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Hybridization of synthetic oligodeoxyribonucleotides to Φ χ 174 DNA: the effect of single base pair mismatch

R.Bruce Wallace, J.Shaffer*, R.F.Murphy* J.Bonner*, T.Hirose and K.Itakura

Division of Biology, City of Hope National Medical Center, 1500 East Duarte Road, Duarte, CA 91010, and *Division of Biology, California Institute of Technology, 1201 East California Street, Pasadena, CA 91125, USA

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ABSTRACT

Oligodeoxyribonucleotides complementary to the DNA of the wild type (wt) bacteriophage Φχ174 have been synthesized by the phosphotriester method. The oligomers, 11, 14, and 17 bases long, are complementary to the region of the DNA which accounts for the am-3 point mutation. When hybridized to am-3 DNA, the oligonucleotides form duplexes with a single base pair mismatch. The thermal stability of the duplexes formed between wt and am-3 DNAs has been measured. The am-3 DNA:oligomer duplexes dissociate at a temperature about 10°C lower than the corresponding wt DNA:oligomer duplexes. This dramatic decrease in thermal stability due to a single mismatch makes it possible to eliminate the formation of the mismatched duplexes by the appropriate choice of hybridization temperature. These results are discussed with respect to the use of oligonucleotides as probes for the isolation of specific cloned DNA sequences.

INTRODUCTION

Specific nucleic acid sequences such as rRNAs, tRNAs, mRNAs, complementary DNA, or cloned DNA sequences have proven invaluable as biological probes when radioactively labeled *in vivo* or *in vitro*. These probes have been used in studies of nucleic acid complexity (see 1 for review), measurement of specific gene frequency (2), examination of sequence divergence (3), studies on transcription of specific genes (1), and most recently in detection and isolation of cloned gene sequences (4,5,6). Of the probes presently available, those for specific single copy genes are receiving the most attention. Unfortunately, only a handful of these probes are currently available. For the most part, single copy gene probes are derived from specific highly abundant mRNAs such as globin (6), ovalbumin (5), immunoglobulins (4) and others. At present, probes for single copy gene se-

quences whose mRNAs are not abundant are very difficult to isolate.

One promising alternative to the use of isolated naturally occurring nucleic acid probes, is the use of chemically synthesized oligodeoxyribonucleotide sequences. Recently, a specific 13 nucleotide sequence complementary to the gene for yeast iso-1-cytochrome c was used to detect and isolate the gene sequence cloned in a bacteriophage λ vector (7). This was possible because the actual nucleotide sequence of a region of the gene could be deduced from genetic information. As a more general approach to the use of oligodeoxyribonucleotides as probes, we propose to use a chemically synthesized mixture of oligonucleotides whose sequences represent all possible codon combinations predicted from a particular peptide sequence within a protein. One of this mixture must be complementary to a region of DNA coding for the protein. Stringent hybridization criteria would then be used to select the single correct sequence from the mixture. As a preliminary investigation, we have chosen to study the hybridization behavior of three oligonucleotides, 11, 14, and 17 bases long, to DNA from wild type (wt) and am-3 bacteriophage Φχ174. The three oligonucleotide sequences are complementary to wt DNA at the region encompassing the am-3 point mutation (8). Duplexes formed between the oligonucleotides and am-3 DNA contain a single mismatched base pair. This system represents a useful model for the study of the effect of mismatched base pairs on duplex formation and stability.

MATERIALS AND METHODS

Synthesis of Oligodeoxyribonucleotides

The oligodeoxyribonucleotides were synthesized by the modified triester method (9). Their use in synthetic DNA directed base change of Φχ174 DNA has been described previously (10). The oligonucleotides were gifts of Genentech, Inc., San Francisco.

Preparation of Phage DNA

The Φχ174 wt and am-3 DNAs were gifts from Dr. Aharon Razin. The DNAs were isolated from purified phage as described (11).

Labeling of Oligonucleotides

The synthesis of oligonucleotides leaved 5'OH. The oligonucleotides are labeled by transferring the ^{32}P from [γ-^{32}P]ATP

(1000 Ci/mmole) with polynucleotide kinase (Boehringer Mannheim) as described (12). Oligonucleotide (0.2 µg) was labeled and separated from [γ-^{32}P]ATP by chromatography on Sephadex G-50. The excluded peak was pooled and used directly in hybridization experiments.

Preparation and Hybridization of DNA filters

The DNA was incubated in 0.2 N NaOH at 37°C for 30 minutes, neutralized with HCl and brought to 6 X SSC at 0°C. Nitrocellulose filters (Sartorious Membranfilter, 2.5 cm, pore size 0.45 µ) were wet in H_2O, washed in 6 X SSC (1 X SSC = 0.15 M NaCl, 0.015 M sodium citrate, pH 7.2) and placed on a filter apparatus. The amount of DNA loaded on the filters depended on the nature of the experiment. For the thermal denaturation experiments, 0.05 µg of wt and am-3 DNA were applied to each filter. For kinetic experiments, 0.005 µg wt DNA and 0.025 µg am-3 DNA were applied to each of ten filters. After application of DNA, the filters were baked at 80°C *in vacuo* for 4 hours.

For hybridization, the filters were placed in 6 X SSC, 10 X Denhardt's [10 X Denhardt's = 0.2% bovine serum albumin (Sigma), 0.2% polyvinylpyrolidone (Sigma), 0.2% Ficoll (Sigma) (13)] at room temperature for 15 minutes. The solution was drained and replaced with 2 ml of hybridization solution (6 X SSC, 10 X Denhardt's, 0.002 µg/ml ^{32}P labeled oligonucleotide). Unless otherwise stated, hybridization was performed at 12°C for 16 hours. The filters were then washed with multiple changes of 6 X SSC at 12°C, until no more radioactivity eluted.

Thermal Denaturation

Filters which had been hybridized and thoroughly washed as described above were used for thermal denaturation studies as follows: 5 ml of 6 X SSC was placed over the filter and the temperature raised to a specific point. Once the temperature had been reached, the filter was kept at that temperature for one minute, the 6 X SSC was then removed for measurement of the radioactivity eluted (in Aquasol 2, New England Nuclear). An additional 5 ml of 6 X SSC was then added and the procedure repeated until the desired maximum temperature was reached.

The radioactivity eluted at each temperature was integrated to determine the fraction of the duplex denatured as a function

of temperature. In order to determine the temperature at which one half of the oligonucleotide dissociates from the filter (T_d), this data was fit to the error function by a nonlinear least squares fitting program as described previously (14,15).

In each experiment, essentially all of the bound oligonucleotide was removed by the highest temperature wash. In addition, none of the Φχ174 DNA bound to the filter was lost during the procedure, since an equal amount of ^{32}P-labeled oligonucleotide will hybridize to the same filter in a second or third experiment.

Agarose Gel Electrophoresis

Vertical agarose gels (SeaKem) were used to separate DNA samples for transfer to nitrocellulose. The gels were 15 cm X 15 cm X 0.2 cm and were electrophoresed at 200 volts for 2 hours. Undigested DNA was separated on 1% agarose gels while Hae III digested Φχ174 am-3 RFI DNA (Bethesda Research Laboratories) was separated on 2% agarose gels. After electrophoresis, gels were stained with 0.1 μg/ml ethidium bromide (Calbiochem) for 30 minutes and photographed through an orange filter under an ultraviolet light source. The DNA was denatured by soaking the gel in 0.4 N NaOH, 0.8 M NaCl for 30 minutes. The gel was neutralized in 0.5 M tris•HCl, pH 7.6, 1.5 M NaCl for 30 minutes in the cold and the DNA transferred to nitrocellulose filters (Millipore HAWP 00010) as described by Southern (16). The filters were baked at 80°C *in vacuo* for at least 4 hours.

The filters were hybridized in 6 X SSC, 10 X Denhardt's, 0.002 μg/ml ^{32}P-labeled oligonucleotide at the temperatures specified in each experiment. After hybridization the filters were washed in 500 ml 6 X SSC at 12°C, blotted dry and autoradiographed using pre-flashed Kodak XR-2 X-ray film exposed between 2 intensifier screens (Cronex Lightning Plus, Dupont) at -80°C for 12-36 hours.

RESULTS

Thermal Stability of Oligonucleotide - Φχ174 DNA Duplexes

In order to study the hybridization of synthetic oligodeoxyribonucleotides to natural DNA, we synthesized three oligo-

nucleotides of chain length 11, 14, and 17 which are complementary to the single stranded DNA (+ strand) of the wild type (wt) bacteriophage ΦX174.

The 11 mer and 14 mer are synthetic intermediated of the 17 mer. The 17 mer is complementary to nucleotides 575 through 591 in the linear sequence of ΦX174 DNA reported by Sanger and coworkers (8) (Figure 1). These sequences represent useful models for the study of single base pair mismatch since duplexes formed with am-3 ΦX174 DNA contain one A-C base pair [amber mutation is a G→A transition at position 587 of the DNA sequence (Figure 1)].

For hybridization of ^{32}P-labeled oligonucleotides to wt and am-3 ΦX174 DNA, the phage DNA was immobilized on nitrocellulose filters. Initially, hybridizations were performed at 12°C in 6 X SSC, 10 X Denhardt's ($[Na^+]$ = 1.2 M) (see Materials and Methods). From previously published results (17-20), the duplexes formed between the 11 mer, 14 mer or 17 mer and wt DNA were expected to be rather stable with Tm's greater than 30°C. Under the conditions of the hybridization or the subsequent washing of the filters (in 6 X SSC), the oligonucleotides do not adhere

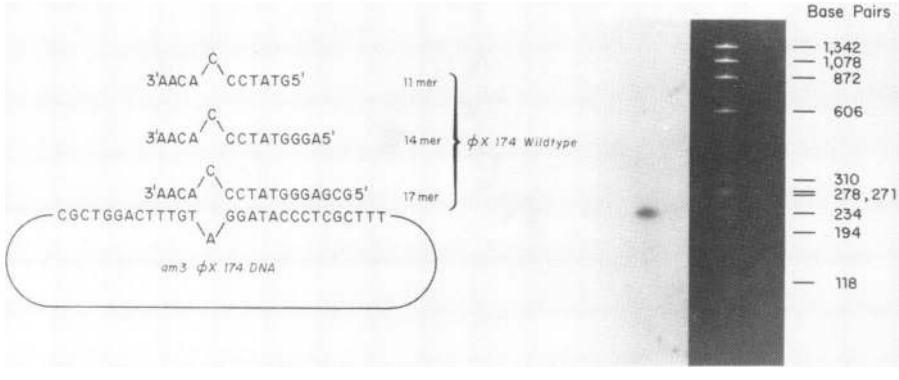

FIGURE 1. A representation of the mismatched duplexes formed between the three oligonucleotides and am-3 ΦX174 DNA. On the right, am-3 ΦX174 RFI DNA, which was digested with Hae III, was electrophoresed on a 2% agarose gel and blotted onto nitrocellulose as described in Materials and Methods. The filter was hybridized to ^{32}P-labeled 14 mer at 12°C, washed at 12°C and autoradiographed. It can be seen that hybridization is to the 234 base pair long restriction fragment which contains the am-3 mutation at nucleotide 587 (8).

nonspecifically to the nitrocellulose (see Figure 1).

The hybridization of all 3 oligonucleotides to wt ΦX174 DNA was quite efficient. Between 13 and 22% of the sites on the phage DNA molecules hybridize with the labeled oligomers (Table 1). The stability of the oligonucleotide-wt DNA duplexes were examined by thermal denaturation. Filters which had been hybridized and washed at 12°C were heated to various temperatures in 6 X SSC and the radioactivity which eluted was measured. The thermal denaturation profiles are presented in Figure 2. The data is summarized in Table 1. Note that the parameter T_d, the

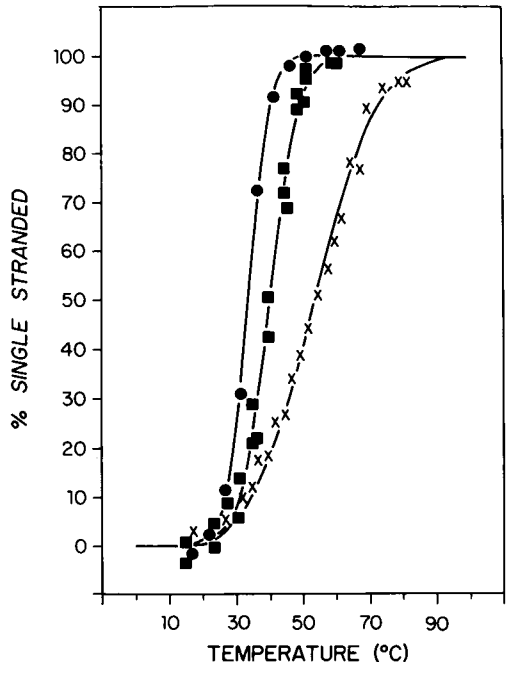

FIGURE 2. Thermal denaturation of oligonucleotide-wild type ΦX174 DNA duplexes. The 11 nucleotide (●), the 14 nucleotide (■), and 17 nucleotide (X) probes shown in Figure 1 were labeled with [^{32}P] in the 5' end and hybridized to wild type ΦX174 DNA immobilized on nitrocellulose filters. The hybridization was performed in 6 X SSC, 10 X Denhardt's (13) and 0.002 µg probe/ml. The filters were washed in 6 X SSC and subjected to thermal denaturation. The radioactivity eluted at each temperature was measured and is plotted as the fraction of the total probe becoming single stranded at each temperature.

TABLE 1.

EFFECT OF MISMATCH ON HYBRIDIZATION OF OLIGONUCLEOTIDES PROBES TO ΦX174 DNA.

Number Nucleotides In Probe	ΦX174 DNA Hybridized	Number Nucleotides In Duplex	% Sites Hybridized	% G+C In Duplex	T_d[a] Observed[b] (C°)
11	wt	11	20	46	33.2 ± 0.4
11	am-3	10	-	-	-
14	25	14	13.6	50	40.6 ± 0.7
14	am-3	13	2.9	43	31.1 ± 0.8
17	wt	17	22.6	59	55.1 ± 0.8
17	am-3	16	11.3	53	43.5 ± 0.3

a T_d is the temperature at which one half of the duplexes are dissociated under the conditions of the experiments.

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

temperature at which one half of the duplexes are dissociated, is used rather than Tm since the experiment does not allow direct measurement of Tm in a thermodynamically rigorous way. As expected, an increase in thermal stability is seen with an increase in duplex length.

Compared to the wt DNA, hybridization of the 3 oligonucleotides to am-3 DNA is much less efficient (Table 1). In fact, the level of hybridization of the 11 mer to am-3 DNA was barely above background and determination of an accurate T_d was not possible. The thermal denaturation profiles of the oligonucleotide-am-3 DNA duplexes are presented in Figure 3. The oligonucleotide-wt DNA melts are plotted for comparison. The data is summarized in Table 1. It can be seen that the thermal stability of the 14 mer and 17 mer duplexes with am-3 DNA is much lower than that of the corresponding wt duplexes.

The substantial difference in the thermal stability of perfectly matched and mismatch duplexes suggests that hybridization of the oligonucleotides to am-3 DNA could be eliminated with little, if any, effect on hybridization to wt DNA by the appropriate choice of either filter wash temperature or hybridization temperature. To test this prediction, wt and am-3 DNAs were electrophoresed in adjacent lanes of a 1% agarose gel. The DNA in the gel was transferred to nitrocellulose essentially as described by Southern (16). The filter strips were hybridized at

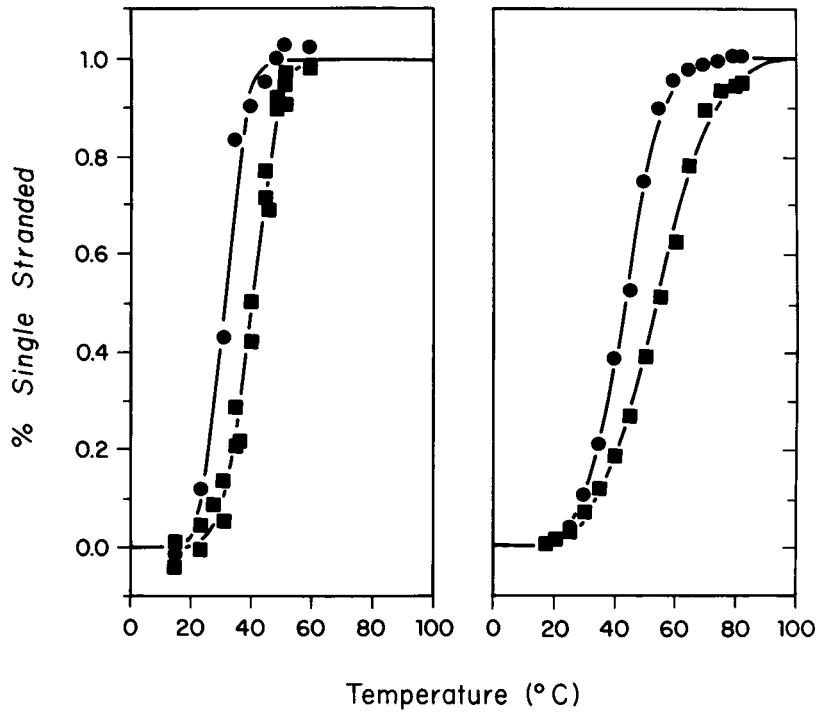

FIGURE 3. Thermal denaturation of oligonucleotide-wild type ΦX174 DNA (■) and oligonucleotide-am-3 ΦX174 DNA (●) duplexes. The hybridization and thermal denaturation were performed as in Figure 2. Left 14-mer and right 17-mer.

12°C with ^{32}P-labeled 11 mer, 14 mer or 17 mer, washed at 12°C and autoradiographed. Figure 4a shows the autoradiograph obtained. Hybridization to wt DNA is approximately equal for all 3 oligonucleotides. The level of hybridization to am-3 DNA, on the other hand, is dependent on the length of the oligonucleotide. Very little hybridization to am-3 DNA is seen for the 11 mer, a slightly greater amount for the 14 mer, while hybridization of the 17 mer to am-3 DNA approaches that to wt DNA (see Table 1 for comparison). After autoradiography, the filter strips were rewashed at higher temperatures, 30°C for the 11 mer, 37°C for the 14 mer, and 50°C for the 17 mer. The autoradiograph of the rewashed filters is shown in Figure 4b. It can be seen

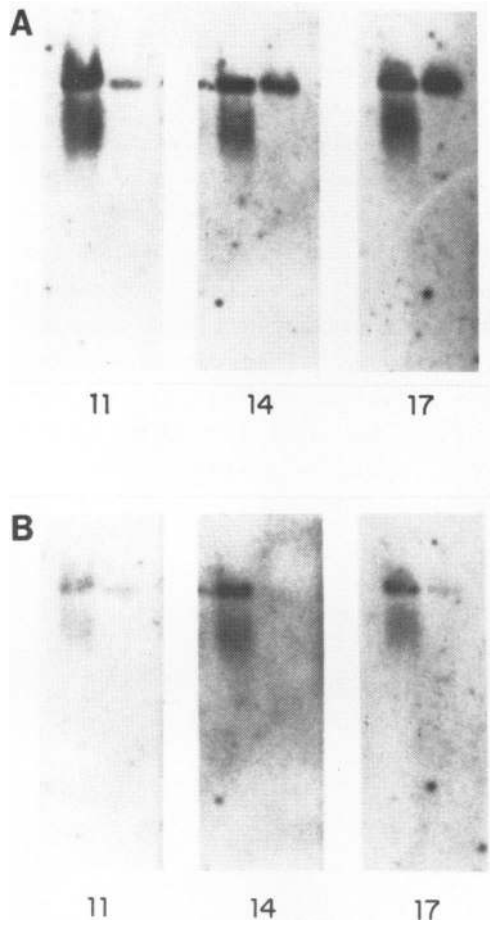

FIGURE 4. Effect of filter wash temperature on hybridization of ^{32}P-labeled oligonucleotides to wt and am-3 ΦX174 DNA. Equal amounts of single stranded wt (on the left) and am-3 DNA (on the right) were electrophoresed on 1% agarose gels and blotted onto nitrocellulose filters as described in Materials and Methods. Three filters, each containing one band of wt and one of am-3 DNA, were hybridized with ^{32}P-labeled 11-mer, 14-mer, and 17-mer at 12°C. The filters were washed at 12°C and autoradiographed overnight with preflashed X-ray film between two screens at -80°C (A). The filters were then rewashed at 30°C (11-mer), 37°C (14-mer) and 50°C (17-mer) and re-autoradiographed (B).

that hybridization to the am-3 DNA is virtually eliminated in each case while hybridization to wt DNA is only diminished

slightly.

Oligonucleotide Hybridization at Elevated Temperatures

In order to examine the effect of hybridization temperature on the formation of non-mismatched or mismatched duplexes, wt and am-3 DNAs were electrophoresed in alternate lanes of a 1% agarose gel and the DNA transferred to a nitrocellulose filter. The filter was cut into strips containing one band of wt and one of am-3 DNA each. The strips were hybridized in 6 X SSC, 10 X Denhardt's, 0.002 µg ^{32}P-labeled 14 mer/ml at 12°C, 25°C, 30°C, 35°C, and 40°C, washed at 12°C, and autoradiographed. The results are shown in Figure 5. It can be seen that hybridization to am-3 DNA is dramatically reduced at 25°C and higher. Hybridization to wt DNA is not affected between 25°C and 35°C with only a slight decrease in hybridization at 40°C (1°C below

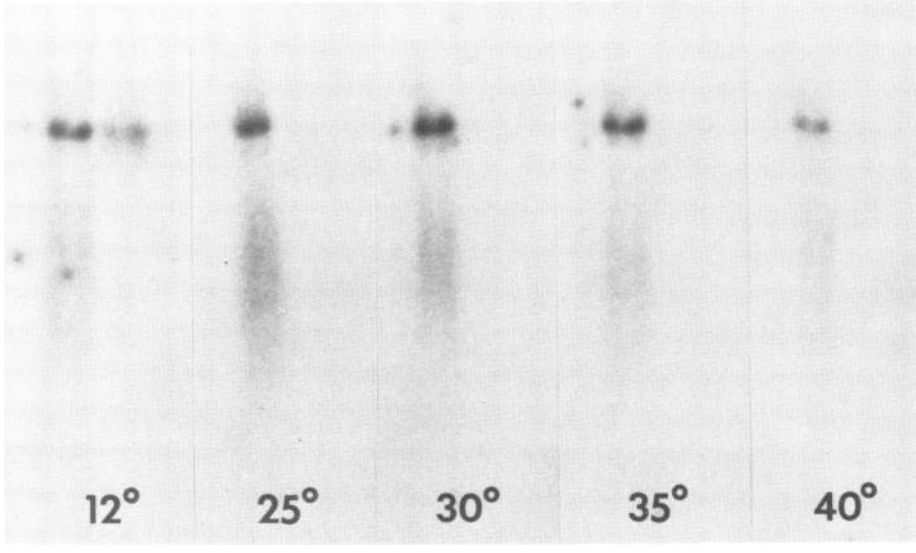

FIGURE 5. Effect of hybridization temperature on the formation of duplexes between ^{32}P-labeled 14-mer and wt (on the left) and am-3 DNA (on the right). Equal amounts of wt and am-3 DNAs were electrophoresed in alternate lanes of a 1% agarose gel and blotted onto nitrocellulose filters. The filters were cut into strips and hybridized at various temperatures (12°, 25°, 30°, 35° and 40°C) as described in Materials and Methods. The strips were then removed from the hybridization solution, washed briefly at 12°C and autoradiographed.

T_d).

Kinetics of Oligonucleotide Hybridization

In order to determine the optimum time required for oligonucleotide hybridization, the kinetics of duplex formation were measured. Multiple filters containing wt or am-3 DNA were prepared. Individual filters were hybridized for various time, washed immediately to remove unbound probe and the radioactivity measured. Figure 6 shows the kinetics of hybridization of ^{32}P-labeled 14 mer to wt and am-3 DNA at 12°C and wt DNA at 37°C. The data is plotted as equivalent $C_o t$ versus fraction hybridized. The data has been fit to a first order rate equation (14). The rate constants and $C_o t$ ½ are presented in Table 2. The measured rate constant is within a factor of 3 (for 37°C) that calculated by the equation of Wetmur and Davidson (22).

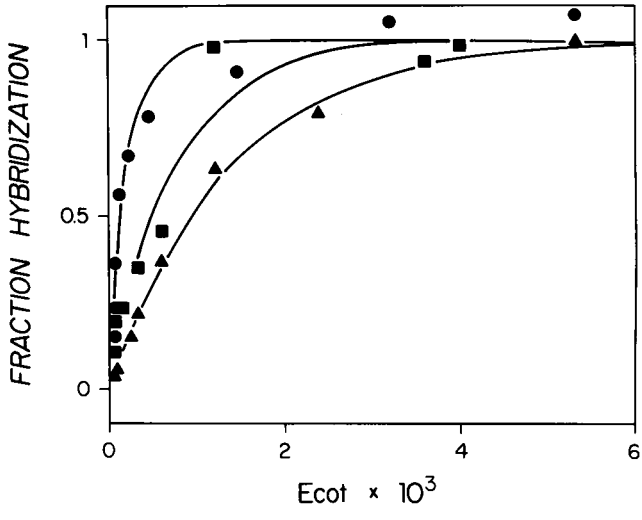

FIGURE 6. Kinetics of hybridization of ^{32}P-labeled 14-mer to wt DNA at 12°C (■) and 37°C (●) and to am-3 DNA at 12°C (▲). Individual filters containing 0.005 µg wt DNA or 0.025 µg am-3 DNA were hybridized for various times in 6 X SSC (1.2 M Na^+), the filters washed, and the radioactivity determined. The $C_o t$ was corrected for hybridization in 0.18 M Na^+ as described (21). The data was fit to a first order rate equation by a non-linear-least-square fitting program as described (14,15).

TABLE 2.

KINETICS OF HYBRIDIZATION OF ^{32}P-LABELED 14 MER TO ΦX174 DNA.

ΦX174 DNA	Temp. (°C)	k[a] ($M^{-1} \cdot sec$)	Cot½[b] (mole nucleotide·$\ell^{-1} \cdot sec^{-1}$)
wt	12	1251 ± 321	5.5×10^{-4}
wt	37	4037 ± 960	1.72×10^{-4}
am-3	12	722 ± 79	9.6×10^{-4}
Calculated[c] kinetic parameters		10440	6.6×10^{-5}

a Rate constant calculated for a first order reaction as described (14,15).

b Cot½ is calculated from k ($\ln 2/k$). All data has been corrected for hybridization in 0.18 M Na^+ (21).

c Rate constant is calculated as described by Wetmur and Davidson (22) and corrected for 0.18 M Na^+ as described (21).

DISCUSSION

This study has taken advantage of the naturally occurring base substitution in ΦX174 DNA resulting in the am-3 point mutation (8). As such, the effects of single base pair mismatch examined here refer only to this single example, an A-C base pair flanked on one side by four base pairs and on the other by 6 to 12 base pairs. Many previous studies have examined the effects of mismatch in model RNA (23,24) and DNA homo- and copolymers (25,26). Very few studies, however, have examined the effects of single base pair mismatch on duplexes formed between oligodeoxyribonucleotides and naturally occurring DNA sequences. It was the main purpose of this study to examine the effects of such mismatches for a defined naturally occurring DNA in order to establish conditions under which the formation of mismatched duplexes could be eliminated. Such conditions are necessary in order to use the specificity of oligonucleotide hybridization as a probe for defined DNA sequences.

Montgomery, *et al.* (7) have recently reported the use of an oligodeoxyribonucleotide of defined sequence as a probe to isolate the yeast gene for cytochrome c. While the 13 nucleotide probe was used successfully in the isolation of the cloned cytochrome c gene, the authors pointed out many difficulties which were encountered. Among these were the poor reproducibility of hybridization of the ^{32}P-labeled 13 mer from experiment to ex-

periment and the poor signal obtained with the labeled probe in the plaque screening assay. Model studies such as this one are very useful, therefore, in establishing conditions which optimize signal and minimize nonspecific interactions.

The single base pair mismatch in the duplexes formed between the 11-, 14- and 17-nucleotide oligomers and am-3 ΦX174 DNA has a significant destabilizing effect. The single base pair mismatch in the case of the 17 mer and the 14 mer resulted in complexes with a thermal stability approximately equivalent to a non-mismatched duplex three base pairs shorter (Table 1). A similar degree of destabilization has been reported for various single and multiple mismatched base pairs in oligonucleotide duplexes by Dodgson and Wells (26). The lower thermal stability of the am-3 DNA:oligomer hybrids make it possible to differentiate between the mismatched and non-mismatched duplexes by the appropriate choice of filter wash temperature (Figure 4) or hybridization temperature (Figure 5). These results demonstrate the high degree of specificity which can be achieved using oligonucleotide probes.

In addition to the effects on thermal stability, a single base pair mismatch has a significant effect on the level of hybridization achieved. This is most clearly seen in the autoradiograph shown in Figure 4. At lower temperature (12°C), the level of hybridization of oligomer to wt DNA is approximately equal for all three (11 to 17) while the level of hybridization to am-3 is dependent on the length of the oligomer. The reason for this phenomenon is not known, however, an examination of the rates of hybridization of the 14 mer to wt and am-3 DNAs (Figure 6) demonstrate that the difference in the levels of hybridization are not due to a lower rate of formation of the mismatched duplexes (at low temperatures). It is conceivable that the rate of dissociation of the mismatched duplexes is greater than those of perfectly matched duplexes and that this is responsible for the lower levels of hybridization to am-3 DNA over wt DNA.

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