Biochemical and Biophysical Research Communications **260,** 273–279 (1999)
Article ID bbrc.1999.0897, available online at http://www.idealibrary.com on IDEAL®

Characterization of Five Human cDNAs with Homology to the Yeast SIR2 Gene: Sir2-like Proteins (Sirtuins) Metabolize NAD and May Have Protein ADP-Ribosyltransferase Activity

Roy A. Frye[1]
Pittsburgh V.A. Medical Center (132L), Department of Pathology, University of Pittsburgh, Pittsburgh, Pennsylvania 15240

Received May 24, 1999

The yeast Sir2 protein regulates epigenetic gene silencing and as a possible antiaging effect it suppresses recombination of rDNA. Studies involving *cobB,* a bacterial SIR2-like gene, have suggested it could encode a pyridine nucleotide transferase. Here five human sirtuin cDNAs are characterized. The SIRT1 sequence has the closest homology to the *S. cerevisiae* Sir2p. The SIRT4 and SIRT5 sirtuins more closely resemble prokaryotic sirtuin sequences. The five human sirtuins are widely expressed in fetal and adult tissues. Recombinant *E. coli* cobT and cobB proteins each showed a weak NAD-dependent mono-ADP-ribosyltransferase activity using 5,6-dimethylbenzimidazole as a substrate. Recombinant *E. coli* cobB and human SIRT2 sirtuin proteins were able to cause radioactivity to be transfered from [^{32}P]NAD to bovine serum albumin (BSA). When a conserved histidine within the human SIRT2 sirtuin was converted to a tyrosine, the mutant recombinant protein was unable to transfer radioactivity from [^{32}P]NAD to BSA. These results suggest that the sirtuins may function via mono-ADP-ribosylation of proteins.

Epigenetic mechanisms create and maintain regions of a structurally altered form of chromatin called heterochromatin in which genes are largely silenced. Some components of the epigenetic regulatory apparatus such as Sir2 and proteins with SET-domains and chromo-domains have been highly conserved during the evolution of eukaryotes (1–3). In *Saccharomyces cerevisiae,* Sir2p participates in the establishment and maintenance of a heterochromatin-like state of suppression of genes located within or adjacent to the silent HM mating type loci, the telomeres, and the nucleolar chromatin (4–6). The other three Sir (Silent information regulator) proteins (Sir1p, Sir3p, and Sir4p) function by controlling the distribution of Sir2p; they form multimeric complexes with Sir2p and target Sir2p to specific chromatin sites (4, 5, 7, 8). In addition to these epigenetic gene-silencing effects, the yeast Sir2p functions in Ku-dependent double-strand DNA break repair and telomere maintenance (9, 10). Furthermore the yeast Sir2p suppresses recombination of nucleolar DNA and this could represent an antiaging effect (11–13).

Sir2-like proteins (sirtuins) are present in prokaryotes and in all eukaryotes including mammals (1). Studies in *Salmonella typhimurium* have shown that the *CobB* gene (a SIR2-like gene) enables bacteria with loss of function mutations in the *CobT* gene to survive (14). The *CobT* gene encodes nicotinate mononucleotide:5,6-dimethylbenzimidazole phosphoribosyltransferase, an enzyme that transfers the 5-phospho-α-D-ribosyl moiety from nicotinate mononucleotide onto 5,6-dimethylbenzimidazole; this produces N^1-(5-phospho-α-D-ribosyl)-5,6-dimethylbenzimidazole (also called α-ribazole-5′-phosphate), a precursor for the nucleotide loop assembly step in cobalamin biosynthesis (15, 16). Because the CobT enzyme acts as a pyridine nucleotide transferase enzyme and because the CobB protein appears to be able to compensate for loss of function of CobT this raises the possibility that the CobB protein may perform some type of pyridine nucleotide transferase function.

In this paper five human Sir2-like cDNAs are described and these sirtuin-encoding genes are found to be widely expressed. The *E. coli* CobB sirtuin is shown to have weak NAD-dependent ADP-ribosyltransferase activity using 5,6-dimethylbenzimidazole as a substrate. Purified recombinant *E. coli* CobB sirtuin and the human SIRT2 sirtuin are each able to cause bovine serum albumin (BSA) to be labeled in the presence of

[1] To whom correspondence should be addressed. Fax: 412-688-6872. E-mail: frye01@pitt.edu.

0006-291X/99 $30.00

[^{32}P]NAD and alteration of a conserved histidine residue in SIRT2 sirtuin abrogates this reaction; these results suggest that sirtuins may function by utilizing NAD to modify substrate proteins via ADP-ribosylation.

MATERIALS AND METHODS

Characterization of human sirtuin cDNAs. The *S. cerevisiae* Sir2 amino acid sequence was used to probe the GenBank EST databank and fragments from five different human cDNAs were obtained. To facilitate analysis of the numerous human sirtuin cDNA EST fragments, the "EST assembly machine" (http://gcg.tigem.it/cgi-bin/uniestass.pl) and "ESTblast" (http://www.hgmp.mrc.ac.uk/ESTBlast/) programs were utilized. SIRT1 had only scant fragments represented in the EST databank and this cDNA was cloned by PCR from the human testis Marathon cDNA library using the Marathon AP1 primer and sense and antisense primers derived from the EST sequence AA461259. For all five SIRT cDNAs the sequence of the coding sequence was verified by sequencing clones from Clontech Marathon or MTC cDNAs obtained using the high fidelity polymerases Advantage-HF, (Clontech) or pfu-Turbo, (Stratogene) to produce amplicons which were subcloned into the TOPO TA pCR2.1 vector (Invitrogen). The pattern of expression of sirtuin genes in various fetal and adult human tissues was characterized with multiple tissue cDNA (MTC) panels (Clontech). For this experiment the human sirtuin PCR primers were designed to generate amplicons in the range of 500 to 600 bp. Primers used correspond to the following cDNA sequence intervals (sense, antisense): *SIRT1*(1021–1045, 1559–1585), *SIRT2* (698–723, 1210–1232), *SIRT3* (800–825, 1283–1307), *SIRT4* (457–482, 951–972), and *SIRT5* (701–726, 1247–1273). Pilot experiments were done to establish appropriate numbers of PCR cycles for each cDNA in order to achieve a sensitive dynamic range of band intensity.

Expression of recombinant proteins. The pHEX vector is a modified form of Pharmacia's pGEX-2T vector in which the GST-encoding sequence was replaced with a hexahistidine-encoding sequence; hence the recombinant proteins expressed in this vector contain the N-terminal sequence MSPIHHHHHHGS fused to the native sequence. Primers (the sense primers incorporated BamH1 sites upstream of the ATG start codons) were used to amplify the full coding sequences of *E. coli* CobB sirtuin (GenBank AE000212) and *E. coli* CobT (GenBank U33333) genes and the human *SIRT2* cDNA. These amplicons were subcloned into TOPO TA pCR2.1 from which the BamH1-EcoR1 cDNA inserts were ligated into the pHEX vector. The site-directed mutagenesis of the *SIRT2* cDNA was done using overlap extension PCR and confirmed by sequencing the TOPO TA pCR2.1-subcloned mutated cDNA. Bacteria (TOP10F') were induced with 0.5 mM isopropyl β-D-thiogalactopyranoside (IPTG) for 3 hr then pelleted and lysed with BPER solution (Pierce). After centrifugation of the lysate, the supernatant was applied to a Ni-nitrilotriacetic acid (Ni-NTA) agarose (Qiagen) column, it was rinsed and eluted with a gradient of imidazole. Fractions containing purified recombinant proteins were concentrated by ultrafiltration, desalted by gel filtration, and after BCA protein assay (Pierce) dithiothreitol was added and the recombinant proteins were aliquoted and stored at −80°C in 10 mM DTT, 10 mM Tris pH 7.5 buffer.

TLC assay of pyridine nucleotide metabolism. The assay buffer was 50 mM glycine/KOH buffer (pH 9.0), 10 mM DTT, 0.5 mg/ml histone (Sigma cat. # H4380). The pyridine nucleotides (Sigma) and the 5,6-dimethylbenzimidazole (Sigma) were present at 5 mM, the 5,6-dimethylbenzimidazole was added added from a 0.5 M solution in Me_2SO. The assay volume was 20 μl and after addition of 0.5 μg of enzyme (cobT or sirtuins) incubation was at 37°C for 3 hr. For thin layer chromatography 10 × 20 cm silica gel HPTLC plates with fluourescent indicator (Whatman) were used with a solvent system of ethanol:2.5 M ammonium acetate (80:20). After spotting 2 μl of each assay sample, the plates were run for 30 min. The plates were examined and photographed under short wave UV illumination.

Protein ADP-ribosylation assay. The assay buffers contained 10 mM DTT and 50 mM Tris/HCl (pH 8.0) or 50 mM glycine/KOH (pH 9.0). The assay volume was 10 μl, 5 μM NAD, with [^{32}P]NAD 2.5 μCi per sample, and bovine serum albumin (Sigma cat. # A3803) present at 0.5 mg/ml. After addition of recombinant cobT or sirtuin proteins (0.5 μg/sample) incubation was at 37°C for 1 hr. Laemmli gel-loading buffer was added and after heating to 90°C for 5 min the samples were separated on 12% SDS–PAGE gels and autoradiography (overnight exposure with no intensifying screen) was performed on the undried gel.

RESULTS

Identification, characterization, and pattern of expression of human sirtuin cDNAs. To identify cDNAs that encode human Sir2-like proteins a combination of *in silico* and PCR-cloning techniques was used to obtain cDNA sequences of five human sirtuin genes SIRT1 (GenBank accession number AF083106), SIRT2 (AF083107), SIRT3 (AF083108), SIRT4 (AF083109), and SIRT5 (AF083110). Regarding the SIRT2 gene product, partial rat and human amino acid sequences have previously been described (1). EST sequence fragments of the SIRT1 cDNA have been grouped as the Unigene cluster Hs.42587 and mapped to chromosome 10. EST sequence fragments of the SIRT2 cDNA have been grouped as the Unigene cluster Hs.112306 and mapped to chromosome 19q. The SIRT3 gene is contained within the yac clone AF015416 from chromosome 11p15.5 region and the SIRT4 gene is contained within yac clone AC003982 from chromosome 12q.

PCR analysis of gene expression in multiple tissues showed that the five sirtuin genes are widely expressed in fetal and adult human tissues (Fig. 1). The five human sirtuin proteins vary in M_r from 33,881 (SIRT5) to 61,957 (SIRT1) and each contains a domain homologous to a sequence present in Sir2 proteins of *S. cerevisiae, C. elegans* and *Drosophila* (Fig. 2). SIRT1 appears to be the human homologue of yeast *SIR2* because of the five human sirtuins, it is the one with the closest amino acid sequence homology to Sir2 (Fig. 2). Furthermore the human SIRT1 protein contains a potential nuclear localization signal (KRKKRK) in amino acids 41-46; this is consonant with the known intranuclear localization of the yeast Sir2p. The SIRT1, SIRT2, and SIRT3 proteins are each more similar to the yeast Sir2 protein than are the SIRT4 and SIRT5 proteins (Fig. 2). The SIRT4 and SIRT5 sequences strongly resemble prokaryotic sirtuin sequences including the sirtuins encoded by the *cobB* genes of *E. coli* and *S. typhimurium* (Fig. 3).

Pyridine nucleotide transferase activity of E. coli CobT and CobB using 5, 6-dimethylbenzimidazole as a substrate. The *cobB* gene was identified in genetic experiments that demonstrated that *cobB* could partially substitute for the function of *cobT*, the

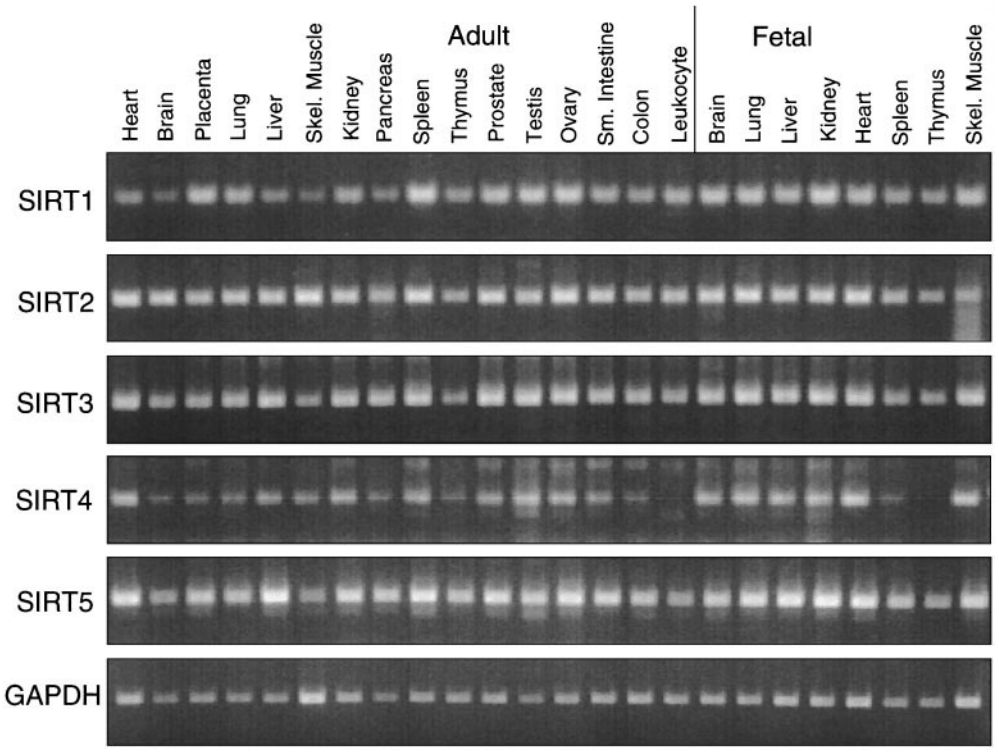
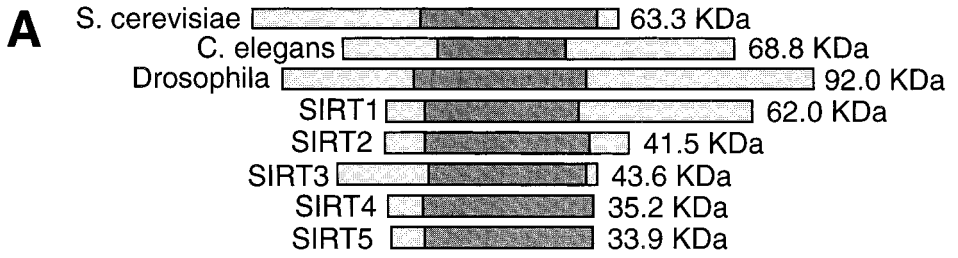


FIG. 1. Expression of human sirtuin genes in normal adult and fetal tissues. PCR was performed on tissue specific cDNA samples (Clontech MTC panels) pre-normalized for six housekeeping genes including glyceraldehyde phosphate dehydrogenase (GAPDH). To generate bands within a sensitive dynamic range of signal intensity the number of PCR cycles was adjusted as follows: SIRT1 (36 cycles), SIRT2 (32 cycles), SIRT3 (32 cycles), SIRT4 (32 cycles), SIRT5 (32 cycles), and GAPDH (23 cycles).

gene for nicotinic acid mononucleotide:5,6-dimethylbenzimidazole phosphoribosyltransferase (14, 17). The *cobT*-encoded enzyme transfers phosphoribose (PR) from the donor molecule, nicotinic acid mononucleotide (NaMN), to the acceptor molecule, 5,6-dimethylbenzimidazole (DMB), to form the product N^1-(5-phospho-α-D-ribosyl)-5,6-dimethylbenzimidazole (PR-DMB), a compound that is required for the synthesis of cobalamin (15, 16). This notion that *cobB* may encode a protein with pyridine nucleotide transferase activity combined with the fact that cobB is a sirtuin, raised the possibility that sirtuins might perform some type of transferase activity that utilizes pyridine nucleotides as donor molecules.

To test this hypothesis, three recombinant hexahistidine-tagged proteins were expressed and purified: the *E. coli* cobT, the *E. coli* cobB sirtuin, and the human SIRT2 sirtuin. In assays utilizing DMB as a substrate, all four major types of pyridine nucleotides (nicotinate mononucleotide, NaMN; nicotinamide mononucleotide, NMN; nicotinate adenine dinucleotide, NaAD; and nicotinamide adenine dinucleotide, NAD) were tested as potential donor molecules. As expected from prior work done with the *S. typhimurium* cobT enzyme (16), the *E. coli cobT* gene product was able to transfer PR from NaMN or from NMN to form the product PR-DMB (Fig. 4). Interestingly, the *E. coli* cobT enzyme was also able to act as a mono-ADP-ribosyltransferase. The cobT enzyme used NaAD (efficiently) and NAD (much less efficiently) as donor molecules to transfer ADP-ribose (ADPR) onto DMB to form ADPR-DMB (Fig. 4). At a low level of efficiency the *E. coli* cobB sirtuin was also able to act as a mono-ADP-ribosyltransferase by utilizing NAD to form trace amounts of the ADPR-DMB product (Fig. 4).

Protein ADP-ribosylation activity of the E. coli cobB sirtuin and the human SIRT2 sirtuin. To determine if sirtuins could possibly represent protein ADP-ribosyltransferases, the *E. coli* cobB sirtuin and the human SIRT2 sirtuin proteins were incubated with [^{32}P]NAD and bovine serum albumin (BSA). This resulted in transfer of label from the [^{32}P]NAD to the BSA protein (Fig. 5A). The *E. coli* cobT protein and heat denatured *E. coli* cobB and human SIRT2 sirtuin proteins were unable to transfer label to the BSA (Fig. 5A). To gain further assurance that this protein ADP-ribosylation was a specific sirtuin-mediated effect, site-directed mutagenesis was used to generate a point mutation that altered a conserved histidine residue in the human SIRT2 sirtuin. Near the middle of the sirtuin homology domain the residues "HG" are present in all eukaryotic and prokaryotic sirtuins (Figs. 2, 3). This histidine at codon 171 of SIRT2 was converted to a tyrosine. The



B

```
S.Cer RKILVLTGAGVSTSLGIPDFRSSE.GFYSKIKHLG..LDDPQDVFNYNIFMHDPSVFYNIANMVLPP..EKIYSPLHSFIKML 332
C.Ele KHILVLTGAGVSVSCGIPDFRSKD.GIYARLRSEFPDLPDPTAMFDIRYFRENPAPFYNFAREIFPG..QFVPSVSHRFIKEL 225
D.Mel QKIIVLTGAGVSVSCGIPDFRSTN.GIYARLAHDFPDLPDPQAMFDINYFKRDPRPFYKFAREIYPGEFQPSPSPCHRFIKML 301
SIRT1 KKIIVLTGAGVSVSCGIPDFRSRD.GIYARLAVDFPDLPDPQAMFDIEYFRKDPRPFFKFAKEIYPG..QFQPSLCHKFIALS 141
SIRT2 RRVICLVGAGISTSAGIPDFRSPSTGLYDNLEKYH..LPYPEAIFEISYFKKHPEPFFALAKELYPG..QFKPTICHYFMRLL 139
SIRT3 QRVVVMVGAGISTPSGIPDFRSPGSGLYSNLQQYD..LPYPEAIFELPFFFHNPKPFFTLAKELYPG..NYKPNVTHYFLRLL 216
SIRT4 KRLLVMTGAGISTESGIPDYRSEKVGLYARTDR....RPIQHGDFVRSAPIRQ..RYWARNFVGWPQFSSHQPNPAHWALSTW 131
SIRT5 KHIVIISGAGVSAESGVPTFRGAG.GYWRKWQAQD..LATPLA.FAHNPSRVW..EFYHYRREVMG...SKEPNAGHRAIAEC 124
                                  *       ------

S.Cer QM....KGKLLRNYTQNIDNLESYAGISTDKLVQCHGSFATATCV..TCHWNLPGERIFN..................... 386
C.Ele ET....SGRLLRNYTQNIDTLEHQTGIKR..VVECHGSFSKCTCT..RCGQKYDGNEIRE..................... 277
D.Mel ET....KGKLLRNYTQNIDTLERVAGIQR..VIECHGSFSTASCT..KCRFKCNADALRA..................... 353
SIRT1 DK....EGKLLRNYTQNIDTLEQVAGIQR..IIQCHGSFATASCL..ICKYKVDCEAVRG..................... 193
SIRT2 KD....KGLLLRCYTQNIDTLERIAGLEQEDLVEAHGTFYTSHCVSASCRHEYPLSWMKE..................... 195
SIRT3 HD....KGLLLRLYTQNIDGLERVSGIPASKLVEAHGTFASATCT..VCQRPFPGEDIRA..................... 270
SIRT4 EK....LGKLYWLVTQNVDALHTKAGSRR..LTELHGCMDRVLCL..DCGEQTPRGVLQERFQVLNPTWSAEAHGLAPDGDVF 206
SIRT5 ETRLGKQGRRVVVITQNIDELHRKAGTKN..LLEIHGSLFKTRCT..SCGVVAENYKSPICPALSGKGAPEPGTQDA...... 197
           ----

S.Cer ....KIRNLELPLCPVCYKKRREYFPEGYNNKVGVAASQGSMSERPPYILNSYGVLKPDITFFGEALPNKFHKSIREDILECD 465
C.Ele ....EVLAMRVAHCKRCE.....................................GVIKPNIVFFGEDLGREFHQHVTEDKHKVD 321
D.Mel ....DIFAQRIPVCPQCQPNKEQSVDASVAVTEEELRQLVEN............GIMKPDIVFFGEGLPDEYHTVMATDKDVCD 421
SIRT1 ....DIFNQVVPRCPRCPADEPL.....................................AIMKPEIVFFGENLPEQFHRAMKYDKDEVD 242
SIRT2 ....KIFSEVTPKCEDCQ.........................................SLVKPDIVFFGESLPARFFSCMQSDFLKVD 239
SIRT3 ....DVMADRVPRCPVCT.........................................GVVKPDIVFFGEPLPQRFL.LHVVDFPMAD 313
SIRT4 LSEEQVRSFQVPTCVQCG.........................................GHLKPDVVFFGDTVNPDKVDFVHKRVKEAD 254
SIRT5 ....SIPVEKLPRCEEAGCG.....................................GLLRPHVVWFGENLDPAILEEVDRELAHCD 243

S.Cer LLICIGTSLKVAP.VSEIVNMVPSHVPQVLINRDP.....................VKHAEFDLSLLGYCDDTAAMVAQKCGW 526
C.Ele LIVVIGSSLKVRP.VALIPHCVDKNVPQILINRES.....................LPHYNADIELLGNCDDTIRDICFSLGG 382
D.Mel LLIVIGSSLKVRP.VAHIPSSIPATVPQILINREQ.....................LHHLKFDVELLGDSDVTINQICHRLSD 482
SIRT1 LLIVIGSSLKVRP.VALIPSSIPHEVPQILINREP.....................LPHLHFDVELLGDCDVTINELCHRLGG 303
SIRT2 LLLVMGTSLQVQP.FASLISKAPLSTPRLLINKEKAGQSDPFLGMIMGLGGGMDFDSKKAYRDVAWLGECDQGCLALAELLGW 321
SIRT3 LLLILGTSLEVEP.FASLTEAVRSSVPRLLINRDLVGPLA.....................WHPRSRDVAQLGDVVHGVESLVELLGW 379
SIRT4 SLLVVGSSLQVYSGYRFLLTAWEKKLPIAILNIGPT.....................RSDDLACLKLNSRCGELLPLIDPC... 314
SIRT5 LCLVVGTSSVVYPAAMFAPQVAARGVPVAEFNTETT.....................PATNRFRFHFQGPCGTTLPEALACHEN 306
```

FIG. 2. Comparison of the yeast Sir2 protein sequence with nematode, fruitfly, and human Sir2-like proteins. (A)*S. cerevisiae*, *C. elegans*, and *Drosophila* Sir2 proteins are compared with the five human sirtuins SIRT1, SIRT2, SIRT3, SIRT4, and SIRT5. The sirtuin homology domains are shaded dark. The sequence-predicted molecular mass of each protein is noted. (B) The amino acid sequences from the sirtuin homology domains of the sirtuin proteins. The conserved "HG" is designated (*). The putative zinc finger domain motifs previously noted in sir2-like proteins (1), are designated with overlying dashed lines. GenBank accession numbers are: *S. cerevisiae* Z74090, *C. elegans* Z70310, *D. melanogaster* AF068758.

wild type SIRT2 and the $^{H}171^{Y}$-SIRT2 showed identical levels of IPTG-induced expression in *E. coli* and the two proteins were purified in parallel under identical conditions. Whereas the wild type SIRT2 protein transfered label from the [^{32}P]NAD onto the BSA, the $^{H}171^{Y}$-SIRT2 was unable to catalyze this reaction (Fig. 5B). The sirtuin-mediated labeling of BSA is enhanced at an alkaline pH and in the presence of salt (Fig. 5B).

DISCUSSION

The yeast Sir2 protein contains a domain comprising approximately 275 amino acid residues that is conserved in Sir2-like proteins (sirtuins) found in both prokaryotes and eukaryotes (1). Here it is shown that there are at least five human sirtuin genes, of these five human sirtuins the SIRT1 sirtuin shows the most similarity to the yeast Sir2 protein. The SIRT4 and SIRT5 sirtuins are quite similar to prokaryotic sirtuins. All of the sirtuin proteins contain the ~275 residue sirtuin homology domain. In many instances a highly conserved protein domain represents a conserved functional binding site for a metabolite or biomolecule and such conserved binding site domains are often found within enzymatic catalytic domains. Previous genetic studies (14, 17) of the cobalamin synthesis pathway in *S. typhimurium* have shown that the *CobB* gene (a SIR2-like gene) can compensate for loss of function mutations in the *CobT* gene (a gene that

```
SIRT4  KRLLVMTGAGISTESGIPDYRSEKVGLYARTD.....RRPIQHGDFVRSAPIRQRYWARNFVGWPQFSS. 118
SIRT5  KHIVIISGAGVSAESGVPTFRG.AGGYWRKWQ.....AQDLATPLAFAHNP..SRVWEFYHYRREVMGS. 111
P.hori KNVIAFTGAGISAESGIPTFRG.KDGLWKKYR.....PEELATPEAFERNP..KLVWDFYKWRIKKILK.  73
A.ful1 KYLVALTGAGVSAESGIPTFRG.KDGLWNRYR.....PEELANPQAFAKDP..EKVWKWYAWRMEKVFN.  73
A.ful2 KHAVVFTGAGISAESGIPTFRG.EDGLWRKYD.....PEEVASISGFKRNP..RAFWEFSMEMKDKLFA.  76
A.aeol LNIVTLTGAGISAESGIPTFRG.KDGLWNKFK.....PEELATPEAFFRNP..KLVWEWYDWKRQLIAK.  64
M.tube MRVAVLSGAGISAESGVPTFRDDKNGLWARFD.....PYELSSTQGWLRNP..ERVWGWYLWRHYLVAN.  62
E.coli PRVLVLTGAGISAESGIRTFRA.ADGLWEEHR.....VEDVATPEGFDRDP..ELVQAFYNARRRQLQQP 102
S.typh PRVLVLTGAGISAESGIRTFRA.ADGLWEEHR.....VEDVATPEGFARNP..GLVQTFYNARRQQLQQP  66
S.aure NRITFFTGAGVSVASGVPDFRS.MGGLFDEISKDGLSPEYLLSRDYLEDDP..EGFINFCHKRLLFVDT.  81
B.subt QRIVVLTGAGMSTESGIPDFRS.AGGIWTEDAS....RMEAMSLDYFLSYP..RLFWPKFKELFQMKMSG  74

SIRT4  .HQPNPAHWALSTWEKL....GKLYWLVTQNVDALHTKAGSRRLTELHGCMDRVLCLDCGEQTPRGVLQE 183
SIRT5  .KEPNAGHRAIAECETRLGKQGRRVVVITQNIDELHRKAGTKNLLEIHGSLFKTRCTSCGVVAENYKSPI 180
P.hori .AKPNPAHYALVELEKM....GILKAVITQNVDDLHREAGTKNLIELHGNIFRVRCTSCEFREHLKESGR 138
A.ful1 .AQPNKAHQAFAELERL....GVLKCLITQNVDDLHERAGSRNVIHLHGSLRVVRCTSCNNSFEVESAPK 138
A.ful2 ..EPNPAHYAIAELERM....GIVKAVITQNIDMLHQRAGSRRVLELHGSMDKLDCLDCHETYDWSEFVE 140
A.aeol .AQPNEGHKILTKMEEE....FPNFYLITQNVDGLHQRAGSKKVIELHGNIWKVRCVECGNERYEYTTPL 129
M.tube .VEPNDGHRAIAAWQD.....HAEVSVITQNVDDLHERAGSGAVHHLHGSLFEFRCARCGVPYTDALPEM 126
E.coli EIQPNAAHLALAKLQDAL...GDRFLLVTQNIDNLHERAGNTNVIHMHGELLKVRCSQSGQVLDWTGDVT 169
S.typh EIQPNAAHLALANLKKRL...AIAFLLVTQNIDNLHERAGNRNIIQMHGELLKVRCSQSGQILEWNGDVM 133
S.aure ..MPNIVHDWIAKLERN....QQSLGVITQNIDGLHSDAGSQHVDELHGTLNRFYCNVCHKSYTKSDVID 145
B.subt SFEPNEGHLLLAELEKQ....GKQVDIFTQNIDGLHKKAGSRHVYELHGSIQTAACPACGARYDLPHLLE 140

SIRT4  RFQVLNPTWSAEAHGLAPDGDVFLSEEQVRSFQVPTCVQCG.......GHLKPDVVFFGDTVNPDKVDFV 246
SIRT5  CPALSGKGAPEPGTQDASIPVEK..........LPRCEEAGCG.....GLLRPHVVWFGENLDPAILEEV 235
P.hori IDEILSED..............................LPKCPKCG........SLLRPDVVWFGEPLPSKELNEA 176
A.ful1 IPP..................................LPKCDKCG........SLLRPGVVWFGEMLPPDVLDRA 171
A.ful2 DFNKGE...............................IPRCRKCGS.......YYVKPRVVLFGEPLPQRTLFEA 177
A.aeol PEI..................................PPKCEKCG........GLLRPGVVWFGESLPVDALSRA 162
M.tube PEPAIEV..............................EPPVCDCG........GLIRPDIVWFGEPLPEEPWRSA 163
E.coli P....................................EDKCHCCQFP.....APLRPHVVWFGE.MPLG.MDEI 200
S.typh P....................................EDKCHCCQFP.....APLRPHVVWFGE.MPLG.MDEI 164
S.aure RT...................................LKHCDNCG........GAIRPDIVLYGEMLDQPTIIRA 177
B.subt RE...................................VPECTAAGNNGDICGTVLKTDVVLFGDAVMH..FDTL 177

SIRT4  HKRVKEADSLLVVGSSLQVYSGYRFILTAWEK.KLPIAILNIGPTRSDDLACLKLNSRCGELLP.LIDPC 314
SIRT5  DRELAHCDLCLVVGTSSVVYPAAMFAPQVAAR.GVPVAEFNTETTPATNRFRFHFQGPCGTTLPEALACH 304
P.hori FKLAKEADVVIVVGTSGLVYPAAYIPYIVKDS.GGVVIEINVQKSGITPIADFFLRGKAGEILPKLVQEV 245
A.ful1 MREVERADVIIVAGTSAVVQPAASLPLIVKQR.GGAIIEINPDETPLTPIADYSLRGKAGEVMDELVRHV 240
A.ful2 IEEAKHCDAFMVVGSSLVVYPAAELPYIAKKA.GAKMIIVNAEPTMADPIFDVKIIGKAGEVLPKIVEEV 246
A.aeol YELSREAHVFIVVGTSGVVYPAAELPFVAKEN.GAQVIEVNPEETPITKIADMHFKEKASTGLKKVYDYL 231
M.tube VEATGSADVMVVVGTSAIVYPAAGLPDLALAR.GTAVIEVNPEPTPLSGSATISIRESASQALPGLLERL 232
E.coli YMALSMADIFIAIGTSGHVYPAAGFVHEAKLH.GAHTVELNLEPSQVGNEFAEKYYGPASQVVPEFVEKL 269
S.typh YMALSMADIFIAIGTSGHVYPAAGFVHEAKLH.GAHTVELNLEPSQVGNEFEEKHYGPASQVVPEFVDKF 233
S.aure LNKIEHADTLVVLGSSLVVQPAAGLISHFK...GDNLIIINKDRTPYDSDATLVIHDDMVSVVKSLMTE. 243
B.subt YEKLDQADLLLVIGTSLEVAPARFVPEDASLIPGMKKVIINLEPTYCDSLFDMVIHQKIGEFARSLGMKK 247
```

FIG. 3. Protein sequence of human SIRT4 and human SIRT5 compared with prokaryotic sirtuin sequences. The prokaryotic sequences were obtained from GenBank: *Pyrococcus horikoshii* AB009498, *Archaeoglobus fulgidus* 1- AE000987 2- AE001098, *Aquifex aeolicus* AE000776, *Mycobacterium tuberculosis* Z95584, *Escherichia coli* AE000212, *Salmonella typhimurium* (*cobB*) U89687, *Staphylococcus aureus* M32103, *Bacillus subtilis* Z99109.

encodes the enzyme nicotinate mononucleotide:5,6-dimethylbenzimidazole phosphoribosyltransferase); this has raised the possibility that sirtuins are enzymes with pyridine nucleotide transferase activity. My experiments indicated that *E. coli* CobB sirtuin is unable to duplicate the previously described enzymatic activity of CobT (i.e., the transfer of the 5-phospho-α-D-ribosyl moiety from nicotinate mononucleotide onto 5,6-dimethylbenzimidazole); instead CobB showed a weak ability to transfer the ADP-ribosyl moiety from NAD onto 5,6-dimethylbenzimidazole. Thus CobB may represent an NAD-dependent mono-ADP-ribosyltransferase. Because CobB shows only a low level of ADP-ribosyltransferase activity with 5,6-dimethylbenzimidazole as a substrate, it is unlikely that 5,6-dimethylbenzimidazole represents the physiological target of the CobB protein's NAD dependent mono-ADP-ribosyltransferase activity. Work on the *cobB* gene in Salmonella has indicated that the actual physiological function of cobB is probably unrelated to the

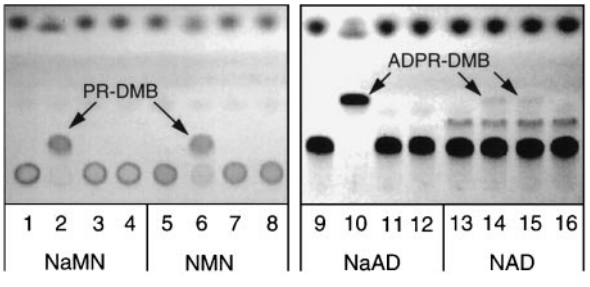


FIG. 4. Transfer of phosphoribose from pyridine mononucleotides (left panel) or ADP-ribose from pyridine dinucleotides (right panel) to 5,6-dimethylbenzimidazole (DMB). No enzyme added (lanes 1, 5, 9, 13), *E. coli* cobT-protein (lanes 2, 6, 10, 14), *E. coli* cobB sirtuin (lanes 3, 7, 11, 15), and the human SIRT2 sirtuin (lanes 4, 8, 12, 16). Purified recombinant hexahistidine-tagged proteins were incubated with 5 mM DMB, and 5 mM pyridine nucleotide (nicotinate mononucleotide, NaMN; nicotinamide mononucleotide, NMN; nicotinate adenine dinucleotide, NaAD; or nicotinamide adenine dinucleotide, NAD) for 3 hr, samples were then separated by TLC. The free DMB and the released nicotinate and nicotinamide comprise the bands near the top edge of the figure.

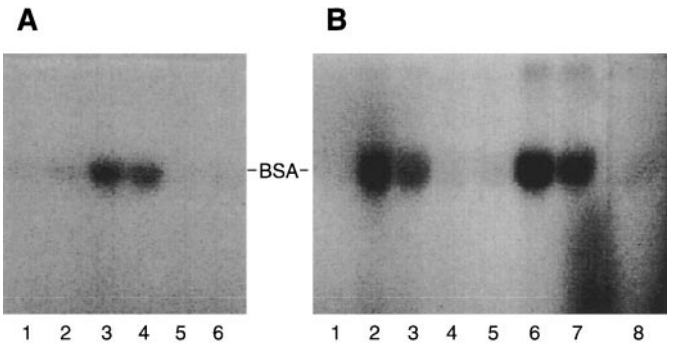


FIG. 5. Labeling of bovine serum albumin in the presence of [^{32}P]NAD by *E. coli* cobB sirtuin and human SIRT2 sirtuin. Samples were separated by 12% SDS–PAGE followed by autoradiography. (A) Reaction buffer of pH 8.0 used. No recombinant protein added (lane 1), *E. coli* cobT enzyme (lane 2), *E. coli* cobB sirtuin (lane 3), human SIRT2 sirtuin (lane 4), heat denatured (5 min at 90°C) *E. coli* cobB sirtuin (lane 5), heat denatured human SIRT2 sirtuin (lane 6). (B) Reaction buffer of pH 9.0 used with absence (lanes 1-4) or presence (lanes 5-8) of 200 mM NaCl. No recombinant protein added (lanes 1, 5), *E. coli* cobB sirtuin (lanes 2, 6), human SIRT2 sirtuin (lanes 3, 7), human $^{H}171^{Y}$ SIRT2 sirtuin (lanes 4, 8).

cobalamin precursor synthesis reaction, instead cobB is thought to function as a regulator of the propionate catabolism pathway (17). The yeast Sir2 protein appears to act as a modulator of chromatin that regulates both epigenetic gene silencing and nonhomologous end joining of double-strand DNA breaks (9, 11). Thus both prokaryotic and eukaryotic sirtuins appear to be regulatory proteins. Regulatory proteins often function by covalently modifying other proteins, e.g., protein kinases and protein acetyltransferases. One process by which proteins can be covalently modified is via ADP-ribosylation, this can occur either by poly-ADP-ribosylation or by mono-ADP-ribosylation. Poly-ADP-ribose polymerases (PARPs) are absent in prokaryotes and yeast but multiple PARPs that share homology in their catalytic domain have been found in higher eukaryotes, e.g., mammals have at least three PARP-like genes (18). However these PARP-like enzymes show no homology to sirtuin sequences. Protein mono-ADP-ribosylation is a potential mechanism for intracellular signal transduction and regulation of protein function in both prokaryotes and eukaryotes. The cloning of eukaryotic *intra*cellular mono-ADP-ribosyltransferase enzymes has not yet been reported despite the cloning of several eukaryotic genes for *extra*cellular mono-ADP-ribosyltransferases (19).

The sirtuin sequences available from prokaryotes, lower eukaryotes and the five human sirtuin cDNAs all appear to encode intracellular proteins as no signal sequences for protein export or transmembrane domains are seen. In an *in vitro* assay, addition of purified recombinant CobB sirtuin and human SIRT2 sirtuin caused transfer of radioactivity from [^{32}P]NAD to a protein (bovine serum albumin). This suggests that sirtuins might have protein mono-ADP-ribosyltransferase activity; however firm proof of this will require characterization of the molecular structure of the labeled adduct(s) within the sirtuin-labeled protein substrate. A point mutation of the human SIRT2 sirtuin, in which a conserved histidine was converted to a tyrosine residue, resulted in abrogation of this putative ADP-ribosyltransferase activity. These results suggest that the sirtuins could represent a family of intracellular regulatory proteins that function as mono-ADP-ribosyltransferases.

Little is known concerning the structure, function, or regulation of intracellular mammalian mono-ADP-ribosyltransferases. In addition to the well known activation of poly-ADP-ribosylation, DNA damage repair in mammalian cells is associated with increased mono-ADP-ribosylation of nuclear proteins (20, 21). Some recent studies have indicated that mammalian cells contain a cytoplasmic brefeldin A-activated NAD-dependent protein mono-ADP-ribosyltransferase activity that can affect the function of the Golgi membrane apparatus (22, 23). The ADP-riboslylation factor (ARF) family of G-proteins are regulated by guanine nucleotide exchange proteins called ARF-GEPs and brefeldin A may directly interact with some ARF-GEPs (24). It remains to be determined if the cytoplasmic brefeldin A-activated protein mono-ADP-ribosyltransferase is a sirtuin protein. Further investigation of sirtuin-mediated NAD-dependent protein modification, including the identification of intracellular proteins which are physiological substrates for modification by sirtuins, could lead to a more complete understanding of the function of sir2-like proteins in both prokaryotic and eukaryotic organisms.

ACKNOWLEDGMENT

This work was supported by funds from the University of Pittsburgh Pathology Department.

REFERENCES

1. Brachmann, C. B., Sherman, J. M., Devine, S. E., Cameron, E. E., Pillus, L., and Boeke, J. D. (1995) *Genes Dev.* **9,** 2888–2902.
2. Singh, P. B., Miller, J. R., Pearce, J., Kothary, R., Burton, R. D., Paro, R., James, T. C., and Gaunt, S. J. (1991) *Nucleic Acids Res.* **19,** 789–794.
3. Cavalli, G., and Paro, R. (1998) *Curr. Opin. Cell Biol.* **10,** 354–360.
4. Loo, S., and Rine, J. (1995) *Annu. Rev. Cell Dev. Biol.* **11,** 519–548.
5. Fritze, C. E., Verschueren, K., Strich, R., and Easton Esposito, R. (1997) *EMBO J.* **16,** 6495–6509.
6. Braunstein, M., Sobel, R. E., Allis, C. D., Turner, B. M., and Broach, J. R. (1996) *Mol. Cell. Biol.* **16,** 4349–4356.
7. Gotta, M., Strahl-Bolsinger, S., Renauld, H., Laroche, T., Kennedy, B. K., Grunstein, M., and Gasser, S. M. (1997) *EMBO J.* **16,** 3243–3255.

8. Smith, J. S., Brachmann, C. B., Pillus, L., and Boeke, J. D. (1998) *Genetics* **149,** 1205–1219.
9. Tsukamoto, Y., Kato, J., and Ikeda, H. (1997) *Nature* **388,** 900–903.
10. Boulton, S. J., and Jackson, S. P. (1998) *EMBO J.* **17,** 1819–1828.
11. Gottlieb, S. and Esposito, R. E. (1989) *Cell* **56,** 771–776.
12. Kennedy, B. K., Gotta, M., Sinclair, D. A., Mills, K., McNabb, D. S., Murthy, M., Pak, S. M., Laroche, T., Gasser, S. M., and Guarente, L. (1997) *Cell* **89,** 381–391.
13. Johnson, F. B., Sinclair, D. A., and Guarente, L. (1999) *Cell* **96,** 291–302.
14. Tsang, A. W., and Escalante-Semerena, J. C. (1998) *J. Biol. Chem.* **273,** 31788–31794.
15. Trzebiatowski, J. R., O'Toole, G. A., and Escalante-Semerena, J. C. (1994) *J. Bacteriol.* **176,** 3568–3575.
16. Trzebiatowski, J. R., and Escalante-Semerena, J. C. (1997) *J. Biol. Chem.* **272,** 17662–17667.
17. Tsang, A. W., and Escalante-Semerena, J. C. (1996) *J. Bacteriol.* **178,** 7016–7019.
18. Smith, S., Giriat, I., Schmitt, A., and De Lange, T. (1998) *Science* **282,** 1484–1487.
19. Okazaki, I. J., and Moss, J. (1998) *J. Biol. Chem.* **273,** 23617–23620.
20. Wielckens, K., Schmidt, A., George, E., Bredehorst, R., and Hilz, H. (1982) *J. Biol. Chem.* **257,** 12872–12877.
21. Kreimeyer, A., Wielckens, K., Adamietz, P., and Hilz, H. (1984) *J. Biol. Chem.* **259,** 890–896.
22. De Matteis, M. A., Di Girolamo, M., Colanzi, A., Pallas, M., Di Tullio, G., McDonald, L. J., Moss, J., Santini, G., Bannykh, S., Corda, D., and Luini, A. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 1114–1118.
23. Mironov, A., Colanzi, A., Silletta, M. G., Fiucci, G., Flati, S., Fusella, A., Polishchuk, R., Mironov, A., Jr., Di Tullio, G., Weigert, R., Malhotra, V., Corda, D., De Matteis, M. A., and Luini, A. (1997) *J. Cell Biol.* **139,** 1109–1118.
24. Sata, M., Donaldson, J. G., Moss, J., and Vaughan, M. (1998) *Proc. Natl. Acad. Sci. USA* **95,** 4204–4208.