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Ruef, Barbara J
Hecht, Jonathan H
Manning, Jerry E

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A method of identifying and isolating a unique member of a multigene family: application to a trypanosome surface antigen gene

Barbara J.Ruef, Jonathan H.Hecht and Jerry E.Manning*
Department of Molecular Biology and Biochemistry, University of California, Irvine, CA 92717, USA

ABSTRACT

A chimeric oligonucleotide was constructed using DNA sequences from two distal regions of a cDNA which encodes a major surface antigen (TSA-1) of *Trypanosoma cruzi*. Conditions were found that allowed the chimeric oligonucleotide to hybridize only to a 5.4 kb EcoRI fragment in a Southern blot of total genomic DNA. The 5.4 kb EcoRI genomic DNA fragment has previously been shown to be located at a telomeric site, thus the studies described here directly demonstrate that the TSA-1 gene is telomeric in location. It is also shown that the chimeric oligonucleotide can be used to selectively identify recombinant λ phage which harbor the TSA-1 gene using standard library screening procedures. Since these studies demonstrate that a chimeric oligonucleotide can be used to identify in both Southern blots and library screens a single member among the more than sixty members of the TSA-1 gene family, it seems likely that chimeric oligonucleotides may be of general use in studies involving repetitive DNA sequence families.

INTRODUCTION

Trypanosoma cruzi, the causative agent of Chagas' disease in humans, is an intracellular parasitic protozoan which can infect a wide range of mammalian species (1). Infection is established by penetration of the vertebrate host cell by the trypomastigote stage of the parasite, and several studies indicate that a trypomastigote specific surface glycoprotein(s) of 83−85 kDa is involved in the adhesion/penetration step (2−6). In an attempt to obtain information on the properties of this protein, we have cloned (7), characterized (8), and obtained the nucleotide sequence (9) of a gene, TSA-1, which encodes an 85 kDa trypomastigote specific surface protein. TSA-1 is a member of a large multigene family whose members are dispersed throughout the genome. A unique feature of TSA-1 is the presence of a 27 bp tandem repeat unit within the coding region. This repeat unit is found in four members of the gene family,

but only one of these members, TSA-1, is transcribed. Earlier studies have indirectly identified the location of the TSA-1 gene to a 5.4 kb EcoRI DNA fragment. However, direct confirmation of the genomic location of the TSA-1 gene has not been possible since the 5.4 kb EcoRI fragment is telomeric in location and has been refractory to cloning by several procedures (8).

In order to directly identify the site of the TSA-1 gene, we explored the use of a chimeric oligonucleotide as a specific hybridization probe. The rationale for this approach is based on the observation that different members of a gene family often share different sequence subsets. It is not unusual for a region of one gene to be homologous to sequences found in some but not all members of the gene family. Likewise, a different region of the same gene might be found to have homology with a different subset of the family. Therefore, a chimeric oligonucleotide containing sequences from two distal regions of a gene might be found to hybridize to only that member of the gene family which contains both sequence subsets. The studies described here identify a chimeric oligonucleotide containing sequences from distal regions of the TSA-1 cDNA that hybridize only to a 5.4 kb EcoRI telomeric fragment in a Southern blot of *T. cruzi* genomic DNA. Therefore, the data obtained demonstrate that the transcriptionally active member of the TSA-1 gene family is located at a telomeric site.

MATERIALS AND METHODS

Parasite strain and culture

Trypanosoma cruzi Peru clone 3 was used in these studies. The isolation and properties of this clonal isolate as well as the growth and maintenance of epimastigote and trypomastigote forms of the parasite are described elsewhere (9).

Nucleic acid isolation, radiolabeling, Southern transfer and restriction enzymes

Parasites were harvested and DNA was isolated as described previously (10). Plasmid DNA was amplified, harvested by lysis in sodium dodecyl sulfate, and purified by banding in cesium chloride as described (11). Synthetic oligonucleotides were

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* To whom correspondence should be addressed

radiolabeled with (γ-^{32}P)ATP as described (7). Agarose gel electrophoresis of DNA, Southern transfer, prehybridization, and hybridization were performed as described (12,13) except that the hybridization buffer contained 5×Denhardt's and the hybridization and wash temperatures were as described in the text. Blots were washed twice in 6×SSC for 10 min at the hybridization temperature. All restriction enzymes were purchased from Boehringer Mannheim (Indianapolis, IN) and used as recommended.

Recombinant plasmids

The DNA used in Figure 1 is pTsa/ES (a 0.9 kb EcoRI/SalI fragment of the surface antigen gene TSA-1 cloned into the plasmid Bluescript (BSSK; Stratagene, Inc. La Jolla, CA)). The DNA used in Figure 2 is pTsa/BS (a 2.7 kb BamHI/SalI fragment of the TSA-1 gene cloned into BSSK). The recombinant DNAs used in the experiment shown in Figure 5 share the following properties: The DNA inserts are cloned into the EcoRI site of BSSK and lack the recognition site for XhoI. The subcloned DNAs are the following: lane a: pTcc-20 (a 154 bp DNA fragment from the 90 kDa surface antigen gene of *T. cruzi* (12)), b: pTcg-1 (a 0.5 kb DNA fragment from the TSA-1 gene (14)), c: pDMlsp 1.4 (a 1.4 kb DNA fragment from the LSPα gene of *Drosophila* (15)), d: pTcg-20 (a 2.3 kb DNA fragment from the 90 kDa surface antigen gene of *T. cruzi* (12)), e: pDMlsp 6.0 (a 6.0 kb DNA fragment from the LSPα gene of *Drosophila* (15)), f: pDMlsp 9.4 (a 9.4 kb DNA fragment from the LSPα gene of *Drosophila* (15)).
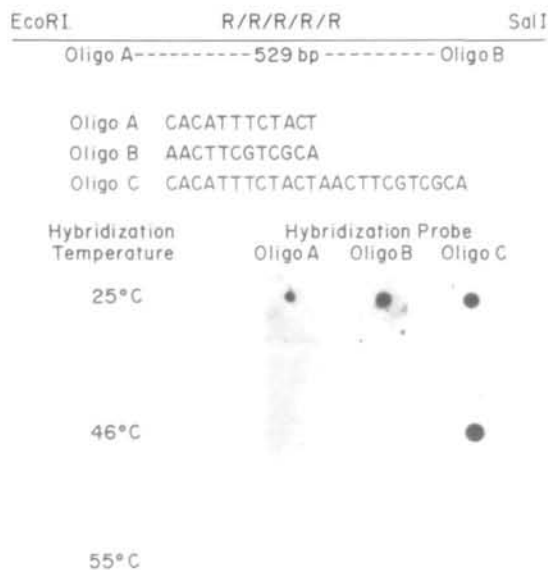

Figure 1. Hybridization of oligonucleotides A,B, and C to a plasmid DNA containing the 0.9 kb EcoRI/SalI DNA fragment of the TSA-1 gene. A schematic diagram of the EcoRI/SalI DNA fragment containing nucleotides 1852—2766 of the TSA-1 gene is shown at the top of the figure. The approximate position of the five copies of the 27 bp tandem repeat motif is denoted by R. The nucleotide sequences of oligonucleotides A, B and C and the approximate location of sequences A and B within the EcoRI/SalI fragment are shown. The 529 bp denotes the number of nucleotides which separate sequences A and B. DNA from subclone pTsa/ES was digested with EcoRI and 0.1 µg was dot blotted to nitrocellulose. Dot blots were then hybridized with either (^{32}P)-labeled oligonucleotide A, B, or C at 25, 46 or 55°C.

RESULTS AND DISCUSSION

Hybridization of a chimeric oligonucleotide to genomic DNA

In an attempt to identify the transcriptionally active member of the TSA-1 gene subfamily, we explored the use of a chimeric oligonucleotide as a gene specific probe. Chimeric oligonucleotide C is 24 bases in length and is a composite of bases 1859—1870 (oligonucleotide A; Fig. 1) and 2400—2411 (oligonucleotide B; Fig. 1) of the TSA-1 cDNA sequence (14). The selection of these two sequences for the construction of the chimeric oligonucleotide is based on the observation that the numerous genomic DNA fragments which share homology with these two regions of the TSA-1 gene are not generally overlapping in size (8; Fig. 3), suggesting that these two regions may be closely linked within only a very small subset of the gene family.

The use of oligonucleotide C to identify genomic DNA fragments which have both the A and B sequence requires that hybridization conditions be determined that will allow oligonucleotide C but not oligonucleotide A or B to form stable duplexes with homologous sequences in genomic DNA. In order to determine these conditions, the three oligonucleotides were hybridized to dot blots containing the EcoRI/SalI fragment of TSA-1 (Fig. 1). The ability of the three probes to form stable hybrids at temperatures in the range of 25°C-55°C was tested at approximately 5° intervals. While all 3 oligonucleotides formed stable hybrids with the EcoRI/SalI fragment at 25°C-41°C, only oligonucleotide C was observed to hybridize at 46°C, and no hybridization was observed with any of the probes at 55°C.

This result suggests that hybridization of Southern blots of genomic DNA with oligonucleotide C at 46°C might allow DNA fragments which contain both sequence A and sequence B to be distinguished from fragments which contain only sequence A or only sequence B. However, since the hybridization signal with oligonucleotide C is less intense at 46°C than at 25°C, the question arises as to whether the decreased signal intensity compromises the ability of oligonucleotide C to detect, in Southern blots of genomic DNA, fragments which might be
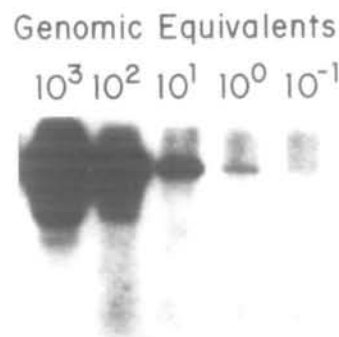

Figure 2. Hybridization of oligonucleotide C to the BamHI/SalI fragment of the TSA-1 gene. DNA from subclone pTsa/BS was digested with BamHI and electrophoresed on a 1% agarose gel in amounts equivalent to 0.1, 1, 10, 100 and 1,000 copies per haploid genome. A Southern blot of the gel was hybridized with (^{32}P)-labeled oligonucleotide C at 46°C.

present only once per haploid equivalent of genomic DNA. This issue was addressed by a genomic DNA reconstruction experiment. Oligonucleotide C was ^{32}P end-labeled and hybridized at 46°C to a Southern blot containing a 2.7 kb BamHI/SalI DNA fragment from TSA-1 equivalent to 0.1, 1, 10, 100 and 1000 copies per haploid genome (Fig. 2). The calculations for copy number determination were based on a haploid genome size in *T. cruzi* of 1.25×10^8 bp (10). Strong hybridization was seen to copy numbers of 10 and above. A modest hybridization signal was observed at one copy per haploid genome, suggesting that it should be possible to utilize this approach to detect a DNA restriction fragment that occurs once per haploid *T. cruzi* genome.

In order to determine whether oligonucleotide C could identify which of the 4 members of the subfamily is the site of transcription of the TSA-1 mRNA, ^{32}P end-labeled oligonucleotide C was hybridized at 46°C to a Southern blot containing EcoRI digested genomic DNA. As shown in Figure 3, hybridization was observed only with a fragment of 5.4 kb. No hybridization was observed with the other 3 members of the subfamily. That the other members of the subfamily are also present in the Southern blot was shown by hybridization of the identical blot with a ^{32}P-labeled 27 base repeat unit (Fig. 3). This result directly shows, therefore, that the transcriptionally active member of the subfamily is also the telomeric member of the subfamily.

Relationship between hybridization efficiency and the spacing between the two hybridization sites

Since it seems likely that the intensity of the hybridization signal is related to the distance separating the two sites which hybridize
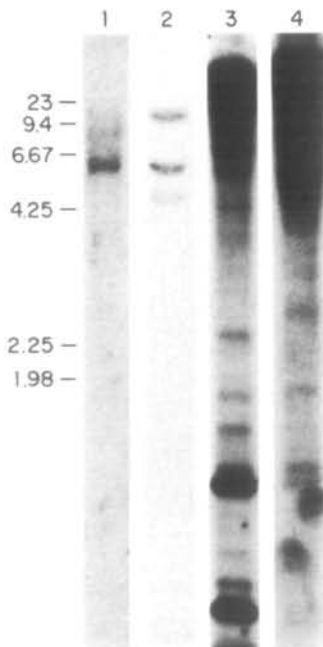
with the chimeric oligonucleotide, a test system was developed to investigate this relationship. As shown in Figure 4, a chimeric oligonucleotide (oligonucleotide F) was constructed containing 13 nucleotides 5′ upstream (i.e. oligonucleotide D), and 12 nucleotides 3′ downstream (i.e. oligonucleotide E), of the EcoRI site in BSSK. Conditions where only oligonucleotide F would hybridize with BSSK were determined by hybridization of the three oligonucleotides at 25°C, 37°C, and 46°C to dot blots containing linear BSSK DNA (Fig. 3). At 46°C only the chimeric oligonucleotide showed hybridization, while all of the oligonucleotides showed hybridization at the lower temperatures.

Separation of sites D and E was accomplished by insertion of DNA fragments of length .15 to 9.4 kb into the EcoRI site of BSSK. The plasmid DNAs were linearized by restriction with XhoI, fractionated by agarose gel electrophoresis, Southern blotted, and hybridized with oligonucleotide F at 46°C. As shown in Figure 5, the hybridization signal is most intense with the BSSK plasmid containing the smallest DNA insert (lane a′) and gradually decreases as the number of nucleotides which separate sites D and E becomes greater. To better define the relationship between signal intensity and the spacing between sites D and E, the relative intensity of each hybridization signal was determined by a densitometric scan of the autoradiograph and expressed as peak area (Table I). In Figure 6 the peak area per fmole of DNA is plotted as a function of the number of nucleotides separating hybridization sites D and E in each plasmid construct. Regression

Figure 3. A Southern blot containing 10 μg per lane of trypomastigote nuclear DNA digested with EcoRI was hybridized with: lane 1, (^{32}P)-labeled oligonucleotide C at 46°C; lane 2, (^{32}P)-labeled 27 nucleotide repeat at 46°C; lane 3, (^{32}P)-labeled oligonucleotide A at 25°C; lane 4, (^{32}P)-labeled oligonucleotide B at 25°C. Size markers on the margin refer to the migration of HindIII fragments of λ phage DNA.

Figure 4. Hybridization of oligonucleotides D, E, and F to BSSK DNA. A segment of the the polylinker region of BSSK DNA showing the approximate position of four restriction enzyme sites is schematically diagrammed at the top of the figure. The nucleotide sequences of oligonucleotides D, E, and F are shown. The approximate location of sequences D and E within the polylinker are shown. The 37 bp denotes the number of nucleotides which separate sequences D and E. BSSK DNA was digested with either EcoRI or KpnI and 0.1 μg was dot blotted to nitrocellulose. Dot blots were then hybridized with either (^{32}P)-labeled oligonucleotide D, E, or F at 25°C, 37°C, or 46°C.
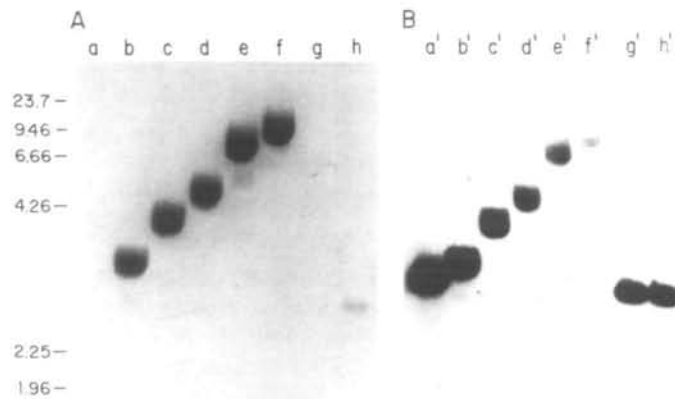
Figure 5. Hybridization of oligonucleotide F to BSSK DNA containing different size EcoRI inserts. A, DNA from subclone pTcc-20 (lane a), Tcg-1 (lane b), pDMlsp 1.4 (lane c), pTcg-20 (lane d), pDMlsp 6.0 (lane e), pDMlsp 9.4 (lane f) and Bluescript plasmid (lane g) were digested with XhoI. Lane h contains BSSK DNA digested with EcoRI. The digested DNAs were electrophoresed on a 1% agarose gel and stained with ethidium bromide. B, Southern blot of the gel shown in A hybridized with (^{32}P)-labeled oligonucleotide F at 46°C. Amounts of DNA electrophoresed are listed in Table I.

Table I.

Nucleotides Separating Sites D and E in kb*	DNA Electrophoresed μg	fmoles+	Peak Area@	Peak Area fmole
.037 (g)	.0075	3.8	640	168
.186 (a)	.08	39	1,318	34
.532 (b)	0.9	390	1,080	2.8
1.43 (c)	1.1	380	670	1.8
2.34 (d)	1.4	400	340	.85
6.01 (e)	2.3	390	80	.21
9.4 (f)	3.1	380	15	.04

* Letters in parentheses denote lane number in figure 5.
+ fmoles of recombinant plasmid electrophoresed in figure 5 was calculated using a value of 660 for the average molecular weight of a nucleotide pair and 2.96 kb for the length of the BSSK plasmid DNA.
@ The relative intensities of the hybridization signals observed in the Southern blot depicted in figure 5 were determined by a densitometric scan of the autoradiogram.

analysis indicates that the intensity of the hybridization signal is logarithmically proportional to the spacing between the two hybridization sites. Using a calculated value of −1.44 for the slope, it is predicted that a 2 fold decrease in the spacing between the hybridization sites will provide an increase of approximately 2.3 in the intensity of the hybridization signal.

Screening recombinant DNA libraries with a chimeric oligonucleotide

To determine whether oligonucleotide C could be effectively used to screen recombinant phage libraries for DNA molecules that include both sequence A and B, a trypomastigote cDNA library constructed in lambda gt 10 was screened with (^{32}P)-labeled oligonucleotide C at 46°C. Approximately 5000 recombinant phage were screened and 2 phage showed positive hybridization signals upon plaque purification (Fig. 7). Both were shown by hybridization analysis to contain the 27 bp repeat motif
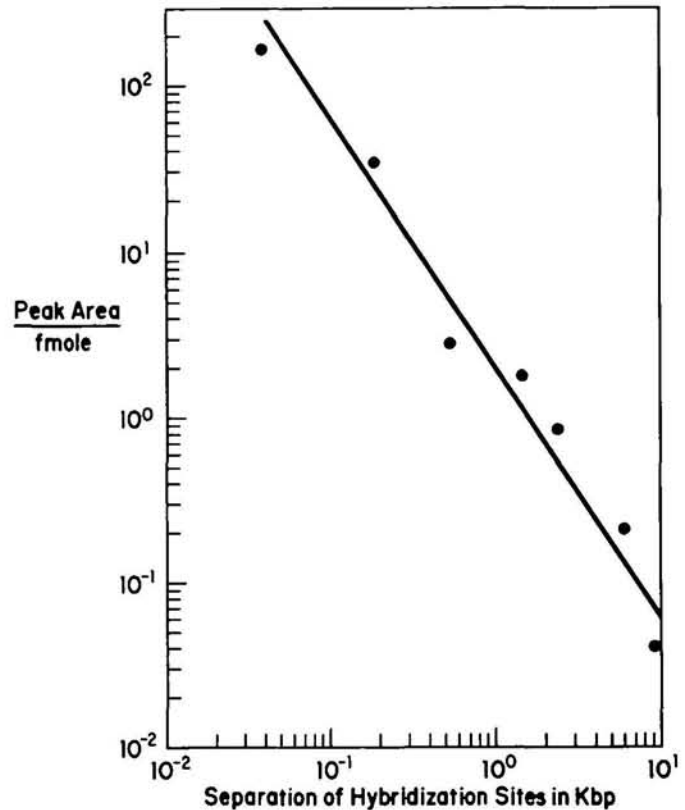

Figure 6. Plot of the relative intensity of the hybridization signals obtained in the Southern blot in figure 5 (ie. peak area/fmole, Table I) as a function of the distance separating the sites of hybridization of oligonucleotide F.

Figure 7. Hybridization of (^{32}P)-labeled oligonucleotide F to (a) λ gt10 which contain the TSA-1 gene and (b) wild type λ gt10.

characteristic of the TSA-1 gene as well as the sequences homologous to oligonucleotides A and B. No phage were selected which contained only sequence A or B.

FURTHER DISCUSSION

Our principal result is that a chimeric oligonucleotide which is composed of two distal sequences of the TSA-1 cDNA hybridizes only with a 5.4 kb EcoRI DNA fragment in a Southern blot of total genomic DNA. Although earlier studies inferred that the TSA-1 gene might reside in this DNA fragment, direct proof was lacking because the 5.4 kb DNA fragment was found to be telomeric and refractory to cloning (8). The hybridization studies shown in figure 3 now provide direct evidence that the TSA-1 gene is present in this DNA fragment, thus confirming the assignment of the TSA-1 gene to a telomeric location.

The successful use of a chimeric oligonucleotide to identify a single unique member from among the more than 60 members of the TSA-1 gene family suggests that this approach may be useful for similar studies in other organisms. Also, it is possible that hybridization studies with chimeric oligonucleotides might be helpful in overcoming other difficulties which arise in studies that involve repetitive DNA sequence families. For example, chromosome walking studies are particularly difficult in areas of the chromosome where extensive regions of repetitive DNA sequences are encountered. While these difficulties have been overcome by genetic means in organisms such as *Drosophila* (16), chromosome mapping studies in organisms which lack similar genetic advantages have required tremendous effort and resources (17). In principle, these difficulties might be overcome by the use of chimeric oligonucleotides. Such oligonucleotides would be constructed from sequences found at distal sites within a cloned DNA fragment and used as described above for screening both Southern blots of genomic DNA and recombinant genomic DNA libraries. If the chimeric oligonucleotide hybridizes to a unique DNA fragment in a Southern blot of genomic DNA, it should be possible to use the chimeric oligonucleotide in conjunction with established procedures to efficiently perform chromosome walking and mapping studies.

A major consideration in utilizing chimeric oligonucleotides as hybridization probes for studies in organisms other than *T. cruzi* is the intensity of the hybridization signal. Using the conditions described in the studies shown in Figure 3, it is unlikely that oligonucleotide C would have detected a DNA fragment which is present only once per haploid genome if the size of that genome were 10 times that of *T. cruzi* (ie. no hybridization signal was seen at 0.1 *T. cruzi* genomic equivalents). However, modification of three parameters which influence the intensity of the hybridization signal should provide the increase in sensitivity required for studies in eukaryotic organisms which have haploid genome sizes $10-20\times$ that of *T. cruzi*. Firstly, an increase in the quantity of genomic DNA electrophoresed might provide a $2-3$ fold increase in the hybridization signal. Secondly, if the chimeric oligonucleotide were radiolabeled with $(\alpha\text{-}^{32}P)dNTP$ using terminal deoxynucleotidyl transferase a $5-10$ fold increase in the specific activity of the probe might be obtained. Finally, selecting hybridization sites which are closer than those chosen for oligonucleotide C would provide a calculable increase in the intensity of the hybridization signal (Figure 6). In this regard, it is interesting to note that assuming a Jacobson−Stockmeyer model for the thermodynamics of loop closure (18,19 and 20), the observed slope of −1.44 in Figure

6 is in close agreement with a predicted slope of approximately −1.5. The slightly lower value would suggest that the longer loops may not be fully extended and might contain internal structures.

Chimeric oligonucleotides may also be useful as probes for screening recombinant DNA libraries. As shown in figure 7, the hybridization signal seen with the chimeric oligonucleotide at the restrictive temperature is readily detectable only in phage which harbor the DNA fragment of interest.

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