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19 ABSTRACT (Continue on reverse if necessary and identify by block number) A cDNA probe was prepared from a calf liver library, identified by synthetic polynucleotide probes and cloned into lambda gt 11 vector. Its hybridization with mRNA of calf liver identified a 4 kb mRNA. <i>Molecular biology and biotechnology</i>			
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Molecular cloning of the bovine liver ADPRT cDNA**by Srinivas S. Sastry, Eva Kirsten, Ernest Kun**

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Materials and Methods:

Screening of a bovine liver cDNA library: Based on the amino acid sequence of the calf thymus ADPRT (Buki & Kun, 1988) the following oligodeoxynucleotide probes were chemically synthesized (courtesy of Cetus Corp. Emeryville, CA). The probes had the following DNA sequence.

Protein Sequence

NH2- leu ile lys met ile phe
 asp val glu ser met lys-COOH

mRNA

5'-CU(G/C) AU(U/C) AA(A/G) AUG AU(U/C) UU(U/C)
 GA(U/C) GU(G/C) GA(G/A) UC(U/C) AUG AA(A/G)-3'

AGC

cDNA

3'-GA(C/G) TA(A/G) TT(T/C) TAC TA(A/G) AA(A/G)
 CT(A/G) CA(C/G) CTC(T) AG(A/G) TAC TT(T/C)-5'

TCG

5'-CT(G/C) ATT(T/C) AA(A/G) ATG AT(T/C) TTT(T/C)
 GA(T/C) GT(G/C) GA(G/A) TC(T/C) ATG AA(A/G)-3'

AGC

The probe was designed based on the mammalian codon utilization data base (Chen et al.). The cDNA probe is underlined. Two of the probes contained either one of the degenerate bases at the wobble

position of each codon. A third probe with inosine at the third position of each codon was also synthesized. A mixture of all these three probes was employed to screen the bovine liver cDNA library.

A bovine liver λ gt11 cDNA library was purchased from Clontech (Palo Alto, CA) and the library was screened with oligonucleotide probes as follows. For the first screening the mixture of oligonucleotide probes was labelled with ^{32}P using T4 polynucleotide kinase and γ ^{32}P -ATP (Maniatis et al., 1982). Duplicate nitrocellulose filter lifts containing a total of $\sim 2 \times 10^6$ plaques were prepared by plating the phage library on E. Coli Y1090. The prehybridization and hybridization of the plaques with the labelled probes was performed in 50% formamide + 5x Denhardt's solution + 5x SSC + 0.1% SDS + 100 $\mu\text{g}/\text{ml}$ denatured Salmon Sperm DNA at 37°C for $\sim 48\text{h}$. After hybridization, the filters were rinsed twice in 6xSSC + 0.1%SDS at 42°C for 5 min each, washed twice in 3xSSC + 0.1%SDS for 20 min at 37°C and finally in 0.1xSSC + 0.1%SDS for 30 min at room temperature. In the first screening 55 positive clones were picked. These clones were replated and rescreened separately (5×10^4 plaques screened for each isolate) with end - labelled oligonucleotide probes as described for the first screening. Two strongly positive clones designated pSKK13 and pSKK34 were isolated. Both the clones were plaque - purified as described in Maniatis et al. (1982) and the DNA isolated. The DNA from pSKK13 & 34 were restricted with EcoRI and the size of the insert determined to be ~ 7.2 kb by agarose gel electrophoresis in both clones. The reported full - length cDNA for poly(ADP-ribose) polymerase is in the range of 3.8 - 4.5 kb (Uchida et al., 1987; Cherney et al., 1987; Kurosaki et al., 1987), indicating that the cDNAs isolated in the present work were almost twice as long as the reported cDNAs.



Availability Codes	
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Subcloning of the insert in pSKK13 : The λ gt11 clone pSKK13 was grown on E.coli Y1090 and a large-scale preparation of the DNA was isolated using the "LambdasorbTM" anti phage λ antibody (purchased from Promegabiotech,WI). The insert in pSKK13 DNA was purified away from the λ arms on a 10 - 30% neutral sucrose density gradient. Two successive runs of the gradient were necessary for a complete purification of the insert DNA. The insert DNA was then digested with *Sau3A* and *Sau3A + EcoRI* separately. Several fragments ranging between 2.5 - 0.3 kb were obtained by these restriction digests. In a separate reaction M13 mp18 & 19 were digested with *BamHI* and *BamHI + EcoRI*, purified on neutral sucrose gradients, and the restricted DNAs were dephosphorylated with calf intestinal phosphatase to prevent self-recircularization during ligation. The fragments from the *Sau3A* and *Sau3A + EcoRI* digests of the insert in pSKK13 were ligated to M13 DNAs obtained from digestion with *BamHI* and *BamHI + EcoRI* and the ligated DNAs were used to transform E.coli JM101. White plaques were selected on YT plates containing X-gal + IPTG. Single isolates from these white plaques were purified and used for DNA sequencing.

DNA sequencing: Single - stranded DNAs (Messing, 1983) were prepared from the plaque - purified subclones and the insert DNAs were sequenced employing dideoxy chain termination sequencing technique of Sanger et al. (1977). Twelve clones were randomly picked and DNA sequencing was performed.

Computer analysis : The DNA sequences were translated in to protein sequences, analysed for restriction endonuclease sites. Alignment of the sequences with the published DNA sequence of poly(ADP-ribose)

polymerase deposited in GenBank™ (Uchida et al., 1987) was performed by the programs of Martinez (1988). Results of this analysis revealed that out of the 12 clones sequenced only one clone (pSKK13 -12) showed an 85% matching region between positions 1207 - 1325. 5' - GGTACAAGGTTGAAGTGAAGGCCATGATTGAGAAACA CGGGGGGATGTTGACGGGGACGGAGGCCAACAAGGCTTCCCTGTGCATCA TCACCAAAAAGGAGGTGGAAAAGATAATAAGAA - 3'. The remainder of the DNA that has been sequenced had a range of 20% - 60% homology with the published sequence (Uchida et al., 1987). These results imply that 1) the insert DNA in pSKK13 contained cDNA sequences that correspond to the published DNA sequence and therefore some parts (in pSKK13 -12) of the insert contain the correct sequences. 2) The DNA sequences that do not correspond to the published sequences may have originated from aberrant splicing of the pre-mRNA for poly(ADP-ribose) polymerase and/or other cDNAs not related to poly(ADP-ribose) polymerase cDNAs that were ligated to parts of the correct sequences of the poly(ADP-ribose) polymerase cDNA during the preparation of the λ gt11 cDNA library. The possibility that the cDNA insert in pSKK13 may represent a Bovine variant of human cDNA poly(ADP-ribose) polymerase (Uchida et al., 1987) can be excluded because both amino acid sequencing (Buki & Kun, 1988) and nucleotide sequencing (Taniguchi et al., 1988) showed that the bovine thymus and human poly(ADP-ribose) polymerase sequences are highly conserved.

Northern blot analysis of mRNA from bovine liver: About 10 - 15 μ g of polyA⁺ RNA from bovine liver (purchased from CloneTech Laboratories, Palo Alto, CA) was loaded on a 0.8% formaldehyde agarose gel (11cm x 5cm) which was prepared and run in MOPS buffer as described by

Ausubel et al. (1987). The gel was run at a constant voltage of 125V for ~ 2hrs until the bromophenol blue dye was about 2cm from the bottom of the gel. RNA ladder from BRL (MD) served as markers. After electrophoresis the markers were visualized by ethidium bromide staining. The RNA from the gel was transferred by capillary action on to HybondTM nylon membranes as described by the manufacturer (Amersham). The purified insert from pSKK13 was labelled with ³²P by nick-translation (2×10^8 per ml) and hybridized to the RNA on the nylon membranes at 42°C for 48hrs in 50% formamide + 5x denhardt's solution + 5x SSC + 5µg tRNA per ml as carrier. After hybridization, the membrane was rinsed twice in 2x SSC + 0.1% SDS at room temperature. The membrane was then washed four times in 2x SSC + 0.1% SDS for 5 min each at room temperature and then finally washed twice in 0.1% SSC + 0.1% SDS for 15 min each at room temperature. The membrane was then exposed to X-ray film overnight with a screen.

As an internal control, a nick -translated β-actin probe (a gift of Dr. Marc Kirschner's lab at UCSF) was also used to locate the β-actin-specific mRNA (Cleveland et al., 1980).

The results of RNA blot analysis shown in figure 1 demonstrate that a single band of ~ 4.5 kb representing the mRNA for poly(ADP-ribose) polymerase was visualized by autoradiography. The size of the mRNA for bovine liver is in agreement with the size of the mRNA reported for human as well as bovine reported by other workers (Kurosaki et al., 1987; Taniguchi et al., 1988). As expected from the results of Cleveland et al., (1980), the β-actin probe hybridized to a single species of mRNA of approximately 2kb from bovine liver.

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pSKK13 (5' → 3') sequences

TCTAGAGATCCTAAAGCCAGAACCACTGGGCAGCGATTTGCTTCACTTGT
TCTGGTAGCGTAGCAGGTCTGCGCGAAGTGCAGTAGTCGTCACACACGCT
GAGTATCCGGATCGATCATGAACGAAGCTTCGTATTAGCTACAAACCGCG
SEQI

GATTCGTTCACTTCGTTCTGGTACGGTTAGCCAGGTCGCCGGGAAAGTT
GCAGTACTGGTCGAACACCGTTGATGATCCGGAT
SEQII

TTCGTTCTGGTCACGGTTAGCCAGGCTGCCGGGAAAGTTGCCAGTAACTG
GCTGAACACACCGTTGATGATCCCCGGGTACCGAGCTCGAATTCGTAAT
SEQIII

TTAGCCAGGCTCGCCGGGAAAGTTGCCAGTAACTGGCTGAACACCACCGTT
GATGATCCCGTCACGAGCTCGAATTCGTAATCATGGTCATACGTCITTTCT
GTTG
SEQIV

GTTGAGGCGAGTTTGTATGTGTCACCCGAGCTGAAATATCCGGACCTTTT
GTATCTACAACGGCATCTTATAGACTACAATAC
SEQV

AGAAATCCCGGTCCCTTTTTGATCTATCAACAGGAGTCATTATGACAAAA
TACACGAAAAATACTCAACTTCGGCAGAGGTAAGTTCGGCAAAGACG
SEQVI

CCCTTTTTATCTATCAACAGGAGTCATTATGACAAATACAGCAAAAATACT
CAACTTGGCAGAGGTAAGTTCGCCGAAAGGACCAGCGCATGTTGCGCAGAC
CCGATATCCACAGAGTCTGAAGAATT
SEQVII

GGGTACGAGTTGAAATTTGG
SEQVIII

GGAATCGTGACAGGTGGCCTTCAGGTTCAATTAGTATAAGCCCCAACTCCA
AAAT TCCCCCGCCCCCTGGTCTTGGTTGTCTC
SEQIX

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ACACGGCAACGCGTCCTAGCCTGTC
SEQX

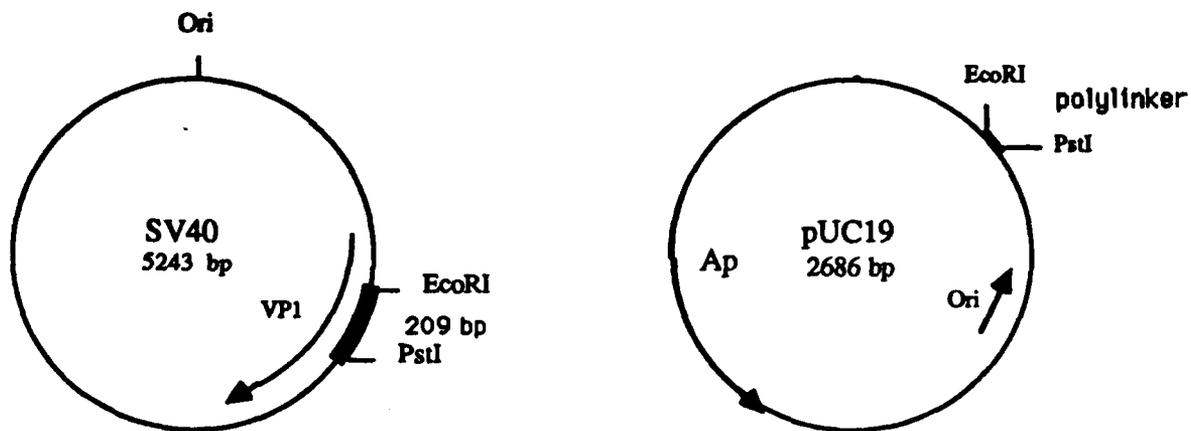
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SEQXI

5'- GGTACAAGGTTGAAGTGAAGGCCATGATTGAGAAACACGGGGGGATGTT

GACGGGGACGGAGGCCAACAAGGCTTCCCTGTGCATCATCACCAAAAAGG

AGGTGGAAGATAATAAGAA - 3'
SEQXII

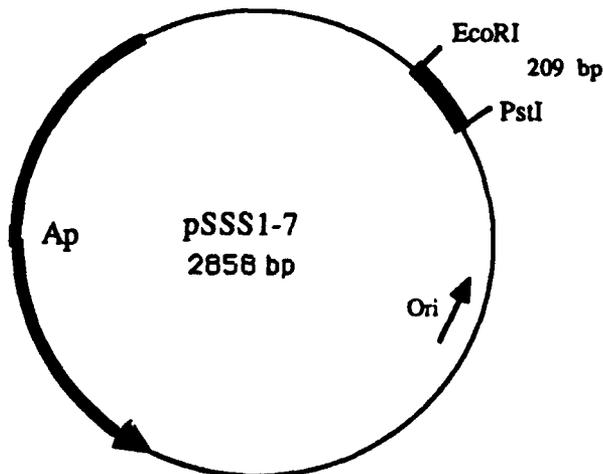
Cloning of the 209 bp EcoRI-PstI fragment of SV40 into pUC19



Cut SV40 DNA with EcoRI + PstI and isolate the 209 bp fragment from a 5% polyacrylamide gel run in 1xTBE buffer & EtOH precipitate the DNA. Total yield of the 209 bp fragment was ~ 0.5 micrograms.

Cut pUC19 (1) with EcoRI + PstI and run the products run on a 1% agarose gel. The large fragment was isolated from the gel and EtOH precipitated. The DNA was dephosphorylated using calf intestinal phosphatase (2), phenol extracted and EtOH precipitated. The total yield of the DNA was ~ 0.5 micrograms.

The 209 bp fragment from SV40 was mixed with pUC19 DNA in equal amounts and ligated overnight at 12°C using T4 DNA ligase. The ligation mixture was used to transform *E. coli* JM109 (1). Transformants were selected on LB plates containing X-gal + IPTG + ampicillin (1). Only white colonies were selected and plasmid DNA isolated by the small scale procedure of Maniatis et al. (2). DNA was restricted with EcoRI + PstI and the products visualized on a 12% acrylamide gel and compared with an SV40 EcoRI + PstI digest.



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β -Actin →



← ADPRT

