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A SIMPLE AND RAPID METHOD FOR THE PREPARATION OF HOMOLOGOUS DNA OLIGONUCLEOTIDE HYBRIDIZATION PROBES FROM HETEROLOGOUS GENE SEQUENCES AND PROBES

E. Stuart Maxwell[1] and Kevin D. Sarge

Department of Biochemistry, Box 7622, North Carolina State University, Raleigh, North Carolina 27695-7622

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SUMMARY: We describe a simple and rapid method for the preparation of homologous DNA oligonucleotide probes for hybridization analysis and/or cDNA/genomic library screening. With this method, a synthetic DNA oligonucleotide derived from a known heterologous DNA/RNA/protein sequence is annealed to an RNA preparation containing the gene transcript of interest. Any unpaired 3'-terminal oligonucleotides of the heterologous DNA primer are then removed using the 3' exonuclease activity of the DNA Polymerase I Klenow fragment before primer extension/dideoxynucleotide sequencing of the annealed RNA species with AMV reverse transcriptase. From the determined RNA sequence, a completely homologous DNA oligonucleotide probe is then prepared. This approach has been used to prepare a homologous DNA oligonucleotide probe for the successful library screening of the yeast hybRNA gene starting with a heterologous mouse hybRNA DNA oligonucleotide probe.

The screening of a gene library, as well as Southern and Northern blot analysis, is greatly facilitated by the utilization of highly homologous probes for hybridization. Frequently however, the only probes available for these analyses are heterologous gene fragments from distantly related organisms or DNA oligonucleotides deduced from protein sequences (1). The low stringency of hybridization conditions required for recognition by these probes often results in high backgrounds or the recognition of sequence-related DNA fragments/RNA transcripts other than those desired.

We have recently used a simple and quick method to generate a completely homologous hybridization probe for the yeast hybRNA gene. Using a heterologous DNA oligonucleotide probe for this same low molecular weight RNA (lmwRNA) gene in mouse, we have sequenced a portion of the yeast hybRNA

[1]To whom correspondence should be addressed.

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transcript with Klenow fragment and reverse transcriptase. From the determined yeast hybRNA sequence, a homologous DNA oligonucleotide was prepared. The resultant yeast probe can be used under very stringent hybridization conditions and thus eliminates any background/non-specific hybridization that results when the heterologous mouse DNA oligonucleotide probe is used. This method should also be useful for the preparation of homologous DNA oligonucleotide hybridization probes for other genes using already available heterologous DNA/RNA/protein gene sequences.

MATERIALS AND METHODS

[γ-^{32}P]ATP (>3000 Ci/mmol) was purchased from New England Nuclear Corporation. T4 polynucleotide kinase, Klenow fragment, AMV reverse transcriptase, deoxyribonucleotides, and dideoxyribonucleotides were obtained from Promega Biotechnologies. Nitrocellulose hybridization filters were purchased from Schleicher and Schuell. DNA oligonucleotides were synthesized on an Applied Biosystems DNA synthesizer.

Total yeast RNA and DNA were prepared from isolated yeast spheroplasts by phenol extraction as previously described (2). Total yeast RNA was fractionated on sucrose gradients (3) to prepare yeast lmwRNA. The heterologous mouse DNA oligonucleotide (39mer) (2) was labeled at the 5' terminus with ^{32}P using T4 polynucleotide kinase (4). For each primer extension/dideoxynucleotide sequencing reaction, approximately 5 ng of DNA oligomer (10^7 cpm/µg) was annealed to 10 µg of yeast lmwRNA. The DNA oligonucleotide and yeast lmwRNA were suspended in 4 µl of H_2O and heated to 90°C for 2 min before being quickly cooled to 65°C. An equal volume of 2.5 x primer extension buffer was added (50 mM Tris-HCl, pH 8.1, 50 mM KCl, 5 mM $MgCl_2$, 10 mM DTT final concentrations in 10 µl). This mixture was slowly cooled to 30°C (approximately 1 h). The mismatched 3' end of the heterologous DNA oligonucleotide was then removed by digestion with 10 units of Klenow fragment for 10 min at 23°C. Appropriate deoxynucleotides and dideoxynucleotides were added to individual reactions to final concentrations of 100 µM and 20 µM respectively. Five to ten units of reverse transcriptase were added and the primer extension/dideoxynucleotide sequencing reactions were then carried out at 42°C for 20 min. Reactions were stopped with an equal volume of 90% formamide, 10 mM EDTA buffer containing bromphenol blue, followed by resolution of extension products on 10% polyacrylamide sequencing gels as previously detailed (5). Screening of a yeast genomic library constructed in lambda bacteriophage gt11 (generously provided by Robert Hamatake and Craig Giroux) was carried out according to standard protocols (4).

RESULTS

The mouse DNA oligonucleotide used for primer extension sequencing of the yeast hybRNA homolog was a 39mer complementary to the last 39 nucleotides of the 3'-terminal region of mouse 4.5S hybRNA (2). Previous Northern blot analysis (2) has shown that this mouse hybRNA probe recognizes the yeast hybRNA homolog of this eukaryotic lmwRNA under low stringency hybridization conditions. However, loss of probe recognition under more stringent hybridization conditions suggested that utilization of this heterologous mouse probe for screening a yeast library, as well as for Northern and Southern blot

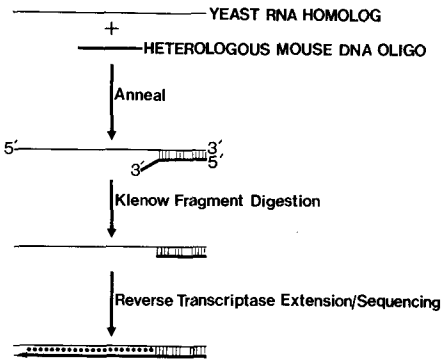
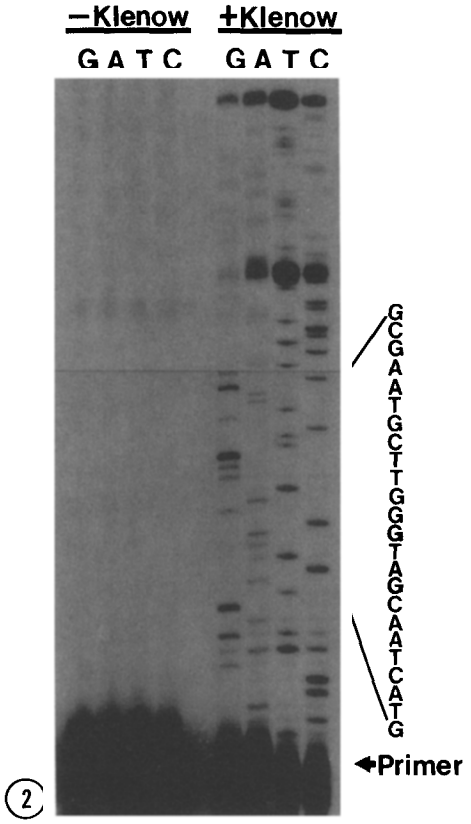


Fig. 1. Primer extension/sequencing protocol utilizing Klenow fragment to trim the heterologous DNA:RNA duplex.

analyses, would be less than ideal. We therefore decided to sequence the yeast hybRNA homolog by primer extension/dideoxynucleotide sequencing using the heterologous mouse 39mer as primer and, from the determined yeast hybRNA sequence, prepare a homologous DNA oligonucleotide.

Initial attempts at primer extension sequencing of this mouse DNA:yeast RNA duplex were unsuccessful because the 3' terminus of the heterologous mouse DNA oligonucleotide was not base-paired with the yeast hybRNA homolog. However, this difficulty was easily overcome by first incubating the heterologous mouse DNA:yeast RNA duplex with Klenow fragment to remove the 3'-terminal mismatched nucleotides before primer extension with reverse transcriptase. A schematic outline of this sequencing protocol is illustrated in Figure 1. The dependence of successful primer extension upon preincubation of the annealed heterologous RNA:DNA duplex with Klenow fragment is shown in Figure 2. We estimate that the yeast hybRNA homolog comprises less than 1% (probably less than 0.1%) of the total yeast lmwRNA fraction used in these primer extension experiments.

The determined sequence of yeast hybRNA was then used to prepare a yeast-specific DNA oligonucleotide probe of 24 nucleotides (indicated in Figure 2). This homologous DNA oligonucleotide was successfully used to screen a lambda phage library of yeast DNA and isolate a yeast hybRNA gene construct (the sequencing analysis of the yeast hybRNA gene will be reported elsewhere). Figure 3 shows the difference in recognition of the isolated yeast hybRNA clones by the homologous yeast 24mer vs. the heterologous mouse 39mer and the clear superiority of the former as a hybridization probe. The homologous yeast 24mer hybridizes strongly to the yeast hybRNA clones even at elevated washing temperatures and reduced salt concentrations. The heterologous mouse 39mer exhibits considerable non-specific hybridization when filters are washed under low stringency conditions and only very weak specific hybridization to the hybRNA clones at intermediate stringency conditions



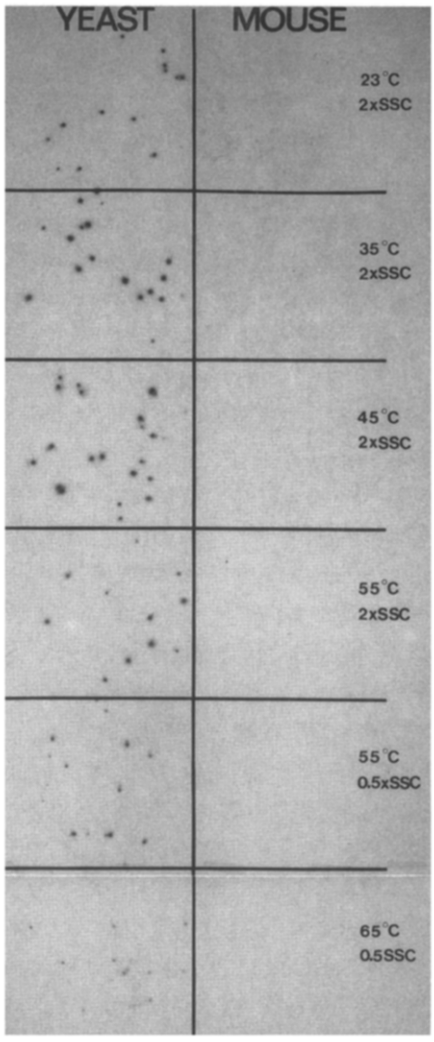


Fig. 2. Primer extension/dideoxynucleotide sequencing of yeast hybRNA. The 5'-radiolabeled mouse DNA oligonucleotide (39mer) was annealed to total yeast lmwRNA. Annealing reactions containing the heterologous mouse DNA:yeast hybRNA hybrids were preincubated in the presence or absence of Klenow fragment before the addition of reverse transcriptase for dideoxy sequencing. The indicated yeast hybRNA sequence was used to synthesize the yeast-specific DNA oligonucleotide probe.

Fig. 3. Differential recognition of the homologous yeast 24mer probe vs. the heterologous mouse 39mer probe for the cloned yeast hybRNA gene. A phage stock of an isolated yeast hybRNA clone and a yeast genomic library stock were mixed to give a lambda phage population of approximately 10% positive hybRNA clones. This prepared phage mixture was plated (approximately 200 plaques per plate), blotted to nitrocellulose filters, and after hybridization with either the radiolabeled homologous yeast or heterologous mouse oligonucleotide probes, washed under different stringency conditions. The stringency conditions used for washing are indicated to the right of each pair of filters hybridized with either the yeast 24mer (yeast) or mouse 39mer (mouse).

(45°C, 2xSSC). These positive clones are seen only at very long exposure times (48 h as compared with 6-8 h exposure times for the yeast 24mer probe) and are completely lost at higher washing temperatures.

DISCUSSION

We describe here a simple method for the generation of homologous DNA oligonucleotide hybridization probes using DNA oligos complementary to related heterologous gene sequences of other organisms. The occurrence of mismatched nucleotides at the 3' terminus of the DNA oligonucleotide primer is likely to be a characteristic of heterologous DNA:RNA duplexes. Therefore, utilization of the Klenow fragment with its 3'-exonuclease activity to remove non-base-paired nucleotides, thus providing a 3'-terminal base-paired nucleotide for subsequent reverse transcriptase sequencing, is particularly critical for this sequencing technique.

Initially, we prepared an homologous yeast hybRNA probe by simply primer-extending the Klenow fragment-trimmed mouse DNA:yeast RNA duplex in the presence of [α-^{32}P]GTP minus added dideoxyribonucleotides. The extended region of the DNA strand was 100% homologous to yeast hybRNA with a specific activity of approximately $6x10^7$ cpm/µg. However, due to the small amount of DNA primer used in each extension reaction (5 ng), the total cpms of homologous primer obtained was less than would be required for library screening (approximately $3x10^5$ cpms/reaction). Multiple extension reactions would have required large amounts of isolated yeast lmwRNA. Therefore, we opted to sequence the yeast hybRNA transcript and, from the determined sequence, synthesize an homologous DNA oligonucleotide probe. This protocol provided a large quantity of hybridization probe which could be radiolabeled to a very high specific activity. The synthesis of the yeast hybRNA-specific probe has allowed us to easily identify and isolate multiple clones of the hybRNA gene from a yeast lambda phage library.

We believe this approach should also be useful for library screening and hybridization analysis of other gene sequences whose partial DNA/RNA/protein sequence is known. The technique may be particularly useful in preparing homologous probes from determined protein/peptide sequences. Oligonucleotide probes derived from protein sequences are often relatively short in size and heterologous in sequence due to genetic code redundancy (1). However, utilization of such a DNA oligo in this protocol as primer for mRNA sequencing would subsequently permit the preparation of a second, larger and completely homologous oligonucleotide probe for hybridization analysis. An oligonucleotide preparation of mixed sequence (1) labeled at the 5' terminus could be used for mRNA sequencing as well. This mixed sequence population would permit the use of higher stringency hybridization conditions during primer extension sequencing, thus reducing possible recognition of related mRNA sequences.

Two advantages of this approach for probe preparation are worth noting. First, sequencing of the RNA transcript does not require purification of that

RNA species. We have easily sequenced portions of the mouse, rat, hamster, Xenopus laevis, and yeast hybRNAs using their respective lmwRNA populations (2,6). Estimates of the hybRNA transcript in these total lmwRNA populations range from 0.5% to less than 0.1%. Thus, one should be able to sequence an mRNA sequence which comprises only a very small fraction of total isolated poly(A+) mRNA. Second, this approach takes advantage of the increased hybrid strength of an RNA:DNA vs. a DNA:DNA duplex. This increased stability facilitates specific hybridization of the heterologous probe to the desired gene sequence. Thus an RNA transcript may be recognized by a weakly hybridizing heterologous DNA probe under conditions where hybridization to the DNA or gene sequence would be considerably weaker or perhaps even impossible to detect.

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