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cDNA Cloning by Amplification of Circularized First Strand cDNAs Reveals Non-IRE-Regulated Iron-Responsive mRNAs

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Currently, the rapid amplification of cDNA ends (RACE) is the most common method for PCR cloning of cDNA. Because RACE uses a gene specific primer and one adaptor primer that is shared by all cDNAs may result in numerous nonspecific products that can hinder the cloning process. Here we report a new method that uses circularized first strand cDNA from mRNA and two gene specific primers to amplify both the 5′ and 3′ cDNA ends in one reaction. A cDNA band of correct size can be obtained on the first pass in this approach. If the correct size is not obtained on the first pass, amplification of cDNA ends can be repeated until the correct size of the cDNA is obtained. We tested this new method on eight mRNAs that we have previously shown to respond to cellular iron levels. We obtained sequences for six mRNAs that were 43 bp to 1324 bp longer than that reported in GenBank and obtained the same length sequence for the other two mRNAs. RNA folding program shows no iron responsive elements (IRE) on these mRNA. In conclusion, our cloning approach offers a more efficient method for cloning full-length cDNA and it may be used to replace the existing method of 5′ end cDNA extension. The data enabled us to exclude the possibility that the expression of these iron responsive genes are regulated by IREs.

Key Words: **cDNA cloning; Polymerase chain amplification; iron responsive element.**

Despite the availability of numerous approaches for cloning cDNA, it is still an arduous task. This is especially true when it is necessary to obtain a complete sequence or when attempting to clone a rare sequence. Polymerase chain reaction (PCR) is an attractive approach for cDNA cloning because of its simplicity and its utilization when starting material is limited. Currently a common method in PCR cloning of cDNA is the rapid amplification of cDNA ends (RACE) (1–3). One of the most distinct advantages of RACE is that a specified cDNA end can be cloned in a short time without the need to construct a cDNA library. However, because RACE uses one gene specific primer and one adaptor primer that is shared by all cDNAs, the resulting non-specific products may seriously hinder cloning the cDNA of interest. In this report we describe a novel approach to PCR cloning of cDNA that uses circularized single strand cDNA and two gene specific primers. This approach can specifically amplify the 5′ and 3′ end of cDNA in one reaction.

Our laboratory is interested in maintenance of cellular iron homeostasis. Iron is known to regulate the expression of genes that contain an iron responsive element (IRE) in their mRNA. However, iron binding sites have been reported on genomic DNA (4–6) and proteins functionally related to iron metabolism have been found in cell nuclei (7, 8). This suggests the possibility that iron may directly regulate expression of genes that do not have an IRE. We have identified a number of known genes that were not known to be iron responsive and a number of novel genes that respond to cellular iron status (9, 10). Cloning the full length of these cDNAs with this method was necessary for determining whether or not an IRE was involved in the response of these genes to iron.

MATERIALS AND METHODS

Cell culture and mRNA isolation. Human astrocytoma cells (SW1088 from ATCC) were cultured in 150 mm petri dishes with 5% carbon dioxide at 37°C. The culture medium for the control group was Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum, 4 mM sodium L-glutamine, 100 U/ml penicillin G and 100 U/ml streptomycin and 0.25 μg/ml amphotericin B. Astrocytoma cells were also cultured in control media plus 100 μg/ml of

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ferric ammonium citrate (Sigma, St. Louis, MO) for 48 h (iron loading condition), control media plus 50 μM deferoxamine mesylate (Sigma, St. Louis, MO) for 6 h (iron depletion condition). Total RNA and mRNA were isolated from cultured human astrocytoma cells as before by using total RNA midi kit and mRNA midi kit (Qiagen) (10). To decrease the number of groups used to prepare circularized single strand cDNA, a mixed total RNA from astrocytoma cells cultured under three different iron conditions (control, iron depletion and iron loading) was used in the isolation of mRNA. Commercial human brain mRNA from Clontech was also used for confirming the novel sequences cloned by our method.

Preparation of circularized single strand cDNA. The first strand of cDNA was generated in 50 μl of reaction buffer consisting of 1000 U RNase H^- Reverse Transcriptase (Gibco/Life Science), 2 mg mRNA, 1 μg phosphorylated oligonucleotide $(dT)_{18}$ N_2, 18.8 mM Tris.HCl, pH 6.9, 90.6 mM KCl, 4.6 mM $MgCl_2$, 10 mM $(NH_4)_2SO_4$, 10 mM DTT, 0.3 μM dNTP and 1 U RNasin (Promega). The synthesis of first strand cDNA was performed at 42°C for 1 h. Subsequently, the mRNA templates were digested with a mixture of the RNase H (6 Unit) (Gibco/Life Science) and RNase A (0.5 μg) (Boehringer Mannheim) at 37°C for 40 min. The first strand of cDNA was purified with a QIAquick spin column (Qiagen). The first strand of cDNA (100 μg) in 100 μl Kin-Lig buffer (50 mM Tris.HCl, pH 8.0, 10 mM $MgCl_2$, 1 mM EDTA, 6 mM 2-mercaptoethanol, 1 mM ATP) (11) was ligated to form a circularized strand by adding 30 U T_4 DNA ligase (Roche) and incubated at 8°C for 16 h and at 15°C for 2 h.

PCR amplification. Two gene specific primers were designed from a segment of known sequence obtained in our previous study (9, 10). The 3′ end of the primers was directed toward the 5′ or 3′ end of cDNA. Advantage Polymerase Mixture 2 (Clontech) was used in the PCR and the PCR reaction consisted of following components: 40 mM Tricine-KOH, 15 mM KOAc, 3.5 mM $Mg(OAc)_2$, 3.75 μg/ml BSA, 0.005% Tween-20, 0.005% Nonidet-P40, 0.2 mM dNTP, 0.3 μM of each primer, 1 μl of a 1:10 dilution of circularized single strand cDNA. PCR amplification was performed under the following conditions: 95°C 1 min; 94°C 15 s, 72°C 2 to 4 min, 5 cycles; 94°C 15 s, 70°C 2 to 4 min, 5 cycles; 94°C 15 s, 68°C 2 to 4 min, 25 to 30 cycles. For PCR amplification of linear single strand cDNA, PCR conditions are same as above except the linear single strand cDNA without circularization served as the template.

The general strategy we used to clone cDNA is illustrated in Fig. 1.

RESULTS

Seven iron responsive mRNAs from our previous screenings (9, 10) and the mRNA for the iron regulatory protein (IRP-1) (12) were selected from mRNA of human astrocytoma cells and human brain for full length cloning with our novel method. The mRNAs were chosen because at least a partial sequence for each of them has been published in GenBank so that we could compare the efficiency of our cloning method to published results.

The PCR reaction product from the first amplification was detected on an agarose gel (Fig. 2A) and the specific DNA band was purified and inserted into the plasmid vector for sequencing. We obtained longer sequences at the 5′ and/or 3′ end for three of the test mRNAs (GAPDH, NEMO and Iron-inhibited ABC transporter) than what had been reported in GenBank. One cDNA (TEXREB107) had the same length as reported in GenBank. The sequence of Thy-1 cDNA is longer than the reported sequence in GenBank but still incomplete compared to the size indicated by Northern blot analysis. Three of the cDNAs (IRP-1, Calpain large polypeptide L2 and NADH dehydrogenase 1 beta subcomplex 9) were incompletely cloned because the sequence we obtained was shorter than that reported in GenBank. In addition there is a possibility that the iron inhibited ABC transporter is encoded by two highly homologous mRNAs because two bands were obtained on Northern blots.

In order to obtain the complete sequence for the four mRNAs that were partially cloned and the homologous mRNA of an iron-inhibited ABC transporter, a second touchdown PCR was performed using new primers designed according to the sequence information from first PCR amplification. The PCR products were analyzed as described above (Fig. 2B). The second PCR amplification resulted in longer sequences at both the 5′ end and 3′ end for Thy-1 mRNA than reported in GenBank and the size of cDNA is consistent with the size indicated on the Northern blot. For the iron inhibited ABC transporter, the second PCR amplification resulted in a specifically amplified product that may represent the difference between two cDNAs corresponding to the two bands that are indicated on Northern blots. The second PCR amplification also resulted in a longer sequence at the 5′ end of IRP-1 cDNA. After the second amplification we obtained the same sequence for NADH dehydrogenase 1 beta subcomplex 9 as that reported in GenBank. From the second PCR amplification for Calpain large polypeptide L2 we obtained same sequence at the 5′ end and a longer sequence at the 3′ end (180 bp) than that reported in GenBank.

Because the first and second PCR amplifications used special templates (circularized first strand cDNAs) and a different primer designation (the 3′ end of primers are toward both ends of the cDNA) we used a third PCR to confirm that the cDNAs from first and second PCR runs are specific PCR products (Fig. 3). A third PCR amplification was also necessary because most of cDNAs cloned by our novel method contained new sequence data. One primer chosen against the novel sequence and the other primer from either a novel sequence or known sequence were used to amplify the specified cDNA sequence from the linear first strand cDNA. The PCR reactions on all seven of the cDNAs produced DNA that corresponded to the size that was predicted with the sequence information obtained from first and second PCR reactions (Fig. 3). The DNA from the third PCR reaction was sequenced and the sequence information was the same as that deduced from the first two PCR reactions.

In GenBank a sequence for NEMO mRNA (see Table 1) has been reported, but our technique results in a sequence that is 74 bp longer. The additional 74 bp that we sequenced for NEMO mRNA have been previously reported on the glucose-6-phosphate dehydrogenase

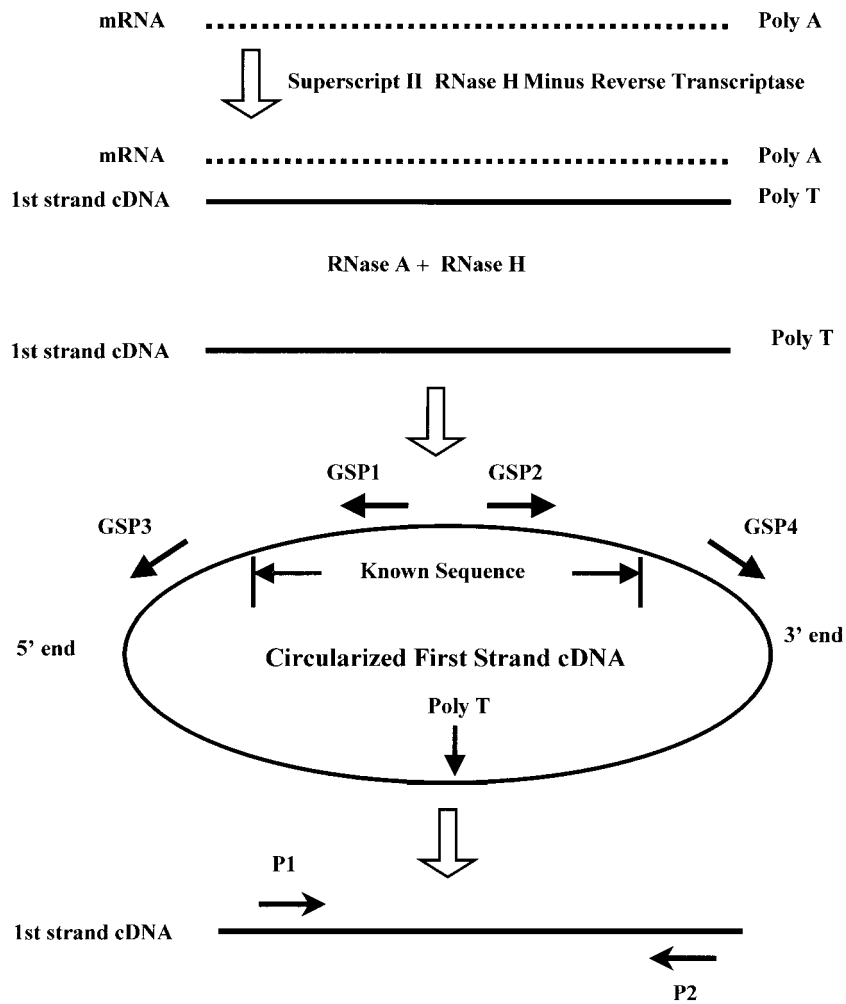

FIG. 1. The principle of cDNA cloning by amplification of circularized first strand cDNA and specific primers. RNA reverse transcriptase without RNase H activity was used to synthesize the first strand cDNA. The mRNA template was degraded by RNases and the remaining first strand cDNA was purified and self-ligated to form circular molecules. Two gene specific primers (GSP 1 and GSP 2) were designed from a segment of known sequence obtained in our previous study (9, 10). Both cDNA ends were amplified by a touchdown PCR reaction by using circularized first strand cDNAs as the template. The specifically amplified DNA was sequenced. To determine if the full length sequence of cDNA ends was obtained, the amplified DNA band was compared to the mRNA size predicted from Northern blot analysis and the sequence was compared to the sequences published in GenBank. If incomplete cDNA sequences were amplified in first PCR, another touchdown PCR could be applied by using circularized first strand cDNAs as templates and another pair of primers (GSP3 and GSP4) that were designed from the sequence information from the first PCR. The novel sequences were confirmed by a third PCR using linear first strand cDNA as a template. One primer of the third PCR was synthesized against the novel sequence (P1) and another PCR primer was from known sequencer novel sequence (P2). The specified amplifications from first and second PCR were confirmed if the size and sequence from a third PCR were consistent with the data from first and second PCR reaction.

gene (G6PDH, Genbank number X55448.1). The G6PDH gene is in close proximity to the locus of NEMO gene on chromosome Xq28. (13) Our PCR and sequencing results prove these 74 bp belong to the first exon of the NEMO gene. The novel cDNA sequences for GAPDH and Thy-1 cloned by our method were also found on their respective genomic DNA (GenBank number J04038.1 and M11749). Thus, we confirmed the accuracy of our cloning method. Our results are compared to the sequences reported in Genbank in Table 1.

In addition to the sequence data, our study revealed two other novel observations. First, our results show that the Thy-1 mRNA may also encode another Thy-1 co-transcripted protein. Because the function of Thy-1 glycoprotein is still unclear but important in regulation of neuritic outgrowth and immune system activity, this new information may provide an important clue for discovering the function of Thy-1. Secondly, for the ABC transporter, two mRNAs were cloned and the sequence information revealed both of them contained the same open reading frame.

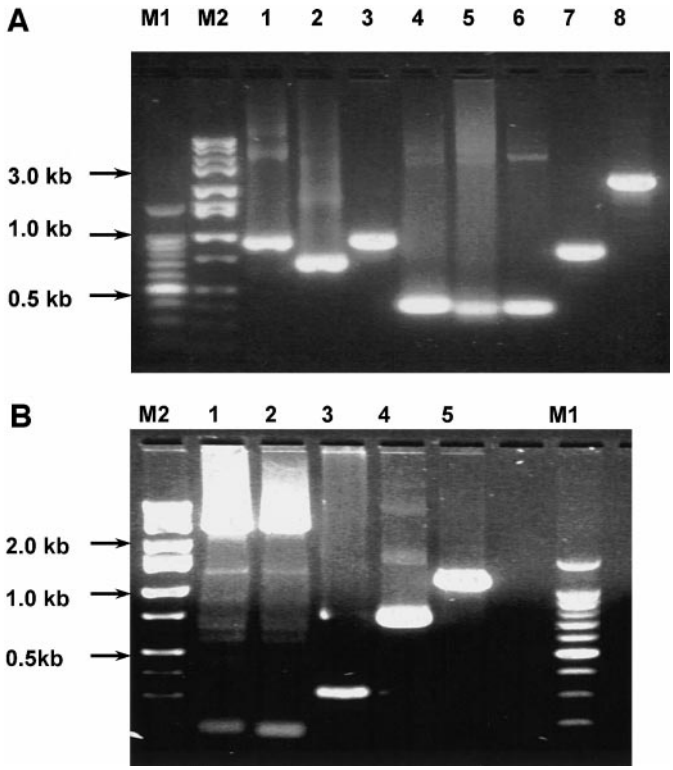

FIG. 2. Amplification of both cDNA 5′ and 3′ ends with specific primers by using circularized first strand cDNA as templates. (A) The first PCR amplifications to determine the size of the selected gene products visualized using ethidium bromide. The products were analyzed on 1% agarose gel. M1 and M2 are DNA molecular weight markers; 1, GAPDH; 2, NADH dehydrogenase 1 beta subcomplex 9; 3, DNA-binding protein, TAXREB107; 4, NEMO protein; 5, IRP-1; 6, calpain large polypeptide L2; 7, Thy-1; 8, iron-inhibited ABC transporter. The calculated sizes for GAPDH, NEMO were longer than that reported in GenBank and the size of the DNA binding protein TAXREB107 was similar to that reported in GenBank. (B) A second PCR amplification using new primers was performed on those genes whose size did not correspond to the size indicated by Northern blot analysis or to the size reported in GenBank. Lane 1, IRP-1; lane 2, calpain, large polypeptide L2; lane 3, NADH dehydrogenase (ubiquinone) 1; lane 4, Thy-1; lane 5, iron-inhibited ABC transporter. Using the second set of primers, we obtained calculated lengths longer than that reported in GenBank for all five of the cDNAs examined. (M2 and M1 are DNA molecular weight markers.)

By using RNA folding program mfold version 3.0 (http://mfold2.wustl.edu/~mfold/rna/form1.cgi), we found that none of the eight cloned iron responsive mRNAs contained an IRE structure. This further supports that iron may influence gene expression through mechanisms other than IRE-IRP interaction.

DISCUSSION

The novel cloning method described in this paper provides not only an alternative to existing methods but represents an improvement in the existing technology. The use of circularized cDNA for cloning is an advantage over existing methods because it minimizes the need to consider upstream and downstream relations in the cDNA template. Thus, two gene specific primers can be used in generating a sequence from unknown cDNA ends. Attempts to circularize double stranded cDNA as PCR templates were not successful because the background was unacceptably high (data not shown). In the development of this technique, we also found that RNA ligase could not be used to form circularized cDNA molecules because the PCR reaction also produced a high background of nonspecific products when circularized single strand cDNAs were ligated by this strategy (data not shown).

Our results also show that this new method provides a powerful alternative to traditional cloning methods for obtaining full length cDNA. Although most of the sequence data for the mRNAs we selected for analysis have been available from a number of entries of GenBank for a relatively long time and have undergone frequent updates, our results showed that their sequences were incomplete. The advantage of cloning full length cDNA with our method is that our approach overcomes two defects that may limit success in full length cloning.

The first problem our technique circumvents is the requirement to synthesize double stranded cDNA following reverse transcription of mRNA to first strand cDNA. It is more difficult to obtain full length double strand cDNA than to obtain full length first strand cDNA. Our novel technique uses only first strand cDNA as the PCR template, so that the longest first strand cDNA could be synthesized by using reverse transcriptase without RNase H activity. The second problem overcome by our approach is that it is difficult to know the exact length of a cDNA insert in a cDNA library until the clone has been separated and it is difficult to know how many clones are needed to get a clone with full length. Our technique provides a mech-

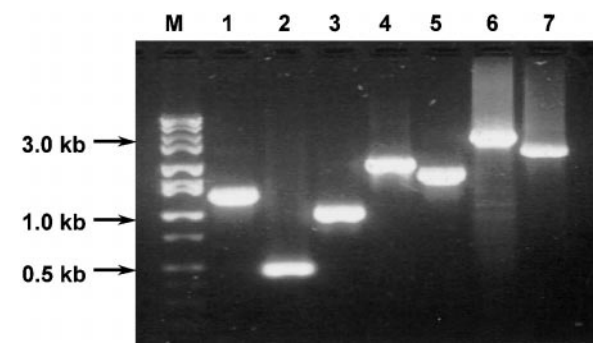

FIG. 3. PCR amplification of cDNAs to confirm the novel cDNA sequences. The products of the PCR reaction were analyzed on 1% agarose gel. M is the DNA molecular weight Marker. Lane 1, GAPDH; lane 2, NEMO; lane 3, IRP-1; lane 4, calpain large polypeptide L2; lane 5, Thy-1; lane 6, ABC transporter (small band); lane 7, ABC transporter (large band). The sequences obtained by this amplification step correspond to the sequences obtained in the previous two PCR amplifications confirming that our cloning method is accurate.

TABLE 1

Comparison of the mRNA Sequence Cloned by Our Novel Method with the Sequence Published in Genbank

Name and GenBank # of our sequence	Genbank # of compared sequence[a]	Compared to GenBank sequence (5′ End)[b]	Compared to GenBank sequence (3′ End)[b]
GAPDH mRNA (GenBank # AF261085)	M 33197.1	43 bp extension	No difference
Nemo mRNA (GenBank # AF261086)	AF 091453	74 bp extension	No difference
TEXREB107 mRNA (GenBank # AF261087)	D 17554	No difference	No difference
IRP-1 mRNA (GenBank # AF261088)	Z 11559	98 bp extension	No difference
Calpain large polypeptide L2 mRNA (GenBank # AF261089)	NM 001748.1	No difference	180 bp extension
Thy-1 mRNA (GenBank # AF261093)	NM 006288.1	91 bp extension	588 bp extension
Iron inhibited ABC transporter mRNA 1 (GenBank # AF261092)	AJ005016.1	312 bp extension	18 bp shorter
Iron inhibited ABC transporter mRNA 2 (GenBank # AF261091)	AJ005016.1	331 bp extension	993 bp extension
NADH dehydrogenase 1 beta subcomplex 9 mRNA (GenBank # 261090)	NM 005005.1	No difference	No difference

[a] If there are several comparable sequences in Genbank, we chose the longest one for comparison. The area of poly-A tail was excluded from analysis.

[b] No difference is defined as less than 6 bp sequence difference between the compared sequences.

anism by which the cDNA band of correct size can be obtained on the first pass or the amplification of cDNA ends can be repeated until the correct size of cDNA is obtained. Another advantage of our method is the special designation of PCR primers. The amplification of cDNA toward the ends, which is contrary to normal gene structure, decreases the possibility of contamination in cDNA cloning from genomic DNA. Obviously, our technique can also be used as a better alternative to existing methods for 5′ end primer extension because of its ability to specifically amplify cDNA ends using a graded series of amplification steps. A major application of the cloning approach outlined in this study is the analysis of the regulatory area on UTRs of mRNAs. In this study, we found no IRE structure on mRNAs that we had identified (9, 10) as iron responsive. Thus cellular iron burden can influence gene expression by mechanisms other than the interaction between iron responsive elements on mRNAs and the cytoplasmic mRNA binding iron regulatory proteins (12).

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