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The use of synthetic oligonucleotides as hybridization probes. II. Hybridization of oligonucleotides of mixed sequence to rabbit β-globin DNA

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ABSTRACT

Two oligonucleotides 14-bases long were synthesized, one complementary to rabbit β-globin DNA (RβG14A) and the other with the same sequence except for a single base change (T for C) (RβG14B). Hybridization conditions were established such that RβG14A would hybridize to globin DNA while RβG14B would not. We also synthesized a mixture of 13-base long oligonucleotides (RβG13Mix), representing eight of the possible coding sequences for amino acids 15-19 of rabbit β-globin. One of the eight is complementary to globin DNA. RβG13Mix was found to hybridize specifically to globin DNA under conditions where oligonucleotides forming single base pair mismatches do not. Furthermore, RβG13Mix was shown to hybridize specifically to colonies containing a plasmid with a globin DNA insert. These results are discussed with respect to a general procedure for screening recombinant clones for those containing DNA coding for a protein of known amino acid sequence.

INTRODUCTION

The isolation of specific cloned DNA sequences depends, to a great extent, on an available assay to allow for the screening of sufficient clones to isolate those containing the desired sequence. Normally, this assay involves the use of specific hybridization probes, either purified from natural sources such as rRNA, tRNA, 5SRNA or abundant mRNAs or prepared from previously isolated clones. It has proven very difficult to isolate clones of low abundance mRNA sequences, such as the cloned cDNA for interferon (1,2), because the available assays are laborious, time consuming, and often very insensitive. It would be desirable to have a general procedure to detect clones of low abundance mRNA sequences which allowed the screening of large numbers of clones with ease.

In a previous study of the effect of single base pair mismatches on the hybridization behavior of oligonucleotides to

ΦX174 DNA (3), we proposed that synthetic oligonucleotides might be useful as specific probes for cloned DNA. We observed that the duplexes with a single base pair mismatch, which were formed when 11-, 14- or 17-base long oligonucleotides were hybridized to ΦX174 DNA, were significantly less stable (dissociated at lower temperature) than their perfectly matched counterparts. This difference in thermal stability made it possible, by the appropriate choice of hybridization temperature, to virtually eliminate the formation of mismatched duplexes without affecting the formation of perfectly matched ones. This specificity of hybridization should allow a mixture of oligonucleotides, whose sequences represent all possible combinations of codon sequences predicted from a short amino acid sequence, to be used as probes for clones coding for the peptide. Stringent hybridization criteria would be used to select the single correct sequence from the mixture.

In this paper we present the results of the hybridization of oligonucleotides to cloned rabbit β-globin DNA sequences. This serves as a model for the general hybridization strategy mentioned above. We show that a mixture of eight different 13-base long oligonucleotides, one of which is complementary to rabbit β-globin DNA, hybridizes specifically to β-globin DNA under conditions where duplexes with single base mismatches will not form. In addition, colony screening experiments with transformed cells containing either pBR322 or pBR322 into which rabbit β-globin cDNA has been cloned demonstrates the use of mixed oligonucleotides as specific probes for screening recombinant clones.

MATERIALS AND METHODS

Source of DNA and Transformants

A genomic clone, RβG1 (4), containing the rabbit β-globin gene inserted into the λ bacteriophage Ch4A (5) was obtained from Dr. T. Maniatis of the California Institute of Technology. DNA was isolated from purified phage by phenol/$CHCl_3$ extraction followed by ethanol precipitation. Plasmid DNA (pBR322 β-globin) (6) containing rabbit β-globin cDNA inserted in the *Hin*dIII site of pBR322 was obtained from Dr. P. Berg of Stanford University.

E. coli strain LS1, a derivative of HB101 (obtained from Dr. A.D. Riggs of the City of Hope Research Institute), was transformed with pBR322 or with pBR322 β-globin (7) and selected for ampicillin resistance.

Chemical Synthesis of Oligodeoxyribonucleotides of Unique Sequence

Oligonucleotides RβG14A and RβG14B (Figure 2) were synthesized in solution by the modified triester approach as described previously (8).

Simultaneous Synthesis of Oligodeoxyribonucleotides of Mixed Sequences

Oligonucleotide RβG13Mix, a mixture of eight different sequences (Figure 2), was synthesized on a solid support (9). The di, tri and tetranucleotide blocks were prepared by the published coupling procedure using either 3'-phosphomonotriazolides (10) or 2,4,6-triisopropylbenzenesulfonyl tetrazolide (TPSTe) (8). The polyacrylmorpholide resin support was derivatized with the 3'-nucleoside (nucleoside-resin) as previously reported (9). The coupling reaction of the oligonucleotide blocks onto the nucleoside-resin was performed as reported (9, 11) with a slight modification: (i) Instead of using a single oligonucleotide coupling unit, a mixture of an equal amount of two different coupling units was used as described in Figure 1; and (ii) for deblocking dimethoxytrityl (DMT) groups from the support, a mild condition was used. The support (250-500 mg) was treated with 2% benzenesulfonic acid (BSA) solution in $CHCl_3$-MeOH (7:3 v/v, 10 ml) for 1 minute at 0°C. This deprotection step was repeated until no color was detected in the reaction. The general operation of one cycle of the coupling reaction is as follows.

Equal amounts of the fully protected oligonucleotide blocks (2-3 fold molar excess of each block to the first nucleoside-resin) were treated with a solution of pyridine-triethylamine-water (3:1:1 v/v, 10 ml) for 20 minutes at room temperature to remove the β-cyanoethyl protecting group. After co-evaporation of the coupling units and the nucleoside-resin with anhydrous pyridine three times, two equivalents of TPSTe in anhydrous pyr-

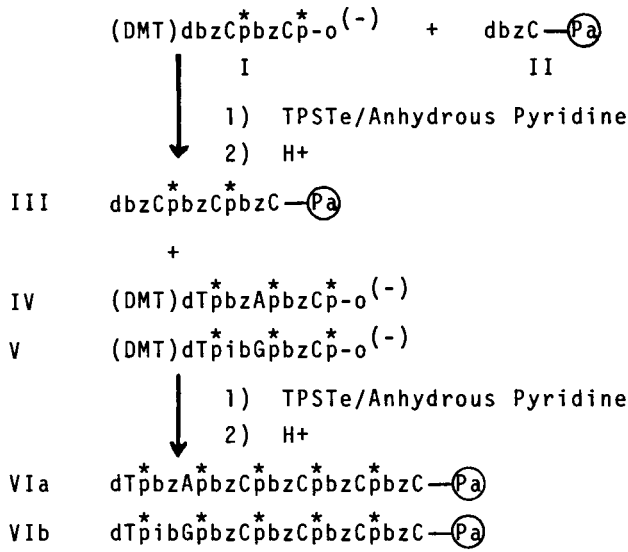

FIGURE 1. Synthesis of a Mixture of Hexanucleotides, dTACCCC and dTGCCCC, is Outlined. Abbreviations: DMT = 4',4'-dimethoxytrityl; p = ortho-chlorophenylphosphate; Pa = $COCH_2CH_2CONHCH_2CH_2NHCO$-polyacrylmorpholide; TPSTe = 2,4,6-triisopropylbenzenesulfonyl tetrazolide.

idine was added to the coupling units and the reaction was shaken for three hours at room temperature. After the reaction, the resin was rinsed twice with pyridine and any unreacted 5'-hydroxyl functionalities were capped by treatment with a solution of acetic anhydride in pyridine (10%, v/v) for one hour at room temperature. After thorough rinsing with chloroform-methanol (7:3 v/v), the 5'-dimethoxytrityl protecting groups were removed and the coupling yield was estimated by spectrophotometric measurement of the released dimethoxytrityl cation in 1% BSA solution (9). The procedure was repeated with the next group of coupling units.

With the liquid-phase approach, a chromatographical purification step on silica gel is necessary to isolate the desired product. The recovery of guanosine-rich oligonucleotides from the silica gel chromatography are generally poorer than that of other sequences. Consequently, the isolated product from the column would not necessarily contain the same population of different sequences in the coupling reaction. In order to cir-

cumvent this problem, the synthesis was performed by the solid-phase method. Needless to say, there is no chromatographical purification step in the solid-phase synthesis since all the products are covalently attached to a solid-phase support. At the end of the synthesis, the products are released from the support and every sequence made by the coupling reaction should be present in the probe.

Purification of Oligodeoxynucleotides

All oligonucleotides were purified after complete deprotection (12) by high performance liquid chromatography on an ion-exchange (DuPont Permaphase AAX) column run at 65°C with a linear gradient of 0.0 to 1.0 M KCl in 50 mM potassium phosphate (pH4.5). The product peak was collected and desalted.

Labeling of Oligonucleotides

Oligonucleotides were labeled at the 5'-end by transfer of ^{32}P from $\gamma[^{32}P]$ATP using T4 polynucleotide kinase (Boehringer-Mannheim) as described previously (3). $\gamma[^{32}P]$ATP was synthesized by the method of Walseth and Johnson (13).

Agarose Gel Electrophoresis

RβG1 DNA was digested with *Eco*R1 endonuclease (Boehringer-Mannheim) at 350 µg/ml DNA in 50 mM Tris·HCl pH7.2, 100 mM NaCl, 5 mM $MgCl_2$, 0.02% NP40, and 7000 units/ml *Eco*R1 for 30 minutes at 37°C. Digested DNA was brought to 13 µg/ml in a gel loading buffer containing 10 mM Tris base, 8.3 mM Boric acid, 0.1 mM EDTA, 0.25% SDS, 0.05% bromocresol green, 5% glycerol. DNA (20 µl) was loaded into individual wells of a horizontal 0.5% or 0.7% agarose gel and electrophoresed at 1 V/cm overnight. The gel was stained with ethidium bromide and photographed under ultraviolet light. The DNA was blotted onto nitrocellulose (Schleicher & Schuell BA85) as described previously (3) and by Southern (14).

Preparation of Colony Filters

Ordered collections of colonies were picked onto 100 mm agar plates containing 20 µg/ml ampicillin (50 colonies per plate). Ten pBR322 β-globin transformants were interspersed among 40 pBR322 transformants. After overnight growth, colonies

were lifted onto Whatman 540 filter circles (82 mm), amplified on agar plates containing 250 µg/ml chloramphenicol, and prepared for hybridization as described by Gergen, *et al.* (15). Location of all colonies on the filter was visualized by staining the filter with 0.25 µg/ml ethidium bromide and photographing under UV light.

Hybridization

Blots were hybridized with ^{32}P labeled oligonucleotides in 6 X NET (1 X NET = 0.15 M NaCl, 0.015 M Tris•HCl pH7.5, 0.001 M EDTA), 5 X Denhardt's (16), 0.5% SDS, 10% Dextran sulfate (Pharmacia Fine Chemicals) at 37°C for 20 hours. Probe concentrations were 1 ng/ml for RβG14A and RβG14B and 8 ng/ml for RβG13Mix. Blots were then washed at 0°C in 6 X SSC (1 X SSC = 0.15 M NaCl, 0.015 M sodium citrate pH7.2) (3), blotted dry and exposed to Kodak XR1 X-ray film with one intensifier screen (Cronex Lightning Plus) for 20-48 hours.

Colony filters were prehybridized in 6 X NET, 0.5% NP40 (Shell Oil), 100 µg/ml single stranded *E. coli* DNA for 2 hours at 55°C. Hybridization was done in 6 X NET, 0.5% NP40, 250 µg/ml tRNA and 1 ng/ml RβG14A or RβG14B or 8 ng/ml RβG13Mix at 37°C for 20 hours. Filters were then washed at 0°C with four changes of 6 X SSC (250 ml) and then at 37°C with two changes of 6 X SSC for 10 minutes. Filters were blotted dry, and exposed to Kodak XR1 X-ray film between two intensifier screens (Cronex Lightning Plus) for 1-5 hours.

Thermal Denaturation

Nitrocellulose filters containing pBR322 β-globin DNA were prepared as described previously (3), hybridized with ^{32}P labeled oligonucleotides as described for the blots above and washed with twenty changes of 6 X SSC (20 ml) at 0°C. The dissociation of labeled probe as a function of temperature in 6 X SSC was measured and the temperature at which half of the probe dissociated (T_d) calculated as described previously (3). Total amount of probe hybridized was determined by summation of the total cpm eluted from the filters during the thermal denaturation. Relative hybridization was corrected for the specific activity of each probe.

RESULTS

Sequence of Oligonucleotides

Figure 2 shows a short amino acid sequence of rabbit β-globin (17) and the corresponding mRNA sequence as determined by Efstratiadis, *et al.* (18). Two oligodeoxyribonucleotides 14-bases long were synthesized, one perfectly complementary to the mRNA (RβG14A) and one with a single base change (T for C) at a position 3 nucleotides from the 5'-end (RβG14B). This latter oligonucleotide is meant to serve as a model probe forming a single mismatch. Since the base change is close to the end of the sequence and because it forms a G-T base pair, it was thought to be a good example of a mismatch with minimal destabilizing effect.

A mixture of eight different 13-base long oligonucleotides was also synthesized (RβG13Mix, Figure 2). This model mixture does not represent all possible combinations of anti-codon sequences (there are 32 possible sequences), but does contain the sequence complementary to the mRNA amongst the mixture of eight. This sequence was only 13-bases long (lacked 3'-deoxyadenosine present in 14 mers) because 3'-deoxyadenosine is incompatible with the method of solid-phase synthesis used (11). The lack of a 3'-deoxyadenosine is not thought to have a significant effect

		15	16	17	18	19	
Amino Acid Sequence		Trp	Gly	Lys	Val	Asn	
mRNA Sequence	5'	UGG	GGC	AAG	GTG	AA	3'
Probe RβG14A	3'	ACC	CCG	TTC	CAC	TT	5'
Probe RβG14B	3'	ACC	CCG	TTC	CAT	TT	5'
Probe RβG13Mix	3'	CC	CCG A	TTC T	CAC T	TT	5'

FIGURE 2. Amino acid sequence (17) and corresponding mRNA sequence (18) for rabbit β-globin were used to design oligonucleotide probes. RβG14A is complementary to the globin mRNA, RβG14B has a single base change (C to T) 3 nucleotides from the 5' end. RβG13Mix is a mixture of eight sequences synthesized simultaneously as described in the Materials and Methods.

on thermal stability.

Thermal Stability of Oligonucleotide-Globin DNA Duplexes

Our previous studies on the hybridization behavior of a 14-base long oligonucleotide to ΦX174 DNA (3) demonstrated that the formation of mismatched duplexes could be virtually eliminated without significantly affecting the formation of perfectly matched duplexes by hybridizing at 37°C (in approximately 1 M Na^+). We therefore hybridized ^{32}P labeled RβG14A, RβG14B and RβG13Mix to rabbit β-globin containing plasmid DNA immobilized on nitrocellulose filters at 37°C. The thermal stability and hybridization efficiency was measured as described in Methods. It can be seen in Table 1 that RβG14B hybridized very poorly under these conditions, giving only 8% the amount of duplex as compared with the RβG14A probe. The temperature at which one half of the labeled RβG14A had dissociated from the filters (T_d) is 46°C compared with 41°C for RβG14B. This 5°C difference is smaller than the effect seen previously for a single A-C mismatch (3). This may be due to a lesser destabilizing effect of G-T mismatches than A-C mismatches, or to a position effect (19).

The ^{32}P-labeled RβG13Mix hybridized at 90% of the level of RβG14A. This is eleven-fold higher than the amount of hybridization seen for RβG14B. More importantly, the T_d of the RβG13-

TABLE 1.

Hybridization of Oligonucleotide Probes to Globin DNA

Probe	Relative Hybridization [a]	T_d [b]
RβG14A	1	45.7 ± 0.2
RβG14B	0.08	40.7 ± 1.0
RβG13Mix	0.90	46.0 ± 0.7

a Specific activity of RβG14A and RβG14B was nearly identical (2.7 X 10^8 cpm/μg), while RβG13Mix was 1.2 X 10^8 cpm/μg. For RβG14A, approximately 6000 cpm of probe were bound per μg of pBR322 β-globin DNA applied to the filter.

b The error estimates represent the error in the T_d parameter as calculated by the least squares fitting program (3).

Mix-globin DNA duplex is 46°C, identical with the RβG14A-globin DNA duplex.

Hybridization of RβG14A and RβG14B to β-Globin Genomic DNA

The difference observed between the thermal stability of RβG14A-globin DNA duplexes and RβG14B-globin DNA duplexes demonstrated that hybridization of RβG14B can be nearly eliminated without affecting hybridization of RβG14A. We can show this specificity more dramatically by hybridizing labeled RβG14A and RβG14B to a blot prepared from *Eco*R1 digested RβG1 DNA (rabbit β-globin gene DNA cloned in Charon 4A (4)). When RβG1 DNA is digested with *Eco*R1 endonuclease, ten restriction fragments are produced. The portion of the gene complementary to the three probes is present in a 2.6 kb fragment (20). Figure 3(A) shows the results of a hybridization of the ^{32}P labeled 14 mers to a blot of *Eco*R1 digested RβG1 DNA at 37°C in 0.9 M Na^+. It can be seen that virtually no hybridization of RβG14B to the 2.6 kb fragment is observed under these conditions while RβG14A hybridized well. Figure 3(B) shows a competition experiment where [^{32}P]RβG14A is hybridized in the presence or absence of a sevenfold excess of an unlabeled competitor, either RβG14A or RβG14B. It can be seen that only RβG14A effectively competes for hybridization of [^{32}P]RβG14A to globin DNA.

Hybridization Specificity of Mixed Probe

In order to determine whether the mixture of eight different 13-base long oligonucleotides could be used as a specific probe to detect globin DNA sequences, a blot experiment similar to that of Figure 3 was performed. When [^{32}P] labeled RβG14A and RβG13Mix were hybridized to a blot of *Eco*R1 digested RβG1 DNA at 37°C in 0.9 M Na^+ (Figure 4), the 2.6 kb fragment is seen to hybridize with both RβG13Mix and RβG14A under conditions where RβG14B does not (Figure 3(A)). The specificity of hybridization of RβG13Mix to the globin DNA, together with the thermal stability data of Table 1, demonstrates the usefulness of oligonucleotides of mixed sequences as probes.

Colony Hybridization

The ultimate application of oligonucleotides of mixed

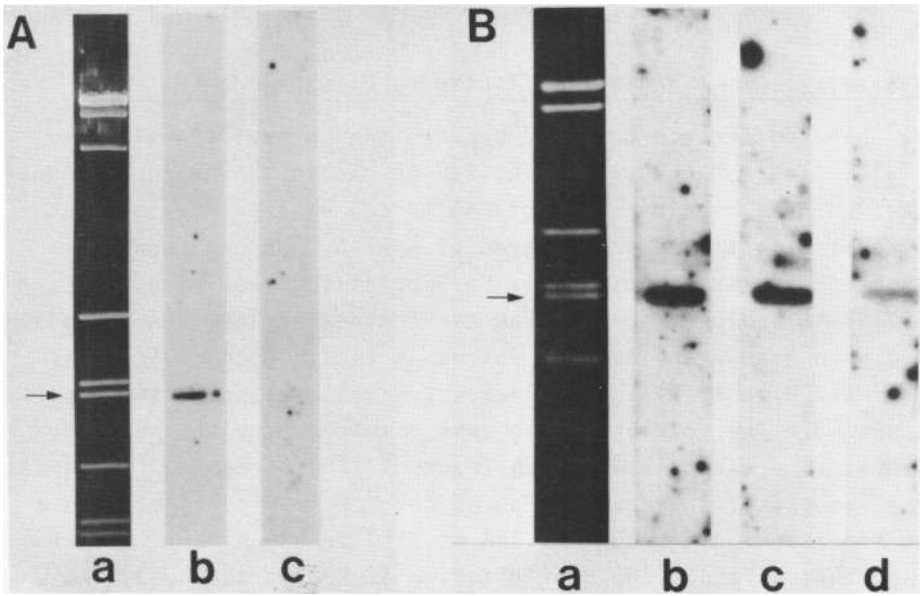

FIGURE 3. Hybridization of oligonucleotide probes to blots of RβG1 DNA. RβG1 DNA was digested with *Eco*R1 endonuclease, the restriction fragments separated by electrophoresis on an agarose gel and transferred to nitrocellulose by the blotting technique described in the Methods.

A. Three lanes of such a blot were hybridized at 37°C with [^{32}P] labeled RβG14A (lane b) or [^{32}P] labeled RβG14B (lane c) as described in Methods. Lane a shows the ethidium bromide stained gel. The restriction map for *Eco*R1 sites has been determined by Lacy, *et al.* (20). The 2.6 kilo base (kb) fragment (arrow) contains the portion of the globin gene complementary to the RβG14A probe.

B. Three lanes of the blot were hybridized with [^{32}P] labeled RβG14A in the presence of a seven-fold molar excess of unlabeled RβG14B (lane c) or RβG14A (lane d) or in the absence of unlabeled probe (lane b). Lane a again shows the ethidium bromide stained gel. The specific activities of the three probes were 4.8 X 10^8 cpm/μg for RβG14A and 3.7 X 10^8 cpm/μg for RβG14B.

sequences would be to use them as probes to screen recombinant clones for those which contain the desired sequence. In order to test the use of the mixed probe in colony screening, transformed cells which contained either pBR322 or pBR322 β-globin were grown and spotted onto a standard 10 cm diameter petri dish. Ten of fifty colonies contained globin DNA sequences. The colo-

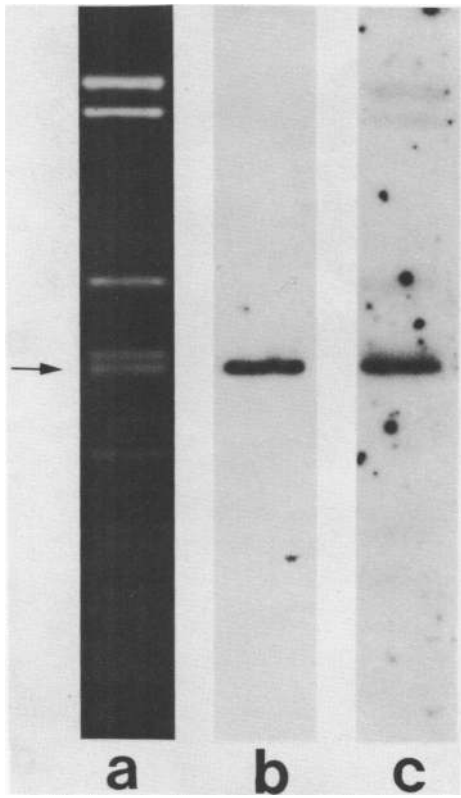

FIGURE 4. Hybridization of mixed probe to globin DNA. Two lanes of a blot of *Eco*R1 digested RβG1 DNA prepared as described in the Methods and Figure 2 were hybridized at 37°C with [^{32}P] labeled RβG14A (lane b) or RβG13Mix (lane c) under the conditions described in the Methods. Lane a snows the ethidium bromide stained gel. The specific activities of the probes were 4.8 X 10^8 cpm/μg for RβG14A and 6.9 X 10^8 cpm/μg for RβG13Mix. The 2.6 kb restriction fragment is marked with an arrow.

nies were grown, transferred to Whatman 540 paper, amplified and prepared for hybridization as described by Gergen, *et al.* (15). Filters were then hybridized with [^{32}P] labeled oligonucleotides as described in the Methods. Figure 5 shows the results of such a screening. As expected, [^{32}P]RβG14A clearly hybridizes to the ten globin DNA containing colonies and not to the others. [^{32}P]RβG14B is not seen to hybridize in this expo-

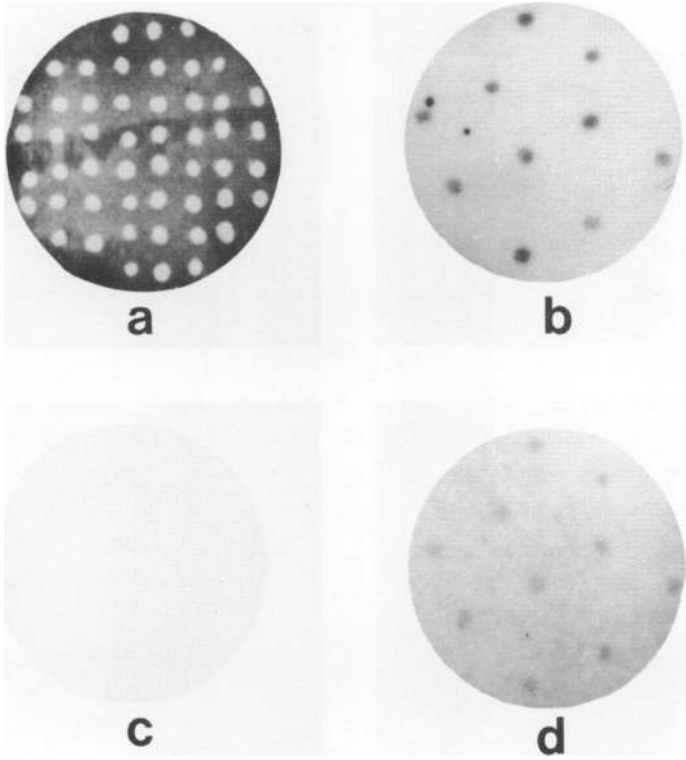

FIGURE 5. Hybridization of the oligonucleotide probes to globin DNA and non-globin DNA containing colonies. Cells transformed with either pBR322 or pBR322 β-globin DNA were picked onto a 10 cm petri dish in an ordered array, and grown overnight. Ten of the colonies contained globin DNA and 40 non-globin DNA. The colonies were lifted onto Whatman 540 filter discs, amplified with chloramphenicol and the filters prepared for hybridization as described (15). The filters were hybridized with [^{32}P] labeled RβG14A (b), RβG14B (c) and RβG13Mix (d) and washed and exposed to X-ray film as described in the Methods. One of these filters was stained with ethidium bromide and photographed under ultraviolet light (a) showing the position of all of the colonies. The positive signal seen for the RβG14A and RβG13Mix coincide with the positions where the globin DNA-containing colonies were placed.

sure, but if the filters are exposed to film much longer, RβG14B also hybridizes to the ten globin DNA colonies. [^{32}P]RβG13Mix is seen to hybridize specifically with the globin DNA colonies,

albeit with a significantly higher background due to the fact that eight-fold more labeled probe was present during the hybridization (15).

DISCUSSION

The results of this model study support the idea that oligonucleotides of mixed sequences can be used as specific hybridization probes in a general procedure for screening recombinant clones for the one desired.

The single base pair mismatch produced when the RβG14B probe is hybridized to globin DNA has a dramatic destabilizing effect. This effect not only allows us to discriminate between the formation of perfectly matched and single mismatched duplexes (Table 1, Figure 3(A)), but also permits hybridization of an oligonucleotide forming a perfectly matched duplex in the presence of a seven-fold molar excess of oligonucleotide with a single base change (Figure 3(B)).

The hybridization behavior of the mixed probe, RβG13Mix, is similar to that of the complementary probe, RβG14A. This is to be expected if the complementary sequence is present in the mixture and if the other members of the mixture do not participate in, nor interfere with, hybridization of the complementary sequence. In this mixture of eight sequences, one is completely complementary to the β-globin DNA (Figure 2), 3 would form a single base-pair mismatch (one A-C and two G-T base pairs), 3 would form two base-pair mismatches, and one would form 3 base-pair mismatches. The hybridization behavior of RβG14B predicts that the seven non-complementary oligonucleotides would have little effect on the hybridization of the single complementary oligonucleotide to globin DNA.

The colony screening experiment shown in Figure 5 demonstrates the potential application of oligonucleotides of mixed sequence to a general assay to screen for recombinant clones present in low abundance. We have previously shown (21) that an oligonucleotide 21-bases long could be used to identify specific clones when used in a colony screening procedure similar to that described by Grunstein and Hogness (22), i.e. where the DNA

from the colonies is immobilized on nitrocellulose filters. In preliminary studies on the hybridization of oligonucleotides of mixed sequence to globin DNA containing colonies, we found that nitrocellulose gave unacceptable results due mainly to high background levels. The use of the Whatman filters was not only found to be convenient and inexpensive (15), but also necessary for the experiments described here.

For the synthesis of oligonucleotides of mixed sequence, our strategy is to break the sequence down into trinucleotide blocks such that the base that differs in the third position of the codon is positioned in the middle of each trimer block. Therefore, the reaction to couple two groups of a trinucleotide mixture occurs between the identical bases for each group of trinucleotides (Figure 1). This design of the coupling reaction is probably essential to obtain an equal amount of the different sequences in the product, since the rate of coupling reactions among the four nucleotides is not identical (23). The fact that the mixture of sequences can be synthesized simultaneously is a great advantage to their use as hybridization probes.

Of the 20 amino acids specified by the genetic code, 11 of them are coded for by either one or two codons. Therefore, there is a high probability of finding peptide sequences 4 or 5 amino acids long within a known protein sequence for which each amino acid is one of these eleven. Once identified, these peptide sequences allow the design of probes, the sequence of which represent all possible combinations of codon (or anti-codon) sequences. The probability of finding a suitable peptide sequence for probe design is further increased by using the fact that 17 of the 20 amino acids are coded for by codons for which the first two nucleotides of the codon are unique. One of these 17 amino acids can be present at the C-terminal position of the peptide sequence being considered for probe design. Therefore, the usual probes designed from short peptide sequences will be 11-, 14- or, rarely, 17-bases long from 4, 5, and 6 amino acid long sequences.

Recently, Goeddel, *et al.* (24), have used an oligonucleotide of mixed sequence as a primer for the synthesis of cDNA from mRNA isolated from human fibroblasts induced to synthesize

interferon. The specifically primed cDNA was then used to screen for recombinant plasmids containing interferon cDNA sequences. Our approach differs, in that the oligonucleotide is used directly as a probe for screening purposes.

In summary, we have demonstrated in this model study that oligonucleotide probes of mixed sequence can be used to specifically identify complementary DNA sequences under stringent hybridization conditions. This demonstrates the feasibility of using oligonucleotides of mixed sequence to screen for clones whose DNA codes for a protein of known amino acid sequence.

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