Proc. Natl. Acad. Sci. USA
Vol. 86, pp. 9747–9751, December 1989
Biochemistry

Isolation of a cDNA clone of the 14-kDa subunit of the signal recognition particle by cross-hybridization of differently primed polymerase chain reactions

(identification of cDNAs/oligonucleotide primers/λ phage gt10)

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Communicated by William J. Rutter, September 20, 1989

ABSTRACT **Using an enhancement of the polymerase chain reaction (PCR) technique, we have isolated a complementary DNA encoding SRP14 (14-kDa subunit), one of six proteins contained in the signal recognition particle (SRP). Several pools of degenerate oligonucleotides encoding different peptide sequences of SRP14 were used to generate amplified DNA by the PCR. A cross-hybridization procedure was developed to identify the authentic SRP14 cDNA clone among the amplified DNA products obtained by PCR. The basis of this approach is the assumption that a partial cDNA of SRP14 should be the only DNA product common to two amplification reactions primed with different degenerate oligonucleotide mixtures. The partial canine cDNA of SRP14 identified by this procedure served as a probe to isolate a complete cDNA clone of SRP14 from a mouse embryonic cDNA library in λ phage gt10.**

Because of the degeneracy of the genetic code, it is not possible to design a DNA probe from peptide sequence information that matches the sequence of the putative cDNA. Therefore, when screening a genomic or a cDNA library, the ability to discriminate between the authentic and the nonspecific signals remains a critical step in isolating cDNA clones. The recently described polymerase chain reaction (PCR) (1, 2) has proven very valuable in providing perfectly matched hybridization probes (3). Mixtures of oligonucleotides, representing all possible nucleotide sequence combinations encoding known peptide sequences, can be used to generate partial cDNA clones that, in turn, are excellent tools for screening at high stringency (3). However, the degenerate oligonucleotide primers used in PCR usually produce a complex pattern of amplification products. The authentic cDNA fragment that encodes the desired protein in the pool of all PCR products has so far been identified by using either an internal oligonucleotide (located between the two primers) as a hybridization probe (3) or size selection (4, 5). Both approaches are limited because either the amino acid sequence or the distance between the peptide sequences encoded by the mixed oligonucleotide primers must be known.

We report here the identification of an authentic cDNA clone by an enhancement of the basic PCR technique. In essence, several sets of oligonucleotide mixtures, derived from different peptide sequences of the protein under study, were used to generate amplified DNA fragments by PCR using cDNA as a template. The authentic cDNA in the pool of PCR products was identified by cross-hybridizing the products of one amplification reaction obtained with one set of primers to the products of another amplification reaction obtained by another set of primers. This approach has the advantage that it does not rely on any additional information other than two relatively short peptide sequences of the protein. This approach was used successfully for the isolation of a partial canine cDNA clone of a 14-kDa protein, SRP14, one of six polypeptides contained in the signal recognition particle (SRP) (refs. 6 and 7 and references therein). The partial canine cDNA clone of SRP14 was used as a hybridization probe to isolate a complete SRP14 clone from a mouse embryonic cDNA library.†

MATERIALS AND METHODS

Sequencing of SRP14. SRP was purified to homogeneity from canine pancreas (8) and two approaches were then used to obtain amino acid sequence information of the SRP14 protein. In one approach, the six SRP proteins were separated by preparative SDS/PAGE. SRP14 was eluted from the gel (9) and sequenced from the N terminus by sequential Edman degradation in a gas-phase sequenator. In the other approach, the six SRP proteins were released from the RNA and separated by hydroxylapatite chromatography followed by further fractionation and concomitant concentration by CM-Sepharose chromatography (10). In this procedure, another SRP protein, SRP9, cofractionates with SRP14. The salt concentration of the protein fraction containing 300–500 pmol of SRP9 and SRP14 was adjusted to 100 mM ammonium chloride (pH 7.8) and 10% acetonitrile and was incubated with 0.25 mg of endoproteinase Glu-C (Boehringer Mannheim) at 37°C overnight. Under these conditions, this enzyme specifically hydrolyzes the peptide bond following glutamic acid residues. After the digest, the peptides were separated on a C8 RP 300 reverse-phase column (Brownlee Lab) using an acetonitrile/H_2O gradient. The peptides were collected and sequenced as described above. This second approach seemed to be most suitable because all attempts to isolate proteolytic peptides of SRP14 after *in situ* digestion of the protein bound to nitrocellulose filters (11) failed because the peptides were not released from the filter (data not shown). Furthermore, this approach was possible because we had already determined the sequence of SRP9 (17) and, hence, resulting peptides could be identified easily.

Amplification of cDNAs. As a template for the amplification reaction, cDNA was prepared from 1 μg of poly(A) RNA from MDCK (Madin–Darby canine kidney) cells using an oligo(dT) adaptor primer as described (12) and diluted to 1 ml. A 20-μl PCR mixture contained 55–65 pmol of each primer set, 5 μl of target cDNA, 0.2 mM each dNTP, 10 mM Tris·HCl (pH 8.3), 50 mM KCl, 2 mM $MgCl_2$, 0.01% gelatin, and 0.6 unit of *Thermus aquaticus* DNA polymerase (*Taq* polymer-

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Abbreviations: PCR, polymerase chain reaction; SRP, signal recognition particle.

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†The sequences reported in this paper have been deposited in the GenBank data base (mouse SRP14, accession no. M29264; partial canine SRP14, accession no. M29265).

ase) (Perkin–Elmer/Cetus). Amplification of the target DNA was performed as follows: denaturation, 94°C for 3 min in the first cycle and 1 min in all subsequent cycles; annealing, 2 min at the temperature indicated in the results; extension, 5 min at 72°C in the first cycle, 3 min in the subsequent cycles, and 12 min in the last cycle. A double-headed arrow (↔) between the primers designates a primer pair used in PCR. A2/A2′ indicates that both primer mixtures have been used in the same PCR reaction.

Cross-Hybridization Procedure. The DNA in 7 μl of a 20-μl amplification reaction mixture was separated on a 2% agarose gel, stained with ethidium bromide, and transferred to N-hybond nylon filters (Amersham) according to the manufacturer's protocol. The PCR products used as hybridization probes were precipitated twice in 2 M ammonium acetate with 2.5 vol of ethanol to remove the unincorporated deoxynucleotides. The labeling reaction was done in 66 mM Tris·HCl, pH 7.5/6.6 mM $MgCl_2$/10 mM dithiothreitol/55–65 pmol of each of the primers used in the amplification reaction/0.2 mM each dATP, dGTP, TTP/50 μCi of [^{32}P]dCTP (3000 Ci/mmol; 1 Ci = 37 GBq; Amersham) using the amplified DNA contained in one-third of a total PCR mixture. The reaction mixture was heated to 95°C for 3 min, quickly cooled on ice for 3 min, 3 units of Klenow fragment of DNA polymerase I was added, and incubation was continued for 45 min at room temperature. The [^{32}P]dCTP-labeled PCR products with an approximate specific activity of 2 μCi/pmol were heated to 94°C for 3 min and then added to 10 ml of hybridization solution containing 50% formamide, 5× NET (1× NET = 50 mM Tris·HCl, pH 8/150 mM NaCl/1 mM EDTA), 0.2% SDS, 0.01% $NaPP_i$, and 100 μg of salmon sperm DNA per ml. Hybridization to the PCR products on the N-hybond nylon filters was done at 42°C for 16 hr. The filters were washed three times in 0.5× NET/0.2% SDS at 42°C for 20 min.

Cloning and Sequencing the Partial cDNA Clone of Canine SRP14. Unless stated otherwise, all procedures followed standard recombinant DNA techniques (13). The PCR products obtained with the S2 ↔ A2 and S2 ↔ A3 primer combinations were precipitated in 2 M ammonium acetate with 2.5 vol of ethanol, and the DNA was subsequently incubated with a 10-fold excess of the restriction enzymes *Xba* I and *Sal* I (New England Biolabs). The DNA fragments were ligated (ligase was from New England Biolabs) at a 10-fold molar excess into the linearized plasmid vector SP64. Typically, this represented 1/10th of the total DNA obtained in a 20-μl amplification reaction mixture. Transformation of bacteria from the *Escherichia coli* strain DH5α with the ligation reactions resulted in ≈300 ampicillin-resistant colonies. A control ligation and transformation with the linearized plasmid vector alone gave only 10 colonies. The colonies were screened by using the labeled products of a S1 ↔ A1 primed amplification hybridization probe. Only 10% of the colonies hybridized with the probe. Plasmid DNA was prepared from five hybridizing colonies and their inserts were sequenced (14) using the enzyme Sequenase (United States Biochemical).

Isolation of cDNAs Encoding Mouse SRP14. A λ phage gt10 mouse embryonic cDNA library was screened using the partial canine cDNA as a hybridization probe. The mouse embryonic cDNA library was prepared by Craig Hauser (Department of Biochemistry, University of California, Berkeley) and was kindly given to us by Michael Frohman (Department of Anatomy, University of California, San Francisco). The hybridization was carried out for 16 hr at 42°C in 50% formamide/5× NET/0.2% SDS/0.01% $NaPP_i$/100 μg of salmon sperm DNA per ml. The filters were washed three times in 1× NET/0.2% SDS at 42°C for 20 min followed by one wash in 0.2× NET. DNA from the λ phages was prepared as described (13) and the cDNA inserts were cloned into the phage M13mp18. The cDNA inserts were sequenced as described above.

RESULTS

The peptide sequence information obtained from canine SRP14 is shown in Fig. 1. Starting from the second amino acid at the N terminus, 17 amino acid residues were identified. No amino acid derivative was observed at position 17 in the internal peptide and we therefore assumed that it was a cysteine for the design of the primers. Two different amino acid residues were equally good candidates at positions 10 and 15 of the internal peptide sequence. The nucleotide sequences of the primers selected for use are outlined in Table 1, and their alignment with the peptide sequences is depicted in Fig. 1. Each primer consists of a mixture of oligonucleotides representing almost all or all possible codons encoding the corresponding amino acid sequence. To limit the number of possible sequence combinations for the primers S2, A2, A2′, A3, and A3′, leucines and arginines are represented by four codons only [CT(N) and CG(N), respectively]. Thus, in five of seven oligonucleotide mixtures the possibility remained that the perfectly matched oligonucleotide was not present. In our nomenclature, "S" designates an oligonucleotide in the sense orientation, whereas "A" designates the antisense orientation. The oligonucleotide mixtures A2 and A2′ were identical, except that at position 15 in the internal peptide sequence A2 encoded an asparagine and A2′ encoded a phenylalanine (Fig. 1 and Table 1). The same was true for the oligonucleotide mixtures A3 and A3′. All primers were synthesized with restriction enzyme recognition sequences at their 5′ ends to facilitate the cloning of the amplified DNA fragments into a plasmid vector.

In two separate sets of experiments, each of the sense oligonucleotide mixtures (S1 and S2) was combined with each of the antisense oligonucleotide mixtures (A1, A2, A2′, A3, and A3′) to prime the amplification reactions. The template for the amplification reaction was a cDNA population generated from poly(A) RNA from MDCK cells. The temperature in the annealing step of the amplification reaction was

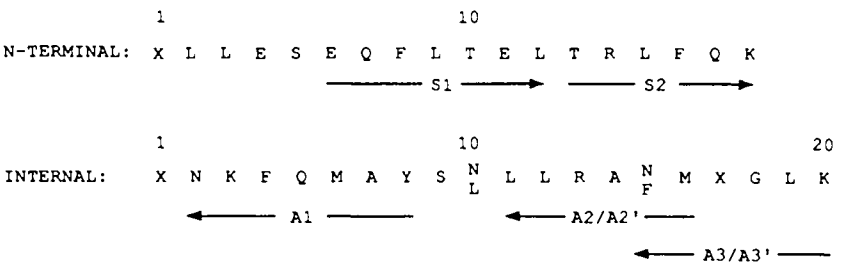

FIG. 1. Protein sequence data of SRP14. Amino acids are designated by the single-letter code. Arrows outline the peptide sequences encoded by the oligonucleotide mixtures. Direction of arrow indicates that the corresponding oligonucleotide represents the sense (right) or antisense (left) strand.

Table 1. Sequences and complexities of the oligonucleotide mixtures used in PCR

Oligonucleotide	Sequence	Complexity of the primer
S1	CTCTA·GAR CAR TTY YTN ACN GAR YT	1024
S2	CTCTAG·ACN CGN CTN TTY CAR AA	256
A1	CAAGCTT·RTA NGC CAT YTG RAA YTT RTT	128
A2	GGTCGA·CAT RTT NGC NCG NAG NAG	512
A2′	GGTCGA·CAT RAA NGC NCG NAG NAG	512
A3	GGTCGAC·YTT NAR NCC RCA CAT RTT	256
A3′	GGTCGAC·YTT NAR NCC RCA CAT RAA	256

Nucleotide sequences encoding SRP14 peptides are separated by a dot from the sequences that contribute to or represent recognition sites for restriction enzymes flanked by one additional nucleotide. Notably, the cutting efficiency of the enzymes *Xba* I and *Sal* I is reasonably high in the presence of only one flanking nucleotide, whereas the cutting efficiency of *Hin*dIII is very low under the same conditions (unpublished observations).

45°C (Fig. 2 *A* and *B*). The same experiment was repeated using 47°C in the annealing step (Fig. 2 *D* and *E*). It is clear from the data shown in Fig. 3 that all combinations of the mixed oligonucleotide primers produced a complex pattern of DNA fragments. Because the expected size of the authentic cDNA of SRP14 was not known, the PCR product encoding SRP14 could not be identified. An additional experimental step was therefore introduced to identify the authentic SRP14 cDNA. This experimental procedure is schematically represented in Fig. 3. It is based on the assumption that the only DNA fragment common to two distinct amplification reactions, primed with oligonucleotide mixtures encoding different portions of the peptide sequences, will be the fragment encoding a portion of SRP14. Thus, the hybridization of one pool of radioactively labeled PCR products, generated by one set of SRP14-specific oligonucleotides, to a Southern blot of another pool of PCR products, obtained using a different set of SRP14-specific primers, should result in the identification of the partial SRP14 cDNA. This procedure takes advantage of the information content of the sequences located between the primers used for PCR, and the cross-hybridization can therefore be done at high stringency.

Two different PCR reactions were used to hybridize to two different sets of amplification reactions. First, the ^{32}P-labeled S2 ↔ A1 primed PCR products were used as a probe to hybridize to a set of amplification reactions, in which the sense primer S1 was combined with all possible antisense primers (Fig. 2*A*). Second, the ^{32}P-labeled pool of S1 ↔ A1 primed PCR products was used to hybridize to the set of amplification reactions generated with the sense primer S2 and all antisense primers (Fig. 2*E*). Both hybridization probes were generated in reactions in which the primers were annealed at 45°C. The autoradiographs of the Southern blot analysis are shown in Fig. 2 *C* and *F*. Although the DNA content of both hybridization probes and of all PCR reactions analyzed was complex, only a few cross-hybridization signals were observed. Moreover, the ratio between the ethidium bromide-stained signal and the hybridization signal is dramatically different between the authentic SRP14 cDNA (see below) (denoted by arrows in Fig. 2 *C* and *F*) and nonspecific

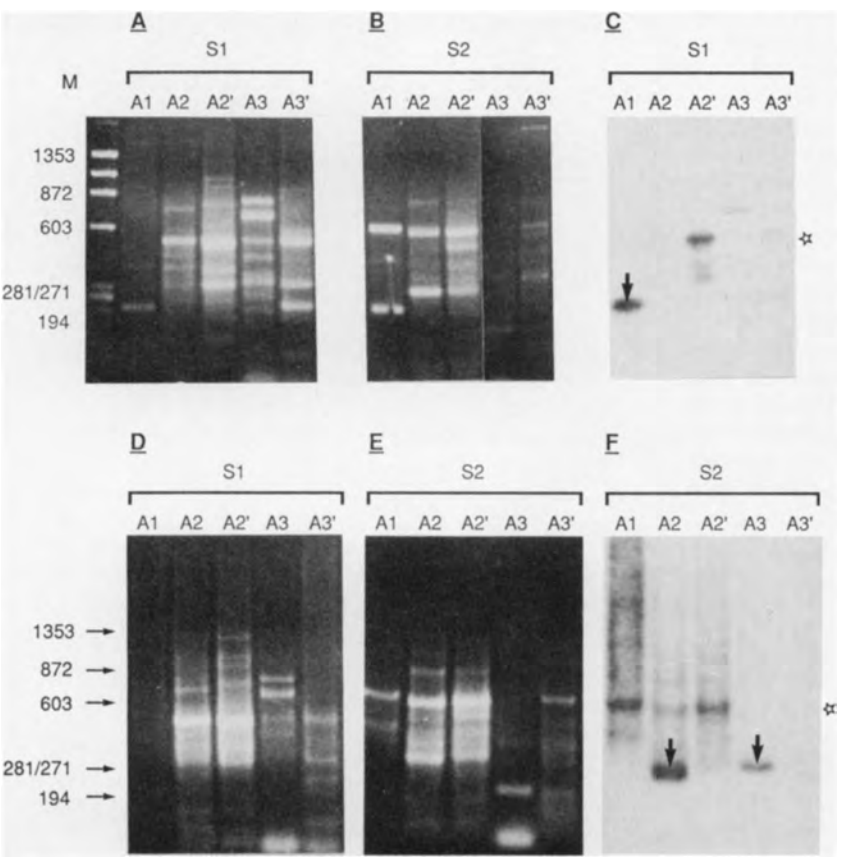

FIG. 2. Identification of the partial canine SRP14 cDNA using the cross-hybridization procedure. One sense primer (indicated on top of each panel) was combined with different antisense primers (indicated on top of each lane) to produce amplified DNA by PCR from a primary cDNA population derived from MDCK cells. The amplification products were displayed on 2% ethidium bromide-stained agarose gels (*A*, *B*, *D*, and *E*). The primer was annealed at 45°C in the amplification reactions shown in *A* and *B* and at 47°C in the amplification reactions displayed in *D* and *E*. (*C*) Autoradiograph of a Southern blot of gel *A* probed with ^{32}P-labeled S2 ↔ A1 (*B*, lane A1) primed PCR products. (*F*) Autoradiograph of a Southern blot of gel *E* probed with ^{32}P-labeled S1 ↔ A1 (*A*, lane A1) primed PCR products. Arrows indicate authentic SRP14 cDNA and stars denote nonspecific hybridization products. Lane M, DNA size markers.

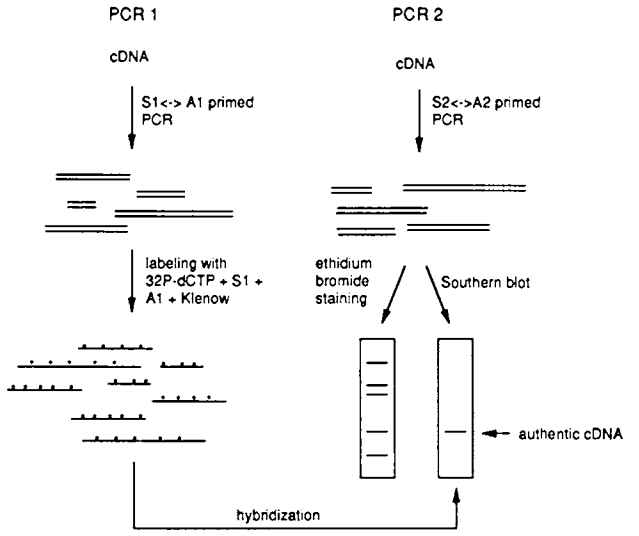

FIG. 3. Schematic representation of the cross-hybridization procedure to identify authentic cDNA clones. S1, S2, A1, and A2 designate oligonucleotide mixtures encoding different portions of the protein of interest. Asterisks denote ^{32}P-labeled products.

hybridization, observed exclusively in regions with high DNA content (stars in Fig. 2 *C* and *F*). Notably, only the primer combination S1 ↔ A1 produced the authentic SRP14 cDNA, while all the other primer combinations using S1 as a sense primer failed to do so (Fig. 2*C*). This result was later confirmed by probing the same Southern blot with the isolated SRP14 cDNA (data not shown). We also observed that the S2 ↔ A1 primer combination failed to produce the SRP14 specific DNA fragment when the amplification reaction was performed using the higher annealing temperature. As expected, the size of the three putative SRP14 cDNA fragments increased when the antisense primer that was used was located further downstream in the internal sequence (Fig. 2 *C* and *F*).

The S2 ↔ A2 and the S2 ↔ A3 primed PCR products were cloned into the plasmid vector SP64 and the putative SRP14 clones were identified by colony hybridization using an S1 ↔ A1 primed PCR pool as a probe. A positive signal was obtained from 10% of the colonies. Five positive clones were sequenced and found to be identical except for one silent base change from a G to an A at position 138 (Fig. 4*A*). Most importantly, the 13 amino acids preceding the sequence encoded by the A3 primer in the predicted protein sequence of SRP14 (Fig. 4*A*) were identical to the internal peptide obtained from SRP14 (Fig. 1). We therefore concluded that we had isolated a partial canine SRP14 cDNA clone. Furthermore, the observation that all five clones identified by colony hybridization contained the SRP14 cDNA supports the previous interpretation that the hybridization signals denoted with asterisks in the Southern blot resulted from nonspecific hybridization of the probe to regions with high DNA content.

The SRP14 cDNA fragment generated by PCR was subsequently used as a probe to screen a λ phage gt10 mouse embryonic cDNA library. Five positive λ phages were isolated and found to have the same-sized cDNA insert. One of the inserts was subcloned and sequenced. The insert is 706 nucleotides long and contains a poly(A) tail (Fig. 5). A 7-nucleotide-long sequence is followed by an open reading frame of 110 amino acids that contains all the peptide sequences obtained previously from SRP14. Hence, we have identified a full-length mouse SRP14 cDNA clone.

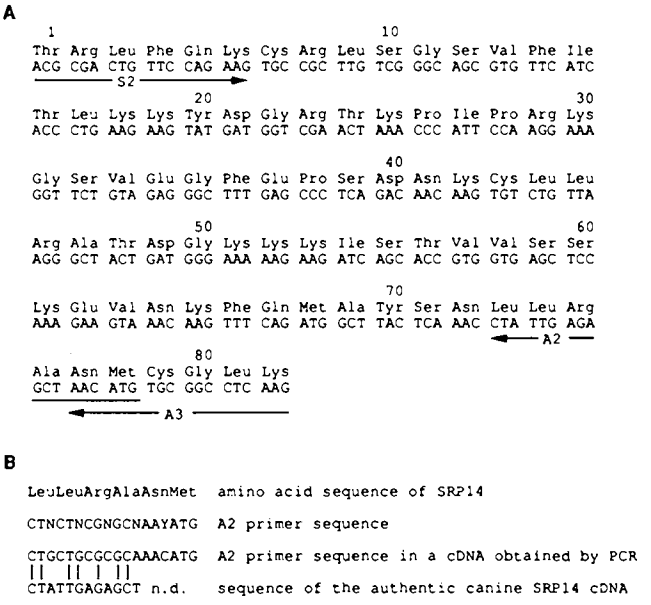

FIG. 4. Characterization of partial canine SRP14 cDNA clones isolated from PCR products. (*A*) The sequence of a partial cDNA clone isolated from the pool of S2 ↔ A3 primed PCR products. Arrows delineate the locations of the primers used in PCR. (*B*) The first and second lines show the peptide sequence of SRP14 as determined by protein sequence analysis and the oligonucleotide sequence of the mixed oligonucleotide primer A2 derived from it, respectively. In the third and fourth lines, the sequence of the A2 primer that was recruited into the SRP14 cDNA clone isolated from S2 ↔ A2 primed PCR products is compared to the sequence of the authentic SRP14 cDNA as derived from an SRP14 cDNA clone isolated from S2 ↔ A3 primed PCR products.

DISCUSSION

We have previously isolated a SRP9 cDNA clone (17) by using two unique oligonucleotides as hybridization probes to

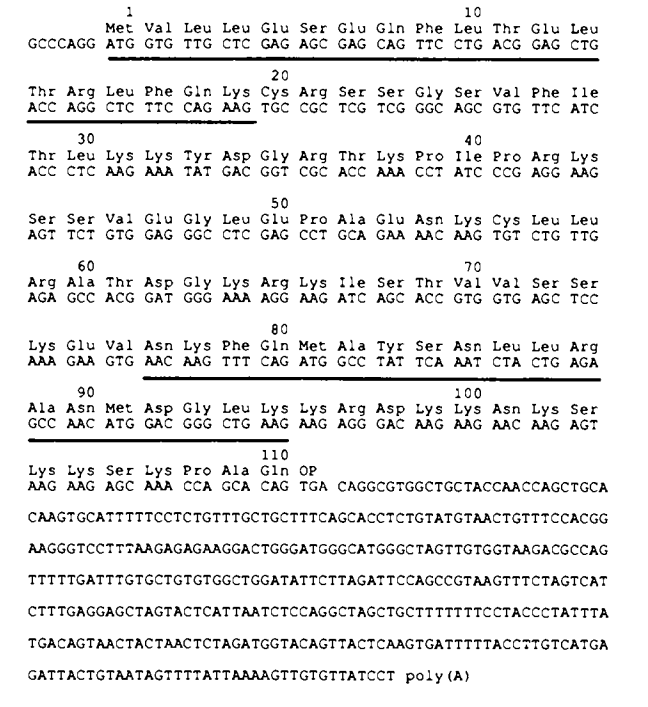

FIG. 5. Sequences of the mouse SRP14 protein and cDNA. The amino acid sequence determined by protein sequence analysis is underlined. The predicted molecular mass of the SRP14 protein is 12,511 Da.

screen a λgt10 library. With this same approach and by using pools of short oligonucleotides representing all possible codon choices in combination with base composition-independent hybridization procedures (15), we were unsuccessful in isolating a cDNA clone of SRP14. Several cDNA clones with significant homology to the hybridization probes used were obtained, but none was an authentic SRP14 cDNA clone.

An approach was developed in which cross-hybridization between PCR products, primed with mixed oligonucleotides derived from different SRP14 peptide sequences, was used successfully to identify the partial SRP14 cDNA in the pool of PCR products. This approach takes advantage of the information content of the cDNA sequence located between the two primer sets used in PCR. Thus, it requires no other information than the amino acid sequences of two peptides from the protein under study. The peptide sequences should have a length of 10 or more amino acids. In principle, this approach can be used to isolate a partial cDNA clone of any protein. However, in the case of a very large protein, the primers may be located far apart and the generation of full-length PCR products might therefore become more difficult. In the SRP14 cDNA, the PCR primers turned out to be located ≈200 nucleotides apart. Once a partial cDNA clone is obtained with this approach, there are at least two ways for isolating a full-length cDNA. First, the partial cDNA can be used as a probe to screen a cDNA library. Second, a recently developed protocol, the rapid amplification of cDNA ends (RACE) (12), can be used to generate full-length cDNA by PCR. The minimum information required in this protocol is a short stretch of sequence within the cDNA from which the region to the 5′ or 3′ end is amplified by PCR. The latter approach is particularly advantageous when no cDNA library is available.

In retrospect, we found that the A2 primer pool lacked the perfectly matched oligonucleotide. In fact, the specific A2 primer recruited from the oligonucleotide pool by the S2 extension product on the cDNA template to produce an authentic SRP14 cDNA contained five mismatches when compared to the authentic SRP14 cDNA sequence (Fig. 4*B*). This suggests that, under the conditions used, numerous mismatches between the primer and the authentic cDNA sequence can be tolerated. It was previously observed that at low stringency the binding of the perfectly matched primer is favored relative to a primer differing by a single base (16). However, this competition was found using 12- to 16-nucleotide oligonucleotides, whereas in our experiments the primers ranged in size from 17 to 20 bases. By using unique primers and very stringent conditions in the annealing step of the amplification reaction, the formation of nonspecific amplification products would be prevented and a single major DNA species would be obtained (2). We observed, using one set of mixed oligonucleotides, A1 ↔ S2, that increased temperature in the annealing reaction resulted in the loss of the desired fragment without otherwise reducing the complexity of the PCR products. We also noticed that not all primer combinations generated high yields of the SRP14 cDNA. Only one of the primer combinations using S1 as a sense primer produced detectable amounts of SRP14 cDNA. The failure of all other S1 combinations to generate SRP14 cDNA is possibly due to the fact that A2 and A3 primers are shorter and have a more diverse composition than A1.

We conclude that a perfect match between primer and cDNA was not required to generate amplified SRP14 DNA. Without relying exclusively on the fidelity of the initial primer template interactions, the cross-hybridization procedure allows the identification of the desired DNA product within the complex mixture of the PCR products.

The primary protein sequence of SRP14 as derived from the cDNA sequence shows no apparent similarity to any protein sequence in the data bank. However, it indicated that SRP14 is very polar with an overall basic character (pI 11.06). Nine of the 26 basic amino acid residues are clustered in the 16 C-terminal amino acids. Of the 70 amino acid residues identified from canine SRP14, only 6 differ from the amino acid sequence determined for the mouse SRP14. In 5 of 6 cases, amino acid residues with similar chemical properties replace each other, indicating the extreme evolutionary conservation among these cellular household proteins.

We thank Jim Kealey for his special effort in performing the protein sequencing, Michael Frohman and Sascha Kamb for their advice in PCR, Mila D. Morrical for her technical assistance, and Gail Martin for providing access to the DNA thermal cycler. We also thank Jodi Nunnari and Mark Poritz for critical reading of the manuscript. This work was supported by fellowships from the European Molecular Biology Organization and from the Juvenile Diabetes Foundation International to K.S. and by a grant from the National Institutes of Health to P.W.

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