Metastable Single-strand DNA Conformational Polymorphism Analysis Results in Enhanced Polymorphism Detection

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Single-strand DNA conformational polymorphism (SSCP) makes use of sequence-dependent folding of single-stranded DNA (ssDNA), which alters the electrophoretic mobility of the fragments, to detect sequence differences between closely related molecules. In this study ssDNAs were purified by depletion of the complementary strand and PCR reactants on magnetic M-280-streptavidin beads. It was found that SSCP profiles created by purified ssDNAs differ from the profiles created by more usual SSCP methods. Under some conditions, SSCP profiles using whole PCR reaction products may result from the interaction between residual PCR primers and ssDNAs. We observed that the ratio of conformers revealed by band position and band intensity may vary between the assay techniques and misinterpretation of sequence variants may result. Another observation of this study was the formation of metastable conformational isomers with bead-purified ssDNAs by eliminating the thermal treatment used in conventional SSCP methods. The metastable SSCP (mSSCP) represents a novel and sensitive system for detection of sequence variation between closely related DNAs. The technique used here for the preparation of the purified ssDNAs is potentially useful for automated PCR-SSCP analysis using capillary electrophoresis or other methods.

The development and application of nonselective techniques for the detection of single base variations in DNA sequence is a major pursuit of molecular genetic research whether directed toward identification of human inherited disorders or human pathology, to the typing of pathogens, or the description of strains and isolates of organisms for ecological or biochemical purposes. Single-strand DNA conformational polymorphism (SSCP) is one of the techniques used most widely for detection of small sequence changes or point mutations because of its rapidity, ease, and generally high sensitivity.⁽¹⁻⁷⁾ The technique relies on the electrophoretic polyacrylamide gel (PAGE) fractionation of distinctive conformational isomers (conformers) of single-stranded DNAs (ssDNAs) generated by denaturation of short PCR products.⁽¹⁾ Theoretically, single base changes may alter the mobility of one or both of the ssDNA strands; however in practice, some changes do not alter the mobility of either strand and thus are not detectable.^(2,3) The sensitivity of SSCP analysis is high (frequently >90%) for short DNA fragments (<200 bp), although the conventional use of polyacrylamide gels with simple heat dissipation can contribute to anomalies and variation in the mobility of DNAs.^(2,7,8) Although isotopic labeling of DNA strands is recommended for detection of low abundance conformers, several studies have suggested that non-isotopic detection of SSCP can be sufficiently sensitive.^(9,10) The direct use of unlabeled PCR products for SSCP analysis is attractive because cost is low and

handling is minimal. However, the presence of both complementary DNA strands following denaturation requires rapid handling to prevent strand reannealing.^(1,2) Furthermore, although in conventional SSCP two bands representing the two ssDNAs are anticipated, many reports show evidence that more than two ssDNA bands are present.^(1-4,8) Although these data have been published, little attempt has been made to interpret the source of the additional ssDNA bands.⁽¹¹⁾

We have explored the possibility of enhancing the sensitivity of SSCP by the use of isolated, ssDNA. PCR products were made using one biotinylated primer and one unmodified primer to allow selective purification of the unmodified strand by removal of the biotinylated strand using magnetic bead techniques. To identify low-abundance molecules the SSCP conformers were also 5'-end labeled using γ -³⁵S-labeled ATP and were analyzed on conventional polyacrylamide gels and detected by autoradiography. We found that SSCP analysis of ssDNA, freed from its complementary strand, had several advantages. The detection of true ssDNA conformers was greatly enhanced, and novel metastable DNA conformers that are not seen by conventional SSCP were readily detected by autoradiography. The ssDNAs were stable for subsequent electrophoretic analysis after ≤ 12 hr storage at room temperature. This would be advantageous for the automated handling and electrophoresis of SSCP by high-performance capillary electrophoresis (HPCE)⁽¹²⁾ or by conventional PAGE

analysis. We also found evidence that SSCP profiles of unpurified PCR products may not contain easily detectable true ssDNA bands because of the interaction between PCR primers and the ssDNA. Removing the primers from the amplified PCR products could eliminate the spurious bands from the SSCP profiles. The use of isolated ssDNA for SSCP analysis could therefore minimize the potential for false-negative and false-positive bandshifts.

MATERIALS AND METHODS

DNA Isolation

Briefly, DNA was isolated from mycelial cultures of European *Heterobasidion annosum* genotypes⁽¹³⁾ as described previously.⁽¹⁴⁾

PCR Amplification of Ribosomal ITS Region

Separate PCR reactions were performed in a total mixture of 25 μ l, containing 5 ng of template DNA and 225 nM of the fungal rDNA primer pairs HA1/ITS4^(14,15) which amplify part of the internal transcribed spacer region (ITS2) flanking the 5.8S rRNA gene. Amplification was as described previously⁽¹⁴⁾ using either *Taq* DNA polymerase or Dynazyme. *Taq* DNA polymerase was obtained from Boehringer and Dynazyme from Flowgen Labs. The PCR products were separated on agarose gels and were purified by Ultrafree-MC filters (Millipore). Oligonucleotide primers were 5'-end labeled using γ -³⁵S-labeled ATP (Amersham) and T4-polynucleotide kinase (T4-PNK) (Boehringer). T4-PNK was removed by phenol extraction and the primers were recovered by ethanol precipitation.⁽¹⁶⁾ Magnetic M-280-streptavidin Dynabeads were purchased from Dynal (UK).

Conventional ssDNA

The HA1/ITS4 products were synthesized as described above. For conventional SSCP the PCR products were boiled for 3 min with STOP buffer (95% formamide, 20 mM EDTA, 0.05% each of bromophenol blue and xylene cyanol)⁽¹⁶⁾, snap-frozen in a dry ice-acetone bath, and brought to 0°C on ice before loading onto polyacrylamide gels that were run at 18°C.

Purification of ssDNA

Biotin-5'-labeled primers were used in conjunction with unmodified primers for PCR amplification of products for ssDNA isolation. M-280-streptavidin beads (100 μ l) were aliquoted into a 1.5-ml Eppendorf tube and washed twice with 200 μ l of PBS, 0.1% BSA (pH 7.5), on the magnetic particle concentrator (MPC). Beads were resuspended in 50 μ l of washing buffer (1 $\times$ TE, 1 M NaCl) (1 $\times$ TE = 10 mM Tris-HCl, 1 mM EDTA at pH 8.0), and 15 μ l of PCR-amplified DNA was diluted to 150 μ l with water and mixed gently with the beads for 30 min at room temperature on a rotator. After binding of the biotin to the streptavidin, the beads were concentrated on the MPC, the supernatant was removed, and the beads were washed three times with washing buffer. The unmodified ssDNA was released by the addition of 40 μ l of 120 mM NaOH for 15 min with occasional mixing. Beads retaining the biotinylated DNA strand were removed using the MPC and the supernatant was collected. The supernatant was neutralized by the addition of 3.6 μ l of 1.7 M acetic acid, and the ssDNA was collected by ethanol (150 μ l) precipitation without carrier at -70°C. Purified ssDNA was dissolved in 15 μ l of 1 $\times$ TE and stored at 4°C. Samples of ssDNA could be analyzed directly for SSCP after storage periods of up to 1 week without a change in profile.

DNA Labeling

To create singly labeled DNA strands in duplex DNA, individual primers were 5'-end labeled using 10 units of TF-PNK prior to PCR amplification of the HA1/ITS4 products, in conjunction with an unlabeled second primer. The Tf-PNK reaction was carried out in a total volume of 15 μ l for 2 hr at 37°C. An equal volume of STOP gel loading buffer (U.S. Biochemical) was added, and 5- μ l amounts, containing ~13 ng of DNA, were loaded onto polyacrylamide gels following denaturation. For the primer interaction experiments, additional ³⁵S-end-labeled oligonucleotide primer (HA1 or ITS4) was added to unlabeled, purified, denatured DNAs to a concentration of 225 nM immediately prior to electrophoresis.

In experiments with biotin-isolated ssDNA, 10 μ l of biotin-isolated ssDNA was 5'-end-labeled with γ -³⁵S-labeled

ATP by 10 units of T4-PNK in a total volume of 15 μ l for 2 hr at 37°C. An equal volume of STOP gel loading buffer (U.S. Biochemical) was added, and 5- μ l amounts, containing ~13 ng of DNA, were loaded onto polyacrylamide gels following denaturation.

PAGE-SSCP

SSCP were electrophoresed on 7% non-denaturing polyacrylamide gels (89:1 acrylamide/bisacrylamide) containing 5% glycerol in 0.5 $\times$ TBE buffer (TBE = 90 mM boric acid, 90 mM Tris base, 2 mM EDTA at pH 8.5) at 18°C, 8 W for 3–4 hr. DNA was visualized by autoradiography, or by ethidium bromide staining accompanied by image integration (0.4 sec) on an ImageStore 5000 video imaging system (UVP, Cambridge).

RESULTS

DNA fragments from *H. annosum* strains S_{Fi} and F_{It} that differ by 1 base substitution and 1 base deletion (1D + 1S/1B) were considered (see Fig. 1).

Autoradiographic Detection

Detection of SSCP using ³⁵S autoradiography (Fig. 2A) and ethidium bromide staining (Fig. 2B) were compared. The SSCP of agarose gel purified HA1/ITS4 products of S_{Fi} and F_{It} (Fig. 2A, lanes 1 and 6, respectively) show reannealed duplex DNA (marked d) as well as several strong bands and multiple weaker bands that migrated in a small region of the gel. The major lower (marked l) and upper (marked u) SSCP bands of the ssDNA were identified as HA1 strand and ITS4 strand, respectively, by specifically labeling each strand (Fig. 2A, lanes 2,3). Reannealing between residual unincorporated labeled primer and its complementary ssDNA produced the additional weak signals in each lane.

S_{Fi} : HA1-----T-----A-----ITS4 (173bp)

F_{It} : HA1-----C-----X-----ITS4 (172bp)

FIGURE 1 Representation of the ITS2 ribosomal region PCR products. Nucleotide differences between S_{Fi} and F_{It} are 1 deletion, 1 substitution, 1 base pair length = 1D + 1S/1B.

FIGURE 2 PAGE-fractionated SSCP of purified single-strand DNA detected by ^{35}S autoradiography (A) or by ethidium bromide staining (B). The ITS4/HA1 PCR products were 5'-end labeled with $\gamma\text{-}^{35}\text{S}$ -labeled ATP or were unlabeled, as indicated. The reannealed duplex DNA (d) and the major lower (l) and upper (u) SSCP conformers of the ssDNA are identified. (M) Metastable conformer region. (Lane 11) pUC₁₉, *Hae*III marker; (lanes 1–5, 6–10) S_{Fi} and F_{It} products, respectively. (Lane 1) Gel-purified S_{Fi} product, both strands labeled; (lane 2) gel-purified S_{Fi} product, labeled HA1 primer strand; (lane 3) gel-purified S_{Fi} product, labeled ITS4 primer strand; (lane 4) biotin isolated, labeled ITS4 primer strand alone; (lane 5) biotin isolated, labeled HA1 primer strand alone; (lane 6) gel-purified F_{It} product, both strands labeled; (lane 7) gel-purified F_{It} product, labeled HA1 primer strand; (lane 8) gel-purified F_{It} product, labeled ITS4 primer strand; (lane 9) biotin isolated, labeled ITS4 primer strand alone; (lane 10) biotin isolated, labeled HA1 primer strand alone.

Magnetic Bead-purified ssDNAs

Considering the S_{Fi} profiles closely, ssDNAs were purified by magnetic beads and were run separately as SSCP without heat denaturation and snap cooling (Fig. 2A, lanes 4,5). The SSCP profiles were completely different than profiles when both strands were present (Fig. 2A, cf. lanes 1–3). Reannealed duplex DNAs were not detected, indicating the purity of each strand. Doublets of two closely migrating major bands were observed for both ssDNAs and a number of slow migrating metastable species (region marker m). The most informative polymorphic SSCP profiles were thus observed with bead-purified ssDNA. The doublet bands are believed to represent stable conformations assumed by a single DNA strand, which present different profiles to the sieving matrix. Neither of these pairs of doublet bands was present as major conformers in the SSCP of gel-purified DNA (lane 1) nor in SSCP of individually labeled strands (lanes 2,3). Thus, true ssDNA conformers are not usually abundant in a conventional

SSCP assay, even of gel-purified PCR product.

If the F_{It} profiles of magnetic bead purified ssDNA are also considered, similar observations can be made (Fig. 2A, lanes 9,10). The SSCP profiles show a pair of closely spaced doublet bands as well as several metastable bands of low mobility. Notably, although S_{Fi} and F_{It} PCR fragments vary by a base substitution plus a base deletion, the ITS4 primer strand SSCP profiles are identical (Fig. 2A, cf. lanes 4 and 9), whereas the HA1 primer strand profiles differs for each conformer band (Fig. 2A, cf. lanes 5 and 10). Magnetic bead-purified SSCPs thus generate a greater number of scorable bands which attest to the DNA sequence differences.

Ethidium Bromide-stained polyacrylamide gels

When the ethidium bromide-stained profiles of the same polyacrylamide gel was examined (Fig. 2B) the major reannealed duplex band and the major SSCP

bands were detected, whereas the majority of the weaker SSCP conformers were not visible. Importantly, the low mobility metastable conformers in SSCP of bead-purified ssDNA that were readily detected by autoradiography were not seen in the ethidium bromide-stained profiles (Fig. 2B, lanes 1,2,7,8).

SSCP Formation

Further information pertinent to the understanding of the nature of metastable conformers can be seen in Figures 3 and 4. Magnetic bead-purified ssDNAs were given the same treatment as duplex DNAs—boiled and then snap-cooled, prior to loading onto the polyacrylamide gel. In Figure 3 the SSCP of gel-purified duplex S_{Fi} product, end-labeled on the HA1 (lane 2) or ITS4 (lane 3) strands [similarly for F_{It} : HA1 strand (lane 7), or ITS4 strand (lane 8)] also show identical autoradiographic (Fig. 3A) and ethidium bromide-stained profiles (Fig. 3B). Notably the multiple, low-mobility (metastable) bands seen in Figure 2A are absent,

FIGURE 3 PAGE-fractionated SSCP of purified ssDNA detected by ^{35}S autoradiography (A) or by ethidium bromide staining (B). The lanes are as shown in Fig. 2. The ITS4/HA1 PCR products were 5'-end labeled with γ - ^{35}S -labeled ATP or were unlabeled, as indicated in Fig. 2. The reannealed duplex DNA (d) and the major lower (l) and upper (u) SSCP conformers of the ssDNA are identified. (M) Metastable conformer region.

suggesting melting and subsequent snap-cooling prevented their formation. These low-mobility bands are also absent from other lanes presumably because the heating/snap-cooling process for formation of the SSCP does not allow for their formation. The bead-purified ssDNAs still display the doublet of two major closely migrating bands that can be detected by both ethidium bromide staining and autoradiography. These major bands represent the stable conformers described above and must therefore form rapidly during quenching of the denatured ssDNA on dry ice.

Autoradiographic Detection

Figure 4 also shows the SSCP of heated and snap-cooled S_{Fi} products. The DNAs were either end-labeled on both strands (lanes 1,4), the HA1 strand (lane 2), or the ITS4 strand (lane 3). If the crude PCR products of S_{Fi} (lane 4) were electrophoresed, three major bands were seen: the reannealed duplex band and two conformers that also do not correspond to SSCPs of true ssDNA.

Selective Hybridization between Primers and ssDNAs

The addition of ^{35}S -labeled oligonucleotide primers complementary to the purified single strands allowed identifica-

tion of conformers resultant from hybridization between primer and DNA strand. Thus, in Figure 4, the addition of excess radiolabeled PCR primers HA1 (lane 5) and ITS4 (lane 6) to unlabeled, gel-purified DNA can be seen to result in different profiles than gel-purified DNA (lane 1), and that the presence of complementary PCR primers increased the mobility differences between SSCP fragments. Because the PCR primers reanneal to common regions of ssDNAs, distinct from the region of sequence variation, the effect of the presence of residual PCR primers in this case is to potentiate conformational differences inherent in the ssDNA from *H. annosum* ITS2 region. The SSCP also shows a different mobility to the true ssDNAs that were depleted of residual PCR primers during bead isolation (Fig. 4, cf. lanes 2 and 5 and 3 and 6). Reexamination of the SSCP of gel-purified PCR products of S_{Fi} (lane 1) now permits many conformers to be identified. The SSCP profile obtained using total PCR reaction products is a mixture of many molecular species, and true ssDNA conformers are among the least abundant. Rather, conformers resulting from interaction between primers and ssDNA are at least as abundant as true ssDNA SSCP (Fig. 4, cf. lanes 1 and 4). Notably, the ratio of interacting DNA species can be seen to have a marked effect on both the detection and

on the mobility of species that may be found in an SSCP profile. This observation is important because the interpretation of sequence difference between two related DNAs is frequently based on weakly detected bands.

A model of the metastable structures formed between bead-purified ssDNA is shown in Figure 5. Although it might be possible for some unpaired, single-stranded regions of folded ssDNA conformers to associate weakly in the formation of multimeric structures, a more likely model would involve intermolecular base-pairing between the regions of two (or more) ssDNA molecules that would normally be capable of contributing to the intramolecular duplex-stabilized regions that ssDNAs are believed to form and are considered the basis of the SSCP assay.

DISCUSSION

Single-strand Conformation Profiles

In a SSCP mixture of PCR product, three DNA fragments are typically present.^(1,2) They include one (reannealed) homoduplex DNA fragment AB and two corresponding single-stranded fragments, A and B. The electrophoretic migration of the ssDNAs in a sieving media is retarded because of conformational distortion created by the folding (loops and bends)

FIGURE 4 PAGE-fractionated SSCP of purified ssDNA detected by ^{35}S autoradiography. The ITS4/HA1 PCR products were 5'-end labeled with γ - ^{35}S -labeled ATP or were unlabeled, as indicated. SSCP mixtures were prepared as described in Materials and methods. All samples were boiled and snap frozen immediately prior to loading on the gel. Additional ^{35}S -end-labeled oligonucleotide primers (HA1 or ITS4) were added to 225 nM when indicated. The reannealed duplex DNA (d) and the major lower (l) and upper (u) SSCP conformers of the ssDNA are identified. (M) Metastable conformer region. (Lane 1) Gel-purified S_{FI} product, labeled both strands; (lane 2) labeled HA1 primer strand alone; (lane 3) labeled ITS4 primer strand alone; (lane 4) crude S_{FI} product, both strands labeled; (lane 5) unlabeled, purified S_{FI} product plus labeled ITS4 primer; (lane 6) unlabeled, purified S_{FI} product plus labeled HA1 primer.

associated with partial duplex formation between complementary regions within each single-stranded molecule. Because the two complementary DNA strands differ in sequence each assumes an independent conformation that has a characteristic mobility. Minor differences in the sequence between DNAs from two sources, such as single point mutations, may affect the conformation assumed by one or the other single strand, allowing detection of the sequence difference as a mobility shift. The number of different conformational isomers (conformers) assumed by each ssDNA depends on the DNA sequence and stability of duplex formed by complementary regions.

For this study, our aim was to identify

factors affecting the fidelity of the SSCP assay and thus to advance the utility and reproducibility of the technique. Furthermore, we were interested in developing a stable conformational assay that would facilitate automation of point mutation detection for large-volume screening, such as by the use of HPCE.⁽¹²⁾ To that end, our aim was to develop a protocol with minimal sample handling following PCR amplification and high sensitivity that would maximize the information of SSCP analysis. The adoption of magnetic bead-purified ssDNA provided a marked increase in the information content of the SSCP analysis. When SSCP of magnetic bead-purified strands of S_{FI} or F_{IT} (1S + 1B/1D) were run, multiple fast-migrating and slow-migrating molecular species were seen that differed markedly in mobility between the two sets of fungal DNA fragments. This suggested that several stable conformations may be assumed by a single DNA strand that presents different profiles to the sieving matrix. The isolated complementary strands of each PCR product (or one alone) provide a novel and reliable source of temperature-stable SSCP, which have characteristic mobility dependent on DNA sequence.

Our experiments also demonstrate that the SSCP experiments that utilize whole, denatured PCR reaction products have conformer profiles resulting principally from interactions between DNA strands and their complementary PCR primers. Similar profiles are seen in the majority of SSCP generated with PCR products directly, or partly purified PCR products. Thus, whereas conventional SSCP profiles are simplified with two major ssDNA bands compared with the four or five major ssDNA bands seen with magnetic bead-purified strands, the conventional SSCPs have a reduced informational content compared with the metastable SSCP assay. The multiple conformers and metastable forms of ssDNA provide additional, novel, and scorable "conformation polymorphisms," which may indicate sequence variation between similar DNAs from two sources.

The bead-purified SSCPs also displayed a remarkable stability over conventional ssDNA generated by the melting of duplex DNA. Because of the absence of the complementary strand, the bead-purified ssDNA can be stored at

room temperature without reannealing to reform fully duplex DNA. This is an advantage for routine or automated analysis, or for situations involving multiple samples in which conformational stability allows sequential processing of samples.⁽¹⁷⁾ HPCE is a new powerful technique that offers speed, sensitivity, and automation in the separation and detection of molecular species. HPCE can provide a semiautomated analytical technique for mass, nonisotopic screening of SSCP samples but requires thermally stable ssDNA.⁽¹²⁾ Furthermore, the bead purification technique could be used for the preparation of ssDNAs that carry different fluorescent tags. The ssDNAs could then be mixed and both SSCP and mSSCP could be detected with high sensitivity by HPCE in a manner similar to a recent report of PCR-SSCP.⁽¹⁸⁾ The utility of these bead-purified DNAs is in marked contrast to conventional SSCPs, which typically show two scorable bands and cannot be used easily for automated genotyping.

Several techniques have sought to improve the efficiency of SSCP for variant detection, and methods for detection of mutations in longer DNA fragments, for improved stability of the SSCP products, and for automation of the analysis procedure have been identified. The detection of SSCP for longer DNA templates was found to be improved by the inclusion of phage RNA polymerase initiation

FIGURE 5 Model of low mobility metastable conformational isomers (conformers). Regions of complementarity allow duplex formation between two or more ssDNAs.

sites to allow synthesis of a single-strand RNA copy of one-half of the DNA duplex^(19,20), or by asymmetric PCR to provide an abundance of one strand.⁽²¹⁾ The high rate of detection of sequence variants by RNA-SSCP was believed to result from a larger repertoire of secondary structure available to RNA than to its corresponding DNA analog and because of the reduced possibility of strand reannealing. The sense and antisense strands of RNA also gave identifiably different mobility profiles.⁽²¹⁾ Recently, Cai and Touitou⁽⁸⁾ found that the presence of residual PCR oligonucleotide primers was a factor that may reduce the efficiency of detection of DNA SSCPs. Residual, unincorporated PCR primers reannealed to the complementary ssDNAs of the cathepsin D gene fragment during gel loading and reduced the separation between the conformers during the SSCP assay. We have also examined the composition of the molecular species formed in SSCP mixtures by the selective radiolabeling of strands, or DNA primers. With the *H. annosum* ITS2 fragments, the mobility differences between the reannealed primer-ssDNA and bead-purified ssDNAs can be clearly seen.

One of the most important considerations arising from our findings is that SSCP seen using whole PCR reaction products may be influenced by the ratio of DNA species in the SSCP mixture. In SSCP profiles produced from PCR amplified, duplex DNA sources, true ssDNA conformers were among the least abundant molecular species (Fig. 2,3). Rather, conformers resulting from interaction between primers and ssDNA are at least as abundant as SSCP of ssDNA. Notably, the ratio of interacting DNA species can be seen to have a marked effect on both the detection and on the mobility of species that may be found in an SSCP profile. For example, in this study, the addition of excess radiolabeled PCR primers produced several lower mobility bands than are conventionally seen in SSCP profiles (Fig. 4, cf. lanes 1, 5, and 6). Annealed primer-ssDNA may result in a reduced separation⁽⁸⁾ or an increased separation (this work) between annealed ssDNA SSCP bands. Overall, variation in the ratio of reagents present in several different SSCP mixtures could bring about differential mobility between the SSCP assays. Observation of different profiles could result in misidentification of the source of altered mobility. This is

an important consideration because the interpretation of sequence difference between two related DNAs is frequently based on weakly detected bands.^(2,5,22) Consequently, we believe that even for conventional SSCP analysis removing residual primers would help to eliminate the uncertainty in band mobility and band identity associated with primer-ssDNA interactions.

The Metastable Bands

Although SSCP using bead-isolated ssDNA has been reported recently,⁽²²⁾ samples were boiled and then cooled on ice in the conventional way and metastable conformers were not observed. In our hands, low-mobility metastable bands were consistently observed only in SSCP of purified ssDNA that were not thermally treated. The metastable conformers were also in low abundance but could be detected by autoradiography. Together, these observations suggest that metastable species represent low stability structures that require sufficient time and low thermal energy for formation. Because boiling/quenching treatment of the bead-purified ssDNAs removed the metastable conformers, they are likely stabilized by partial, or short duplex regions. Boiling disrupts the metastable structures, and the rapid quenching does not allow sufficient time and energy for reformation. The structure of the metastable molecules is not entirely clear, but it is likely that they represent species resulting from bi- or trimolecular self-annealing interactions between single strands at complementary regions. Several lines of evidence support this suggestion. The low electrophoretic mobility suggests larger molecular species, such as multimers. The strands are not fully self-complementary; therefore, multimers would have a partially single-stranded character. Because conventionally, SSCPs are believed to result from intramolecular base-pairing between complementary regions within a single DNA strand, additional intermolecular duplex between complementary regions of two or more molecules is conceivable. Furthermore, the predicted minimum free energy folded structures (MFOLD program)⁽²³⁾ of single strands of the HA1/ITS4 products of F_{it} and S_{Fi} suggest extensive regions of intramolecular sequence complementarity. Evidence⁽²⁴⁾ that duplex

and triplex structures between both folded and linear oligonucleotides can form in solution and are energetically stable would support the formation of intermolecular structures in our ssDNA mixtures.

The failure to resolve between closely related sequences in conventional SSCP is frequently ascribed to the location of sequence differences to unpaired loops or bends that do not participate in formation of the folded monomolecular ssDNA structure. It is possible that complementary, unpaired ssDNA loops on two molecules could also participate in metastable conformer formation. Metastable conformers may therefore provide an additional set of information with which to detect DNA sequence differences in molecules that are not readily observed by conventional SSCP procedures. Low-temperature or stabilizing compounds could also influence the formation and structure of metastable ssDNA species and, hence, would be likely to influence the electrophoretic mobility.

The major bands seen in conventional SSCP can be detected by ethidium bromide.^(9,10) In our hands, ethidium bromide detection of SSCP did not provide the high sensitivity necessary for observation of low-abundance metastable conformers. Ethidium bromide does not intercalate into ssDNA as efficiently as with double-stranded DNA. Efficient, nonisotopic staining might be achieved by use of new fluorescent dyes for ssDNA, such as TOTO-1 or SYBR Green I (Molecular Probes) either in conventional SSCP analysis or using HPCE. The latter dye is claimed to detect ≥ 100 pg of DNA under standard UV illumination, which is $\sim 100\times$ more sensitive than ethidium bromide.

ACKNOWLEDGMENTS

This work was supported by the Natural Environment Research Council (GR9/370), the Scottish Office (SOAFD), and the UFC-Biotechnology Initiative.

NOTE ADDED IN PROOF

Sequence data described in this paper have been submitted to EMBL under accession numbers X70021 and X70025.

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Received August 29, 1994; accepted in revised form December 2, 1994.

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Genome Res. 1995 4: 227-233

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