSMR-mrr*)*173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac* [F′ *proAB lacI*^{q}*ZΔM15* Tn*10*(Tet ^{r})]^c
	BE237	BL21 (DE3) RIL/pET-41a (T7 expression vector without insert)
	BE255	BL21 (DE3) RIL/pNL128 (bovine ATR + MTS)
	BE256	BL21 (DE3) RIL/pNL129 (bovine ATR without MTS)
	BE257	BL21 (DE3) RIL/pNL132 (human ATR + MTS)
	BE258	BL21 (DE3) RIL/pNL133 (human ATR without MTS)
S. enterica serovar typhimurium LT2		
	TR6579	*metA22 metE551 trpD2 ilv-452 hsdLt6 hsdSA29* HsdB$^-$ *strA120* GalE$^-$Leu$^-$Pro$^-$
	BE253	TR6579 *cobA*::dcam Δ*pduO651* yeex::kan^r/pSJS1240 (*ileX, argU*)
	BE254	BE253/pNL121 (pBlueScript with bovine ATR)
	BE266	Δ*pduO651 cobA366*::Tn*10*dCam/pSJS1240 (*ileX, argU*)
	BE263	Δ*pduO651 cobA366*::Tn*10*dCam/pLAC22/pSJS1240 (*ileX, argU*)
	BE265	Δ*pduO651 cobA366*::Tn*10*dCam/pNL135 (human ATR without MTS under *plac* control)

quence was amplified by PCR and cloned into plasmid pLAC22 which allows IPTG-inducible expression in *S. enterica* (28). The primers used for amplification were forward 5′-GCCGCCAGATCTTATGCCTCAGGGCGTGGAAGACGGG-3′ and reverse 5′-GCCGCCAGCTTTCAGAGTCCCTCAGACTCGGCCG-3′. These primers eliminate the putative MTS and provide the needed ATG start triplet such that the first five amino acids of the expressed protein would be MPQGV (Fig. 2). The PCR product was restricted with *Bam*HI and *Hin*dIII and ligated into pLAC22 that had been digested with *Bgl*II and *Hin*dIII. Ligation mixtures were used to transform *S. enterica* strain BE253 (ATR-deficient) by electroporation. Transformants were selected on aldehyde indicator medium supplemented with 1,2-propanediol and Amp. Use of this medium allowed the identification of clones expressing ATR activity. Pure cultures were prepared from selected transformants, and plasmid DNA isolated from these strains was analyzed by restriction digestion and DNA sequencing. Clones having the expected DNA sequences were moved into strain BE266 (*pduO cobA*) via P22 transduction. Pure cultures prepared from the resultant colonies, and determined to be phage-free by cross-streaking against P22 H5, were used for complementation studies.

Growth of ATR Expression Strains and Preparation of Cell Extracts—The *E. coli* strains used for expression of the bovine and human ATRs were grown on LB supplemented with 25 μg/ml kanamycin at 37 °C with shaking at 275 rpm in a New Brunswick Scientific shaker incubator. Cells were grown to an absorbance of 0.6–0.8 at 600 nm, and protein expression was induced by the addition of 1 mM IPTG. Cells were incubated at 37 °C with shaking at 275 rpm for an additional 3 h. Cells were removed from the incubator, held on ice for 10 min, and then harvested by centrifugation at 6,690 × g_{max} for 10 min using a Beckman JLA-10.500 rotor. The cells were resuspended in 3 ml of 50 mM sodium phosphate, 300 mM NaCl, pH 7, and broken using a French pressure cell (SLM Aminco, Urbana, IL) at 20,000 psi. Phenylmethylsulfonyl fluoride was added to the cell extract to a concentration of 100 μg/ml to inhibit proteases. The crude cell extract was centrifuged at 16,000 rpm (31,000 × g_{max}) for 30 min using a Beckman JA20 rotor to separate the soluble and insoluble fractions. The supernatant was decanted (soluble fraction), and the pellet was resuspended in 1 ml of 50 mM sodium phosphate, 300 mM NaCl, pH 7 (inclusion body fraction). Both fractions were used for the ATR assays.

Growth Curves—Cells to be used as inocula for growth curves were grown overnight at 37 °C in 2 ml of LB medium or LB medium supplemented with ampicillin at 100 μg/ml for strains carrying pLAC22. A portion of the LB culture (1.5 ml) was pelleted by centrifugation and resuspended in 1 ml of minimal medium. Then resuspended cells (125 μl) were used as the inoculum. Cells were grown in 16 × 150-mm culture tubes containing 5 ml of minimal media and incubated at 37 °C in a New Brunswick model C-24 water bath with a shaking speed of 250 rpm. Cell growth was determined by measuring the absorbance at 600 nm using a Milton Roy model Spectronic 20D$^+$ spectrophotometer.

Western Blots—Human skin fibroblasts from one normal individual (MCH45) and three patients diagnosed with cobalamin b (*cblB*) disorder (WG1680, WG1879, and WG2127) were obtained from the Repository for Mutant Human Cell Strains at Montreal Children's Hospital. These cell lines were grown in Eagle's minimum essential medium supplemented with Earle's salts, L-glutamine, 1% nonessential amino acids, 10% heat-inactivated fetal bovine serum, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 0.2 μg/ml HO-B_{12} and incubated at 37 °C with 5% CO_2, 95% air mixture. For lysate preparation, each of the cell lines was cultured in 3, T150-cm^2 tissue culture flasks and grown to confluence. Harvested fibroblasts were washed twice with phosphate-buffered saline and lysed in 50 mM Tris, pH 7.3, 100 mM KCl, 5 mM $MgCl_2$, 1.6% Triton X-100, and a protease inhibitor mixture (Roche Diagnostics). Insoluble cellular matter was removed by centrifugation at 12,000 × *g* at 4 °C for 15 min. Protein concentration of the soluble extract was determined using a Micro BCA assay (Pierce). Cell extracts (220 μg of protein) were fractionated by 10% SDS-PAGE and transferred onto a Hybond-P, polyvinylidene difluoride membrane (Amersham Biosciences). The membrane was incubated at room temperature for 1 h with rocking in 10 mM Tris-HCl, pH 8, 150 mM NaCl, 0.05% Tween 20 (TBST) containing 3% bovine serum albumin and 3% mouse serum. The primary antibody used was affinity-purified rabbit IgG anti-human-ATR diluted 1:1000. The secondary antibody was monoclonal anti-rabbit IgG (γ-chain specific) biotin conjugate (Sigma) and was used at a 1:25,000 dilution. All membrane incubations were carried out at room temperature for 1 h with rocking in TBST. The membrane was prepared for detection using streptavidin horseradish peroxidase conjugate (Caltag Laboratories, Burlingame, CA) diluted 1:10,000, and Immun-Star™ horseradish peroxidase chemiluminescent kit (Bio-Rad) following the manufacturer's protocol. Membranes were exposed to x-ray film from Research Products International (Mt. Prospect, IL).

DNA Sequencing and Analysis—DNA sequencing was carried out by University of Florida, Interdisciplinary Center for Biotechnology Research, DNA Sequencing Core facility and University of Florida, Department of Microbiology and Cell Science, DNA Sequencing Facility as described previously (29). Sequence similarity searches were carried out using the Blast family of sequence analysis software (30, 31). Multiple sequence alignments were done with ClustalX using default parameters except as noted below (32). The slow and accurate method was used for pairwise alignments with a gap opening and extension penalties of 35 and 0.75, respectively. Multiple sequence alignment parameters were gap opening and extension penalties of 15 and 0.3, respectively, and delay divergent sequences was set at 25%.

RESULTS

Screening of the Bovine and Human Liver cDNA Libraries for Clones That Express ATR Activity—Our previous studies (20) showed that *S. enterica* strain BE253 forms red/brown colonies on aldehyde indicator medium supplemented with 1,2-propanediol and CN-B12 when it is transformed with an ATR expression plasmid. The basis of the color formation is that ATR activity restores the ability of strain BE253 to convert CN-B12 to Ado-B12, the required cofactor for DDH. Given

Ado-B12, DDH converts 1,2-propanediol to propionaldehyde which reacts with components in the aldehyde indicator medium to form a red/brown precipitate (20). Hence, we expected that BE253 would be useful for screening expression libraries for ATR activity.

Accordingly, we attempted to identify a human ATR cDNA by transforming strain BE253 with a human liver cDNA expression library and screening the resultant colonies for red/brown color formation on aldehyde indicator medium. Approximately 250,000 human cDNAs were screened, but no red/brown colonies were found. Subsequently, a bovine liver cDNA library was similarly screened, and four positive clones were isolated. The DNA sequences of two of the four clones were determined and found to be identical. The clone was 1058 bp in length and included a poly(A) tail indicating that its 3′ end was complete. However, analyses indicated that the 5′ end of the clone was incomplete. The clone included a 228-amino acid open reading frame that would be expressed as an N-terminal β-galactosidase fusion protein (as was expected given the cloning method employed), but it lacked the expected ATG triplet. Hence, the bovine clone apparently lacked a portion of its 5′ end.

After the DNA sequence of the bovine cDNA clones was determined, Blastp searches of the NCBI nonredundant data base were conducted, and these searches showed that the 228-amino acid protein encoded by the bovine cDNA clone was 29% identical (Expect = 8×10^{-15}) to the N-terminal domain (165 amino acids) of the PduO ATR of *S. enterica* (20). The findings that the bovine cDNA isolated both complemented an ATR-deficient mutant of *S. enterica* and encoded a protein with sequence similarity to a known ATR enzyme suggested that the bovine clone also encoded an ATR enzyme.

Identification of a Human Gene Related to the Bovine ATR cDNA—Blastp searches showed that the putative bovine ATR enzyme was 88% identical to a human protein encoded by IMAGE cDNA clone 2822202 (accession number BC005054). The human cDNA clone was 1128 bp in length and included an appropriate ATG triplet and a poly(A) tail indicating that it was a full-length clone. Further sequence analyses showed that the predicted protein encoded by the human cDNA was 250 amino acids in length and was homologous over its entire length to the putative bovine ATR except that it included 19 additional N-terminal amino acids (Fig. 2). This suggested that the bovine clone was nearly full length, lacking only a portion of its N terminus, probably a small region of its MTS (see below). Perhaps more importantly, however, the high identity between the human and bovine sequences indicated that both encoded proteins with similar functions. Furthermore, sequence similarity searches showed human cDNA BC005054 encoded a protein with 26% amino acid identity to the PduO ATR of *S. enterica*. Thus, these results tentatively identified sequence BC005054 as the human ATR cDNA

Subsequent sequence analyses showed human cDNA BC005054 was 99% identical to nine regions of human chromosome XII. The IMAGE clone BC005054 encoded a protein with 2 amino acid substitutions compared with the putative protein encoded by chromosome XII (Arg-19 to Gln and Met-239 to Lys). These changes may represent natural polymorphisms or may have resulted from mutations introduced during cDNA preparation. The nine regions on chromosome XII were the only regions of the human genome that showed significant homology to human cDNA BC005054. Hence, it seems likely that these regions correspond to nine exons that encode the human ATR enzyme. The exon structure of the human ATR is indicated in Fig. 2.

Putative Subcellular Localization of ATR Enzymes—The amino acid sequences of the human and bovine ATR enzymes were aligned with 20 related sequences obtained from GenBank™ (Fig. 2). This alignment showed that the human, bovine, and mouse ATR sequences included about 50–60 additional N-terminal amino acids compared with their prokaryotic homologues. In higher organisms, ATRs localize to mitochondria (6); hence, the additional N-terminal amino acids of the eukaryotic sequences might represent MTSs. Prediction of the subcellular localization of the human and mouse proteins using Predotar[2] and MitoProt II (34) software indicated mitochondrial localization: human, MitoProt and Predotar scores both = 0.89; and mouse MitoProt and Predotar scores = 0.97 and 0.99, respectively. Because the bovine clones were apparently partial sequences, reliable prediction of subcellular localization of the presumptive bovine ATR enzyme was not possible (34).[2]

High Level Expression of the Bovine and Human ATRs in E. coli—E. coli strains were constructed for high level expression of the presumptive bovine and human ATR enzymes. Clones were constructed to allow expression of these enzymes with and without sequences presumed to be involved in mitochondrial targeting. Our concern was that the MTS sequences might interfere with enzyme activity as such sequences are normally removed during mitochondrial localization (35). The *arrows* in Fig. 2 indicate the regions of the bovine and human ATRs that were expressed by clones that eliminated their MTSs. Furthermore, in all cases, the ATRs were produced as fusion proteins with N-terminal glutathione *S*-transferase and His_6 tags that could be removed by enterokinase cleavage if desired.

Production of the presumptive bovine and human ATR fusion proteins was monitored by SDS-PAGE (Fig. 3). Both the soluble (*lanes 2–4*) and insoluble fractions (*lanes 5–7*) were analyzed. Strains BE255 and BE256 produced large amounts of protein with molecular masses of 55 and 52 kDa, respectively (Fig. 3*A*, *lanes 3*, *4*, *6*, and *7*). These values are near the predicted masses (58 and 54 kDa) for the bovine ATR fusion proteins with and without the presumptive MTS and including the expression tags. In contrast, a strain carrying the expression plasmid without insert (BE237) produced little protein near these molecular masses (Fig. 3*A*, *lanes 2* and *5*) indicating that BE255 and BE256 were indeed producing large amounts of bovine ATR fusion proteins with and without the presumptive MTS.

Expression of the human ATR fusion proteins was also monitored by SDS-PAGE (Fig. 3*B*). The predicted molecular masses of the human ATR fusion proteins with the expression tags, and with and without the predicted MTS, are 58 and 55 kDa, respectively. As shown in Fig. 3*B*, large amounts of proteins with calculated molecular masses of 56 and 52 kDa were produced by expression strains BE257 (ATR + MTS) and BE258 (ATR without MTS) (Fig. 3*B*, *lanes 3*, *4*, *6*, and *7*) but not by the control strain BE237 (*lanes 2* and *5*), which carried that expression plasmid without insert.

Assay of the Bovine and Human ATR Fusion Proteins for ATR Activity—The cell extracts analyzed by SDS-PAGE (Fig. 3) were tested for ATP:cob(I)alamin adenosyltransferase activity (Table II). Substantial activity was found in both soluble and inclusion body extracts from strains BE255 (bovine ATR + MTS), BE256 (bovine ATR), BE257 (human ATR + MTS), and BE258 (human ATR). Of the four bovine ATR extracts tested, the highest specific activity measured was 85.7 nmol/min/mg found in the inclusion body fraction from strain BE255 (ATR + MTS). The highest specific activity found in the human ATR extracts tested was 98 nmol/min/mg in the inclusion body frac-

[2] Internet address: www.inra.fr/predotar.

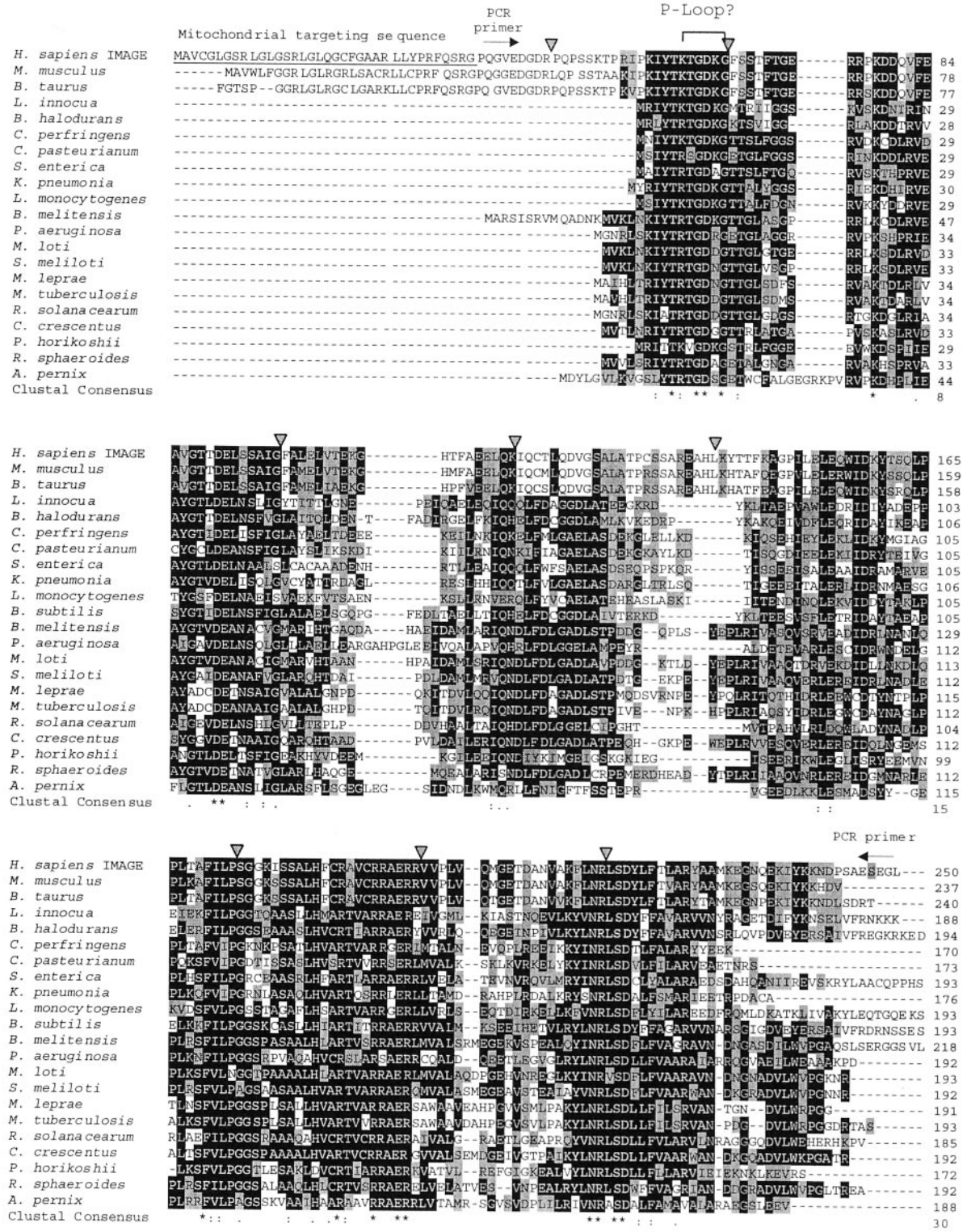

FIG. 2. **Multiple sequence alignment of adenosyltransferase enzymes.** ClustalX was used to align eukaryotic, prokaryotic, and archaeal homologues of ATR enzymes. Identical residues are *shaded black*, and highly conserved residues are *shaded gray*. The predicted mitochondrial targeting sequence determined by MitoProtII software is *underlined* in the human sequence. *Triangles* separate the human ATR into nine regions that correspond to exons on chromosome XII. *Arrows* represent the PCR primers used for amplification of the human ATR without its presumptive MTS. A possible ATP-binding region (P-loop) is also indicated.

tion containing human ATR without its presumptive MTS. For each ATR fusion protein, the majority of the activity was found in the soluble fraction (Table II, 4th column). For both the human and bovine cell extracts, ATR activity was linear with protein concentration (data not show). Furthermore, when the substrates ATP and/or cob(I)alamin were excluded from the assay mixture, no activity was detected. Also, as expected, cell extracts from strain BE237 (T7 expression vector without insert) expressed no measurable ATR activity.

Complementation of an S. enterica Mutant Deficient in ATR Activity by a Human ATR cDNA Clone—We showed previously (20) that an *S. enterica* strain deficient for both the CobA and

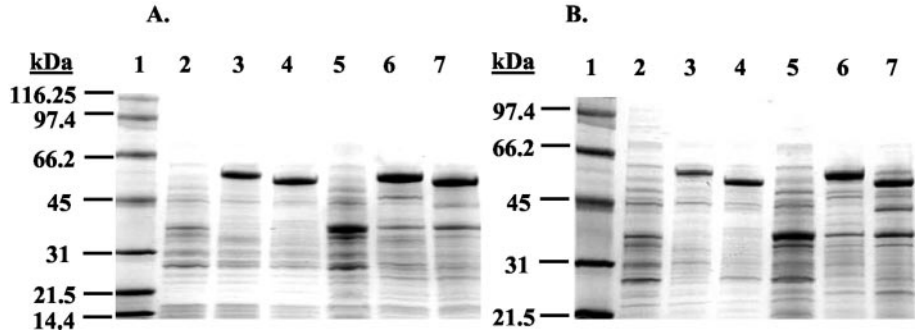

FIG. 3. **SDS-PAGE analysis of cell extracts from bovine (*A*) and human (*B*) ATR expression strains.** *A* and *B*, *lane 1* is molecular mass markers. *A*, *lanes 2–4* are soluble extracts of BE237 (T7 expression vector without insert), BE255 (bovine ATR + MTS), and BE256 (bovine ATR without MTS). *A*, *lanes 5–7* of are inclusion body fractions from strains BE237, BE255, and BE256. *B*, *lanes 2–4* are soluble extracts from BE237 (T7 expression vector without insert), BE257 (human ATR + MTS), and BE258 (human ATR without MTS), respectively. *Lanes 5–7* are inclusion body fractions from strains BE237, BE257, and BE258.

TABLE II
Specific activities of bovine and human ATP:cob(I)alamin ATRs

Strain	S/I[a]	Total activity	Total activity	Specific activity
		nmol/min	*%*	*nmol/min/mg*
BE255 (bovine ATR+MTS)	S	2021	95	47
	I	107	5	85.7
BE256 (bovine ATR without MTS)	S	3364	84	44.1
	I	632	16	30
BE257 (human ATR+MTS)	S	2445	52	37.5
	I	2296	48	60.8
BE258 (human ATR without MTS)	S	5481	57	63
	I	4144	43	98

[a] S, soluble fraction; I, inclusion body fraction.

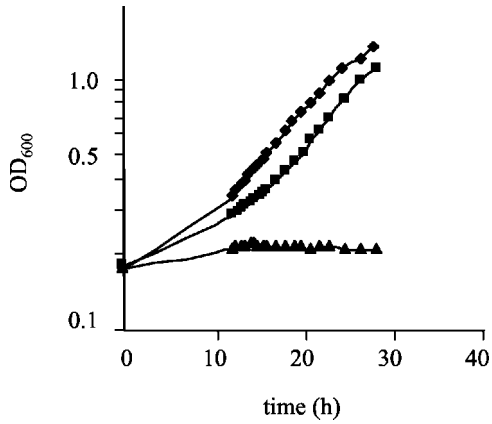

FIG. 4. **Complementation of a ATR-deficient bacterial mutant for Ado-B12-dependent growth on 1,2-propanediol by a plasmid that expresses the human ATR.** Cells were grown in minimal 1,2-propanediol medium supplemented with HO-B12 and 0.1 mM IPTG. Growth was determined by measuring the absorbance of cultures at 600 nm (OD_{600}), and data are shown as a semi-log plot. ♦, wild type (*S. enterica* serovar typhimurium LT2); ■, BE265, ATR-deficient/pNL135(human ATR under *plac* control); ▲, BE263, ATR-deficient/pLAC22 (vector without insert).

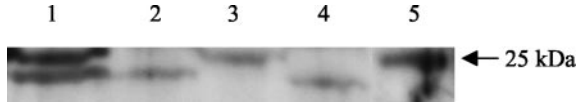

FIG. 5. **Western blot analysis of ATR expression by normal and *cblB* mutant cell lines.** Extracts were prepared from cultured fibroblast lines obtained from a normal individual and three *cblB* patients. *Lane 1*, cell extract from the MCH45 control cell line. *Lanes 2–4*, cell extracts from *cblB* mutant cell lines WG1680, WG1879, and WG2127, respectively. 220 μg of protein was loaded in each of *lanes 1–4*. (It was necessary to load relatively high amounts of protein because of the low abundance of the ATR enzyme in skin fibroblasts.) *Lane 5* contained 100 pg of recombinant human ATR lacking the mitochondrial targeting sequence and affinity tags. The *arrow* shows the location of the recombinant ATR enzyme (~25 kDa). The experiment was performed twice with similar results.

PduO ATRs (*cobA pduO*) was unable to grow on 1,2-propanediol due to an ATR deficiency. To test whether the human ATR cDNA could complement this defect, we compared the growth of the wild type strain, BE265 (*pduO cobA*/pLAC22-human ATR) and BE263 (*pduO cobA*/pLAC22-no insert) on 1,2-propanediol minimal medium (Fig. 4). The doubling times of the wild type and strain BE265 (*pduO cobA*/pLAC22-human ATR cDNA) were found to be comparable; they were 8.4 and 8.9 h, respectively. Control experiments showed that strain BE263, which carried the pLAC22 vector without insert, grew minimally on 1,2-propanediol and that growth of strain BE265 (*pduO cobA*/pNL135-human ATR) required the addition of IPTG as was expected because expression of the human ATR in this strain is under control of the *lacI* repressor (not shown). Thus, clearly, the human ATR cDNA complemented the ATR-deficient bacterial mutant for Ado-B12-dependent growth on 1,2-propanediol. These results provided further evidence that human cDNA BC005054 encodes an ATR enzyme and also showed that this enzyme can function as an ATR under physiological conditions albeit in a heterologous host.

Expression of the Human ATR Enzyme in Normal and cblB Mutant Human Skin Fibroblasts—Above we presented biochemical and genetic evidence that human cDNA BC005054 encodes an ATR enzyme. If defects in this enzyme underlie *cblB* methylmalonyl aciduria, its expression or stability might be altered in *cblB* mutant cell lines. To examine the expression of this enzyme in normal and *cblB* mutant human skin fibroblasts, Western blots were performed on lysates from these cells using antibody against recombinant human ATR described above. Fig. 5 shows a representative experiment. *Lane 5* contained recombinant human ATR lacking both the mitochondrial targeting sequence and the expression tags. In this lane, a single protein band with an approximate molecular mass of 25 kDa was recognized by the anti-ATR antibody as expected. In *lane 1*, which contained cell extracts from normal

skin fibroblasts, the anti-human ATR antibodies recognized two bands. One of these bands was similar in molecular mass to the recombinant human ATR (~25 kDa) and the second band was of lower mass (~23 kDa). Neither the 25- nor the 23-kDa band was observed in blots performed with preimmune serum (data not shown), indicating that these bands most likely represent two processed forms of the human ATR. Interestingly, ATR-reactive polypeptides of these molecular masses were differentially expressed in the three *cblB* cell lines tested (*lanes 2–4*). *cblB* mutants WG1680 and WG2127 did not express detectable levels of the 25-kDa form of the ATR and expressed reduced levels of the 23-kDa form (*lanes 2* and *4*). For *cblB* mutant WG1879, the 25-kDa form was reduced, and the 23-kDa form was not detectable. These results indicated that expression of the ATR was significantly altered in *cblB* mutant human skin fibroblasts. These results provide direct evidence that defects in the ATR identified in this study underlie *cblB* methylmalonyl aciduria.

Conserved Amino Acids and Distribution of ATR Enzymes—The human and bovine ATR enzymes were aligned with 20 related sequences obtained from GenBank™ (Fig. 2). Of the sequences shown, the function of the *Homo sapiens*, *Baus taurus*, and *S. enterica* ATRs is supported by biochemical evidence presented here and previously (20). To obtain additional information about the function of the remaining prokaryotic ATRs, we examined their genomic context. Genes encoding ATR homologues from *S. enterica*, *Listeria innocua*, *Listeria monocytogenes*, *Bacillus halodurans*, *Clostridium perfringens*, *Clostridium pasteurianum*, and *Klebsiella pneumonia* were found proximal to either Ado-B12-dependent enzymes or Ado-B12 biosynthetic genes. Because bacteria frequently cluster genes of related function, these finding suggest that the genes from these organisms do indeed encode ATR enzymes. It is also notable that these are several regions of high conservation in all the ATRs aligned suggesting that many are indeed ATR enzymes (Fig. 2). Thus, PduO-type ATR enzymes appear to have broad phylogenetic distribution, being found in mammals, Gram-positive and Gram-negative bacteria, and the Archaea.

The highly conserved regions found in the ATRs also indicate amino acids likely to be essential for enzymatic activity and may represent sites directly involved in catalysis or in binding the substrates, ATP and cob(I)alamin. There are at least two different ways in which proteins bind B12, the "base-on" and "base-off" modes. Base-off binding involves displacement of the lower axial ligand of B12 (dimethylbenzimidazole) with an imidazole side chain of a histidine residue (36–38). The sequence containing the coordinating histidine (D*X*H*XX*G), which is conserved among proteins that bind B12 in the base-off mode, is absent from PduO-type ATRs examined in this study. This suggests that the ATR enzymes described in this study bind B12 in the base-on mode.

Attempts were also made to identify the ATP-binding region of the human or bovine ATRs. The best candidate ATP-binding region found was a conserved series of amino acids represented by residues 66–60 in the human ATR. This region is similar to the modified P-loop that binds ATP in the CobA ATR (39).

DISCUSSION

In this report we identified human and bovine cDNAs that encode ATR enzymes. The highest specific activities measured in cell extracts from expression strains were 85.7 nmol/min/mg protein for the bovine ATR and 98 nmol/min/mg protein for the human ATR. These values are comparable with those reported previously (20, 40, 41) for purified CobA ATR, and partially purified PduO ATR which were 53 and 312 nmol/min/mg protein, respectively. The bovine ATR cDNA was isolated based on its ability to complement an ATR-deficient *S. enterica* mutant for color formation on indicator medium, and the human ATR cDNA was shown to restore the ability of an ATR-deficient mutant to grow on 1,2-propanediol in an Ado-B12-dependent fashion. This demonstrated that the human and bovine cDNAs described here encode ATR enzymes that are active under physiological conditions, although in a heterologous host. Moreover, the bovine and human ATR cDNAs had 29 and 26% identity to the PduO ATR of *S. enterica* (20), and the human enzyme included a presumptive MTS as expected (10). Thus, genetic, biochemical, and bioinformatic evidence indicate that the bovine and human cDNAs analyzed here encode ATR enzymes.

Prior studies (6, 17) have indicated that ATR defects in humans lead to methylmalonyl aciduria. MCM is known to require Ado-B12 for activity (6), and fibroblast extracts from methylmalonyl aciduria patients with defects in the *cblB* complementation group have been shown to lack ATR activity (17). The ATR cDNA identified in this study corresponded to a single human gene composed of nine exons found on chromosome XII. Furthermore, Western blots indicated that expression of this ATR was altered in *cblB* mutant human cell lines compared with normal cell lines. Thus, we propose that the human gene identified here corresponds the *cblB* complementation group that is involved in methylmalonyl aciduria.

The approach used in this study to identify the human ATR cDNA involved screening mammalian cDNA expression libraries for clones that complemented an ATR-deficient bacterial mutant. This general strategy may have application to the identification of additional cDNAs involved cobalamin metabolism. Human cDNAs encoding the β-ligand transferase and the cob(III)alamin and cob(II)alamin reductases (the *cblACD* complementation groups) have not yet been identified (8, 9). In this regard, it is notable that in this study the human ATR cDNA was not directly isolated, but rather the bovine ATR cDNA was isolated, and sequence similarity searching was used to identify the homologous human gene. The fact that our screen allowed direct isolation of a bovine ATR cDNA, but not a human ATR cDNA, may reflect the relative abundance of ATR mRNA in the human *versus* the bovine liver. Ruminant livers have among the highest levels of B12-dependent enzymes measured in any tissue (42). Thus, ruminant liver libraries may be particularly useful for isolating mammalian cDNAs involved in cobalamin metabolism.

Importantly, the identification of human genes involved in methylmalonyl aciduria should help with the development of improved methods for diagnosis and treatment of this rare but devastating disease. Knowledge of the relevant genes will allow DNA-based methods of diagnosis following amniocentesis or chorionic villi sampling. Such techniques will allow the identification of the specific genetic lesions involved, and this may help guide treatment. Some cases of methylmalonyl aciduria respond to high-dose B12 therapy, and it seems likely that such cases will be associated with specific mutations (33, 43, 44). Moreover, as the needed methodologies become available, knowledge of the genes involved in cobalamin metabolism may also help with the development of gene or enzyme therapies as treatments for methylmalonyl aciduria.

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