Nucleic Acids Research, Vol. 20, No. 4 871–878

Screening for mutations by RNA single-strand conformation polymorphism (rSSCP): comparison with DNA-SSCP

Gobinda Sarkar, Hong-Sup Yoon and Steve S.Sommer*
Department of Biochemistry and Molecular Biology, Mayo Clinic/Foundation, Rochester, MN 55905, USA

Received September 9, 1991; Revised and Accepted January 20, 1992

ABSTRACT

Single-strand conformation polymorphism (SSCP) is a simple method for detecting the presence of mutations in a segment of DNA, but the fraction of all mutations detected is unclear. We have evaluated SSCP for the detection of single-base mutations in the factor IX gene. Multiple conditions were examined including electrophoresis temperature, electrophoresis buffer concentration, acrylamide to bis-acrylamide ratio, and water-cooled versus fan-cooled gel apparatuses. Depending on conditions, 10–11 of 12 known mutations were detected in a 183 bp segment whereas only 11–14 of 22 known mutations were detected in a 307 bp segment. We hypothesized that single stranded RNA should have a larger repertoire of secondary structure because shorter hairpins form stable duplexes and the 2′ hydroxyl group is available for sugar-base and sugar-sugar hydrogen bonds. By incorporating phage promoter sequences into PCR primers, RNA-SSCP (rSSCP) could be compared directly with standard DNA SSCP. rSSCP was generally superior to SSCP, especially for the 307 bp segment. In addition, the abundance of transcript produced as a result of rSSCP allows the rapid, nonradioactive detection of mutations by staining the gel with ethidium bromide. To gauge the utility of the method in a prospective manner, a blinded study was performed in which SSCP, rSSCP, and direct genomic sequencing were compared in 28 patients with hemophilia B. A total of 2.6 kb of factor IX genomic sequence was examined in nine regions ranging from 180 to 497 nucleotides of factor IX sequence. Sequence changes at 20 different sites were detected by direct genomic sequencing; 70% of these were detected by rSSCP while only 35% were detected by SSCP.

INTRODUCTION

Several methods exist for determining whether a single-base change exists in amplified DNA, but they have not been widely accepted because of (a) time-consuming protocols, (b) poor reproducibility, (c) a substantial percentage of undetected base changes, and/or (d) complicated instrumentation (1–4). We have focused on single-strand conformation polymorphism (SSCP) because of its simplicity and potential for high output (5). As implied by the name, SSCP involves the separation of single strands of DNA on a nondenaturing polyacrylamide gel.

SSCP has been applied for the detection of sequence changes in various contexts. For example, it has been used to detect polymorphisms in Alu repeats (6) and in the human dopamine D2 receptor (7), to define mutations in Tay-Sachs disease (8) and in the human p53 gene (9), and to define polymorphism in the KIT proto-oncogene (10).

Although SSCP is being used with increasing frequency, parameters that could influence the informativeness of the method, such as sequence length and composition, have not been thoroughly evaluated. Orita et al. reported that 12 of 12 known mutations in various human RAS genes could be detected as mobility shifts when present in DNA segments of 103 or 162 bp (5). Subsequently, these investigators detected 10 of 12 mutations in a DNA repair gene for DNA segments of 400 bp (6). Our initial application of SSCP for detecting mutations in segments greater than 200 bp of the human factor IX gene was not as promising as we hoped. Therefore, we developed RNA single strand conformation polymorphism (rSSCP) which, in parallel tests, detects a higher fraction of single-base changes.

MATERIALS AND METHODS

Amplitaq was purchased from Perkin-Elmer Cetus. T7 and SP6 RNA polymerases and RNAsin were from Promega Biotech (Madison, Wisconsin). α-^{32}P-dCTP and α-^{32}P-UTP were purchased from Amersham.

PCR (11) was performed from human genomic DNA essentially as described earlier (12) in an automated thermal cycler (Perkin-Elmer Cetus). The denaturation was at 94°C for 1 min, and the annealing was at 50°C for 2 min followed by elongation at 72°C for 3 min for a total of 30 cycles. The PCR mixture contained a total volume of 20 μl: 50 mM KCl 10 mM Tris-Cl, pH 8.3, 1.5 mM $MgCl_2$, 200 μM of each dNTP, 0.1 μM of each primer, 0.5 U of Amplitaq, 200 ng of genomic DNA, and

* To whom correspondence should be addressed

5 μCi of α-^{32}P-dCTP (>3000 Ci/mmole) (Amersham) for SSCP. The PCR primers used for the SSCP and rSSCP are listed in Table 1.

Generation of ^{32}P-Labeled Transcripts

Transcription with either T7 or SP6 RNA polymerases (Promega Biotech) was performed in a 10 μl reaction containing: 40 mM Tris-HCl (pH 7.5), 6 mM $MgCl_2$, 2 mM spermidine, 10 mM NaCl, 0.5 mM of the four ribonucleoside triphosphates, RNAsin (1 U/μl), 10 mM DTT, 10 U of the RNA polymerase, 1.5 μl of the PCR amplified template and 2 μCi of α-^{32}P-UTP (>800 Ci/mmole). The transcription reaction was incubated at 37°C for 1 hr and the reaction was stopped by freezing the samples.

DNA-SSCP and rSSCP Analysis

Usually 1 μl of the ^{32}P-labeled amplified DNA or transcribed RNA was mixed with 50 μl of 95% formamide, 20 mM EDTA, 0.05% bromophenol blue and 0.05% xylene cyanol. The mixture was then heated in boiling water for 4 minutes followed by quick chilling in ice-water for 10 minutes. One μl then was loaded onto a 5.6% nondenaturing polyacrylamide gel containing 45 mM Tris-borate, pH 8.3, 4 mM EDTA and 10% glycerol. Electrophoresis was carried out with a sequencing gel apparatus from either IBI (model STS 45) or the Hoeffer Poker-face at 30 W constant power for 5 to 7 hr with cooling provided by either a water jacket, a fan, or a coldroom. After electrophoresis, the gel was dried and subjected to autoradiography.

If desired, the intensity of the rSSCP signal can be increased by decreasing the amount of 95% formamide dye solution tenfold. Analogous experiments with DNA were unsuccessful, presumably because the complementary strands reanneal.

Non-radioactive rSSCP Analysis

1.5 μl of transcripts as generated above were suspended up in 20% formamide, 5 mM EDTA, 0.05% bromophenol blue and 0.05% Xylene cyanol, heated in boiling water for 4 minutes, then chilled in ice-water for 10 minutes before loading on a 5.6% non-denaturing polyacrylamide gel. Electrophoresis was as above with cooling from a fan. The electrophoresis buffer contained ethidium bromide at a concentration of 100 ng/ml. After electrophoresis, the gel was carefully transferred onto a thin plastic sheet (one side of a regular plastic sheet protector, 8.5″×11″,

Table 1. List of Primers

Abbreviation	Informative Name[a]	Sequence[b]
A	F9(Hs)(T7-6)E8(31007)-22D	GGGAGA TCCTCA CCACAA CTAC
B	F9(Hs)(T7-23)E8(31007)-49D	T7 + ACGAACA TCTTC CTCA
C	F9(Hs)(SP6-6)E8(31177)-21U	GAATAC GCCAGC ACAGAA CAT
D	F9(Hs)(SP6-35)E8(31177)-50U	SP6 + GCCAG CACAG AACAT
E	F9(Hs)(T7-6)E8(30883)-22D	GGGAGA TCCTCA CCACAA CTAC
F	F9(Hs)(T7-29)E8(30883)-45D	T7 + TCCTCA CCACAA CTAC

[a]In routine practice, it is very useful to have informative names for every primer. The detailed description of informative name is provided in Sarkar et al. (18) and Sarkar and Sommer (19). As an example, for oligonucleotide A, F9 = factor IX; Hs = *Homo sapiens*; (T7-6) = six bases of the T7 phage promoter; E8(31007)-22D = 5′ end of factor IX-specific sequence begins at base 31007 in exon 8 (E8) [Yoshitake et al., (20)] and the total length of the oligonucleotide is 22 bases 'downstream' (i.e. in the direction of transcription).

[b]The bases *not* underlined are responsible for amplification of the segments of factor IX genomic DNA. The underlined bases and the complete phage promoter sequences (abbreviated T7 + or SP6 +) serve two functions: (i) generation of RNA transcript for SSCP, and (ii) comparison of rSSCP and SSCP with segments of identical sequence. The T7 promoter sequence is 23 nucleotides: TAATACGACTCACTATA**GGGAGA**. The SP6 promoter sequence is 35 nucleotides: CATACACATACGATTTAGGTGACACTATA**GAATAC**. The bold face bases in the T7 and Sp6 sequences are incorporated into the transcript. These six bases are also present in primers A, C, and E (see underlined bases). In order to obtain DNA and RNA segments of identical sequence, three different PCRs were performed. For DNA SSCP, primers A and C were used to produce a 183 bp segment that contains 171 bp of factor IX sequence (bp 31007 to 31177) flanked by GGGAGA and GAATAC. The amplified segment was diluted, denatured, and electrophoresed. For T7 RNA SSCP, primers B and C were used. The amplified segment contains a T7 promoter sequence (17 nucleotides and GGGAGA). When transcribed with T7 phage polymerase, an identical 183 nucleotide transcript was produced. The RNA was denatured and electrophoresed. To produce a 183 nucleotide transcript that is precisely complementary, PCR was performed with primers A and D and the amplified segment was transcribed with SP6 RNA polymerase. To generate the 307 bp segments, primers E + C were for SSCP and primers F + C and E + D were used for T7 and SP6 rSSCP, respectively.

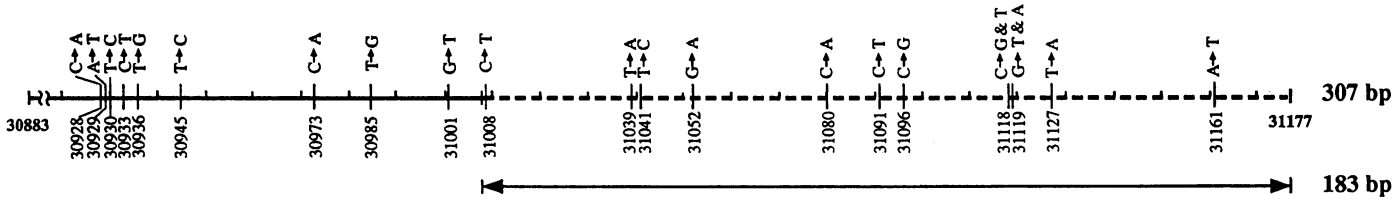

Figure 1. Mutations found in the 183 bp and 307 bp segments—[the lengths include 6 bp of nucleotides at each end derived from the phage promoter sequences (see Table 1)]. The nucleotide numbering is from Yoshitake et al. (20). The broken line indicates the length of the shorter segment (183 bp). The normal nucleotide and the mutated nucleotide are given on the upper side of the line (e.g., CA at base no. 30928). The 22 mutations are consecutively numbered to correspond to the lane numbers in figs. 2–4.

poly-C® standard weight, catalog no. 03213 from C-Line Products, Inc., Des Plaines, IL) and photographed under UV-illumination. Alternatively Ziploc bags may be used for this purpose but the plastic sheet protectors are easier to handle.

RESULTS

We hypothesized that the additional hydroxyl group in RNA might impart a larger repertoire of single-strand structures. To generate material to test this hypothesis, PCR was performed with primers containing phage promoter sequences. These were then transcribed, and the resulting RNA was electrophoresed on nondenaturing gels. RNA SSCP (rSSCP) was compared with SSCP for the detection of mutations in two overlapping segments in the human factor IX gene of lengths, 183 bp and 307 bp, respectively.

Our laboratory previously has delineated many different mutations in patients with hemophilia B (13–16 and Bottema et al., in press). Twelve mutations are located in the 183 bp segment and an additional 10 mutations (22 total) are located in the 307 bp segment (Fig. 1). In the case of SSCP, both strands of DNA were analyzed simultaneously; for rSSCP each strand was generated as single-stranded RNA and analyzed independently. All possible combinations of mutations (A→T, G or C; T→A, G or C, G→C, T or A and C→G, A or T) have been analyzed by both SSCP and rSSCP.

Since SSCP was used as a reference for rSSCP for virtually all experiments, multiple conditions for SSCP were initially evaluated. We found 45 mM Tris-borate, pH 8.3, 4 mM EDTA with 10% glycerol routinely produced as good or better results than the 90 mM Tris-borate used in the Orita *et al.* (5) protocol. Therefore, 45 mM Tris-borate was used as the electrophoresis buffer for all experiments unless otherwise stated.

Analysis of Mutations in a 183 bp Segment by SSCP and rSSCP

Orita *et al.* (5) reported that SSCP readily detects single base changes in segments less than 200 bp. Therefore, the first set of experiments were designed with a 183 bp segment. Genomic DNA was amplified from 12 individuals with different mutations distributed throughout the 183 bp region. Primers for PCR amplification were designed so that the amplified DNA and the RNA transcripts would have the identical 183 bp sequence (see Table 1, footnote B). These DNA and RNA segments were then electrophoresed through a 5.6% nondenaturing polyacrylamide gel (experiment 1, Table 2). The results are presented in Fig. 2. The

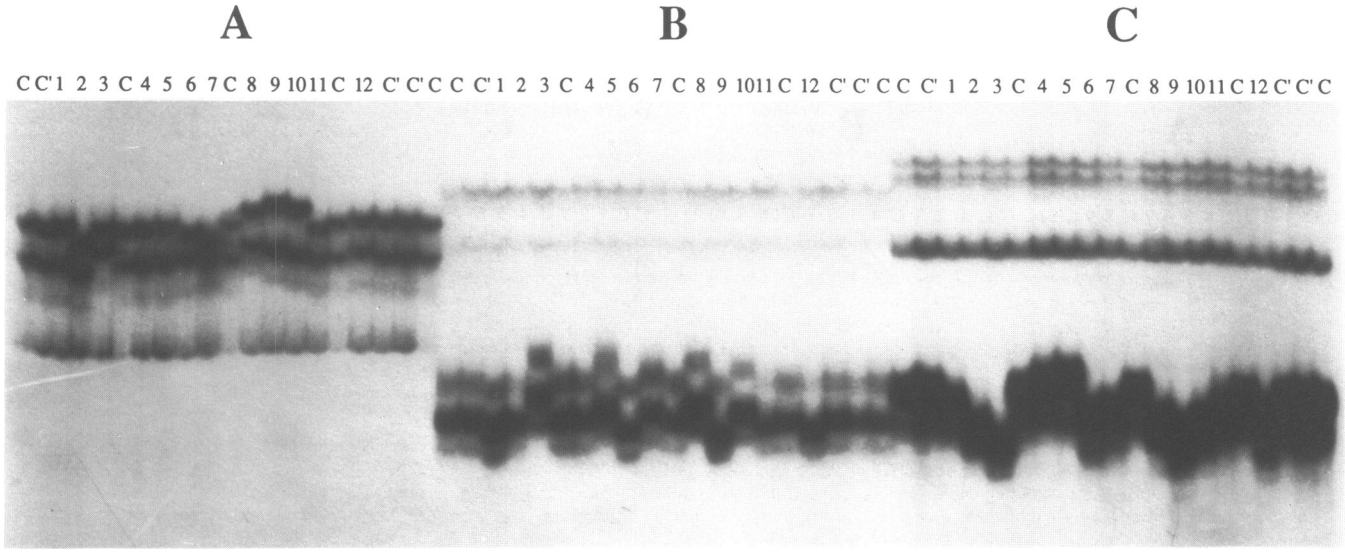

Figure 2. Comparison of SSCP and rSSCP (T7 and SP6) of 12 different mutations contained in the 183 bp segment. **Panels A—SSCP, B—T7-rSSCP, and C—SP6-rSSCP.** *Lanes labeled C*—normal control sample from one individual I (each loading taken from the same transcription reaction). *Lanes labeled C'*—normal control samples from different normal individuals (from left to right in each panel, the samples are derived from individuals II, III, and IV respectively). *Lanes labeled 1–12*—Templates containing mutations 1–12 (see Fig. 1 for precise mutation). The samples were electrophoresed through a 5.6% acrylamide gel in a poker-face apparatus (Hoeffer Scientific) at 10°C for 5 hr, dried and autoradiographed. Exposure time was 16 hr. The following indicates the scoring of each mutant segment in panels A, B, and C. '–' indicate either no difference between control or a minor difference that is not clearly distinct from the minor variation present in different normal controls. '+' indicates an abnormal (mutant) pattern characterized by clear alteration in the migration pattern of one or more bands or the appearance or disappearance of a band. *Note that scoring was performed on the autoradiograms; some loss of resolution may occur in the published figures.* **Mutation 1**—A: –; B: +, the two top bands are slightly discontinuous with the corresponding adjacent control bands and the lower band is substantially darker than the corresponding control band; C: +, the major band migrates faster than the control. **Mutation 2**—A: +, both major bands migrate substantially faster; B: +, hardly any of the top band is present; C: +, the major band migrates faster. **Mutation 3**—A: +, the lower band migrates much slower; B: +, all the bands migrate much slower than in the control; C: +, bands migrate much faster. **Mutation 4**—A: –, B: –, C: +, both the major (upper) and the minor (lower) bands migrate slower; **Mutation 5**—A: –, both major bands conceivably migrate slightly slower, but a difference of this magnitude easily could be overlooked in a blind screening; B: +, the upper band migrates much slower, the lower band migrates somewhat slower; C: +, the upper band is significantly retarded; **Mutation 6**—A: +, the major (upper) band migrates somewhat faster, the second band (second from top) migrates somewhat slower; B: +, all the bands migrate faster; C: +, all the bands migrate much faster; **Mutation 7**—A: +, the uppermost band migrates faster, B: +, all the bands migrate slower; C: +, all the bands migrate faster; **Mutation 8**—A: +, the upper two bands migrate slower, B: +, the bands migrate slower, C: +, the bands migrate much faster; **Mutation 9**—A: +, the diagnostic bands (the major two bands from the top) migrate slower, B: +, all bands migrate faster, C: +, all bands migrate faster; **Mutation 10**—A: +, the uppermost band migrates slower, B: +, the bands migrate faster, C: +, the bands migrate faster; **Mutation 11**—A: +, the uppermost band migrates faster, B: +, the bands migrate slower, C: +, the major band migrates much faster; **Mutation 12**—A: +, the upper two bands migrate somewhat slower, B: +, the major bands migrate faster, C: +, the bands migrate faster.

first panel of lanes (A) represents DNA segments (SSCP), the second and third panel of lanes (B and C) represent T7 and SP6-transcripts, respectively (rSSCP). Normal samples from different individuals migrate in an identical manner. To control for positional effects across the gel, normal samples were loaded periodically. As can be seen, rSSCP with either T7- or SP6-transcripts was more informative than DNA SSCP (see legend to Fig. 2). In fact, 100% of the mutations can be distinguished from the control with the SP6 transcripts. Notice also that lane 1 is uninformative in panel A whereas it is clearly informative in panels B and C. Also compare lane 5 in the three panels (A, B, and C). Although this lane is informative in all three panels, the migration difference is much more pronounced for rSSCP than for SSCP. Another example of this kind is lane 12.

Table 2. Percentage of Mutations Detected by SSCP and rSSCP for the 183 bp Segment

Experiment #	Conditions of Analysis[a]	Type of Analysis	% of Mutations Detected
1	~17.5°C	SSCP	75
	Cooled with water	T7-rSSCP	92
	30W	SP6-rSSCP	100
2	~20°C	SSCP	83
	Cooled with fan	T7-rSSCP	92
	30W	SP6-rSSCP	100
3	8°C (coldroom)	SSCP	83
	no fan	T7-rSSCP	83
	8W	SP6-rSSCP	83
4	10°C	SSCP	92
	Cooled with water	T7-SSCP	100
	30 W	SP6-SSCP	92

[a]SSCP was performed as described in the Methods except as indicated. Cooling with water should give more uniform and consistent temperature control than cooling with a fan. However, in these and subsequent experiments, the method of cooling did not dramatically affect the results. The poker face (Hoefer) apparatus was used with water cooling and the IBI apparatus was used with fan cooling.

Other conditions were tested (e.g., electrophoresis in the cold room, or with water coolant at 17.5°C and 10°C), but rSSCP with either T7 or SP6-transcripts was often more informative than with DNA (although this represents both the strands). The collective results of these experiments are summarized in Table 2. Under the four conditions tested, the efficiency of mutation detection by SSCP was 75%, 83%, 83%, and 92%, respectively. For rSSCP, the minimum detection rate was 83% and, in three cases, 100% of mutations were detected. The average rates of detection for SSCP and rSSCP were 83 and 93%, respectively.

Electrophoresis time is a factor that limits the speed of detection. Therefore, this parameter was investigated for rSSCP by loading samples on the same gel at various times. The result of such an experiment is presented in Fig. 3. Each group of samples were loaded one hour after the previous group. Electrophoresis was performed for up to 5½ hr. The first group

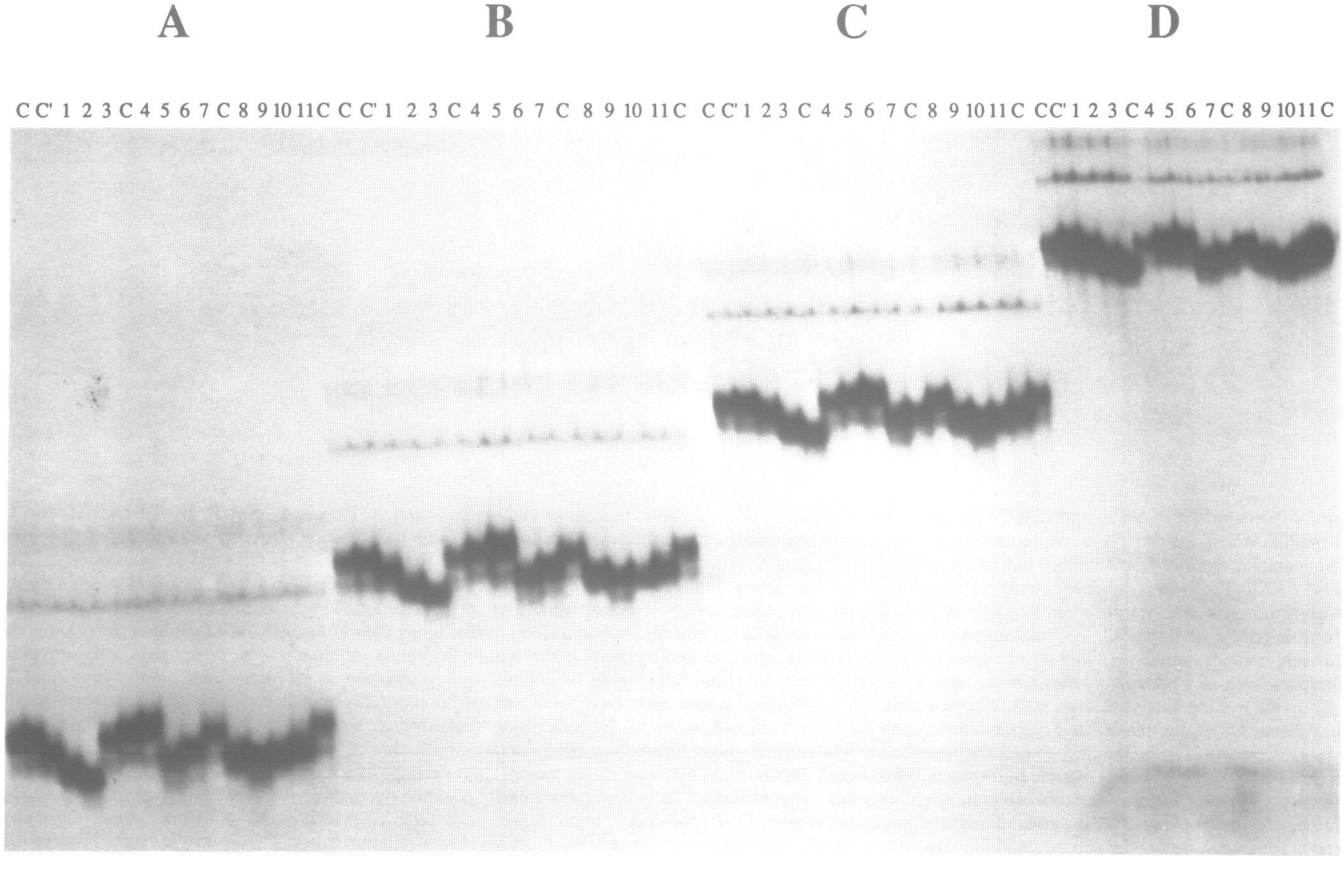

Figure 3. Effect of electrophoresis time on rSSCP. The samples are SP6 transcripts containing various mutations derived from the 183 bp template. The IBI sequencing gel apparatus was used at room temperature (with fan). The electrophoresis was carried out in a 5.6% polyacrylamide gel at 30 W constant. Panel A: 5−1/2 hr; Panel B: 4−1/2 hr; Panel C: 3−1/2 hr; Panel D: 2−1/2 hr. The notations for C and C′ are as in the legend to Fig. 2. Lanes 1−11 represent transcripts containing mutations 1−11 (see Fig. 1).

of samples were electrophoresed for 5½ hr, followed by the second group for 4½ hr, the third group for 3½ hr and the last group for 2½ hr. It can be seen that the RNA samples assume their respective conformations quite early and maintain these conformations throughout electrophoresis. However, desired separation was not achieved until after 3½ hr (third group). This experiment indicates that for an RNA target ~200 b, a 3–5 hr electrophoresis should be optimal.

Analysis of Mutation in a 307 bp Segment by SSCP and rSSCP

The previous experiments suggest that rSSCP (as well as SSCP) detects most mutations in segments of 200 bases. We then examined the detection rate of rSSCP in a larger segment of 307 bp within which 22 mutations were available in the laboratory. Appropriate primers were used to amplify 307 bp DNA segments or RNA segments of 307 bases. The samples were then electrophoresed through a 5.6% nondenaturing polyacrylamide gel for 6 hr. The results of this experiment are presented in Fig. 4. The upper panel of bands shows different DNA samples (SSCP). The lower panel shows corresponding SP6 RNA samples (rSSCP). The T7-transcripts are not illustrated as they gave similar results. The DNA samples were informative in only 13 out of 22 cases (59%), whereas the RNA samples were informative in 21 out of 22 cases (95%). In some cases in which DNA and RNA both were informative, the differences between the control and the sample were more pronounced with the RNA. For example, in lane 7 of SSCP, the sample is scorable only

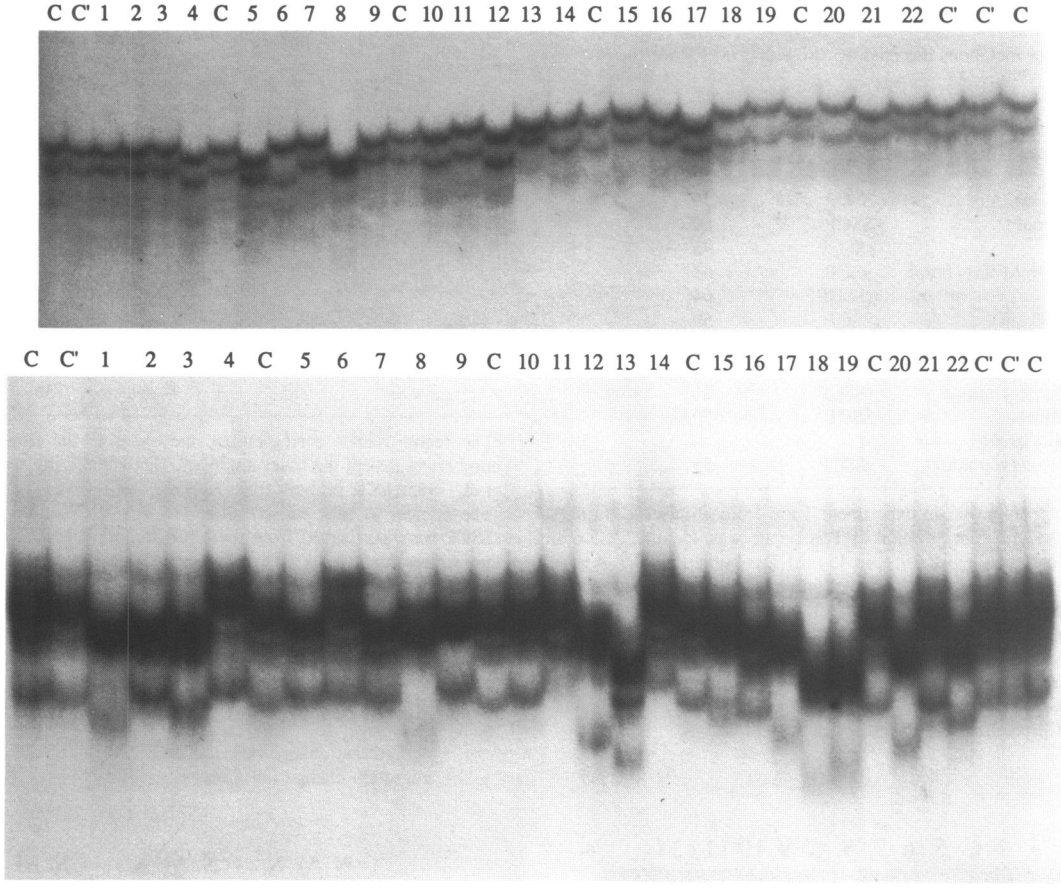

Figure 4. SSCP and rSSCP (SP6) of mutations 1–22 contained in the 307 bp segment (see Fig. 1). Upper panel—SSCP, lower panel—rSSCP. The samples were electrophoresed with an IBI sequencing apparatus for 5–1/2 hr as described in the legend to Fig. 3. All the samples were electrophoresed side-by-side in one gel, however, in this figure the rSSCP portion is placed beneath the SSCP portion to facilitate direct comparison of SSCP and rSSCP in a given sample. The lane numbers correspond to mutation numbers. Controls (denoted by C and C′) are from the four individuals in the legend to Fig. 2. **Scoring of autoradiograms (see criteria in legend to Fig. 2):** Upper panel (SSCP); lanes 1,2,3, '–', no visible difference from the control (C) lane; lanes 4, 5 '+' all the bands migrate faster than in the control; lane 6, '+' the uppermost band migrates slower and the rest of the bands migrate faster; lane 7, '+' the bands migrate somewhat slower; lane 8, '+' the bands migrate much faster (also the gap between the two upper bands has become smaller); lane 9, '+', the upper band migrates slightly faster whereas the next band migrates slightly slower; lane 10, '+' the space between the two upper bands has increased; lane 11, '+' the two upper bands migrate slower; lane 12, '+' the migration of the upper band is unchanged but the second band migrates faster; lane 13, '+', the second band (from top) migrates slower; lane 14, '-'; lane 15, '+' bands migrate slower; lane 16, '+', the second band from top migrates slower; lane 17, '+', the bands migrate faster; lanes 18, 19, 20, '+', the uppermost band migrates slower; lane 21, '–', lane 22, '+' the second band from top migrates slower. Lower panel (rSSCP): lane 1, '+' the bands migrate faster than the control; lane 2, '+' the upper dark band migrates faster; lane 3, '+' the bands migrate faster; lane 4, '+' the bands migrate slower; lane 5, '+' the uppermost band (major band) migrates faster; lane 6, '+' the uppermost band migrates slower; lane 7, '+' the major band migrates faster; lane 8 '+' the major band migrates slower and the minor band migrates much faster; lane 9, '+' the minor band migrates slower; lane 10, '+' the bands migrate slightly slower; lane 11, the major band migrates slower, also note the disappearance of the minor band; lanes 12 and 13, '+' the bands migrate faster; lane 14, '+' the bands migrate slower; lane 15, '+' the upper band migrates somewhat slower but the lower band migrates faster; lanes 16–20, '+', the bands migrate faster; lane 21, '-'; lane 22, '+' the bands migrate faster.

on careful examination, whereas the corresponding RNA lane can be scored with ease. Notice also that lanes 1, 2, 3 (among others) were not informative for SSCP, but were clearly scorable with rSSCP. Mutation number 21 could not be detected by either method. Various conditions did not significantly increase the informativeness of DNA-SSCP method. The results of these experiments are summarized in Table 3. With each condition, rSSCP was at least as informative as SSCP. On average, SSCP detected 58% of the mutations whereas rSSCP detected 77% of the mutations.

To test the informativeness of SSCP and rSSCP for larger segments, preliminary experiments were performed with a 520 segment nucleotide in exon h. The results were disappointing for both SSCP and rSSCP; only a minority of the mutations could be detected (data not shown).

Table 3. Percentage of mutations detected by SSCP and rSSCP for the 307 bp segment

Experiment #	Conditions of Analysis[a]	Type of Analysis	% of Mutations Detected[b]
1	Initial	SSCP	59
2	Initial	SSCP	59
		rSSCP	95
3	90 mM Tris-borate	SSCP	64
		rSSCP	64
4	4°C	SSCP	59
		rSSCP	86
5	50:1:: acrylamide: bis-acrylamide	SSCP	59
		rSSCP	86
6	100:1:: acrylamide: bis-acrylamide	SSCP	50
		rSSCP	55

[a]The initial conditions employed in experiment 1 are from Orita et al. (1989) with the exception that the concentration of Tris-borate was 45 mM. A 5.6% polyacrylamide gel (acrylamide:bis::19:1) in 45 mM Tris-borate, pH 8.3, 4 mM EDTA, and 10% glycerol was electrophoresed in an IBI sequencing apparatus at room temperature with a fan at 30 watts of constant power for approximately 6 hrs. In the subsequent experiments, the deviations from initial conditions are indicated.

[b]The percentages are for SP6 transcripts. rSSCP of T7 transcripts of these samples did not increase the percentage of mutations detected. Twenty-two different mutants were tested in each of the experiments.

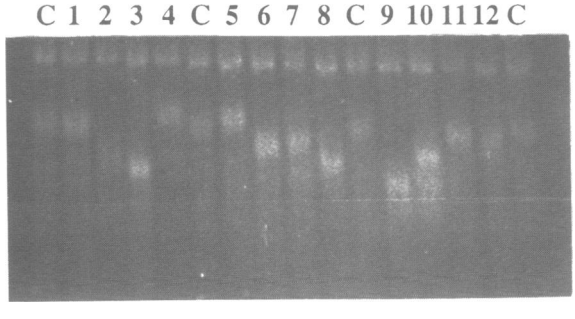

Figure 5. Non-radioactive detection by rSSCP SP6 transcripts of mutations in the 183 nucleotide RNA (see Methods). An IBI sequencing gel apparatus was used in this experiment. The condition of electrophoresis is identical to that for Fig. 3. The lane numbers in this experiment correspond to mutations 1–12 (see Fig. 1). Lane C—control. The scoring criteria are as in Fig. 2. Lanes 1 and 11 are '–', all the other lanes are '+'. The resolution is not as good as in Fig. 2, but the overall migration of mutants relative to controls is similar.

Nonradioactive Detection of Mutation with rSSCP

Nucleic acids are routinely detected by polyacrylamide gels after staining with ethidium bromide. However, relatively large amounts of single stranded nucleic acid is required for staining with ethidium bromide in a gel (>250 ng for a strong signal). For SSCP, the need to retard renaturation of the two strands precludes the loading of such an amount. For rSSCP, reannealing

Table 4. Sequence changes detected by SSCP and rSSCP

Region[a]	# bp[b]	Seq. Δ[c]	SSCP[d]	rSSCP[d]
A: –19–155	180	G→A	–	–
B + C: 6247–6738	497	G→C	–	+
		T→C	–	+
		C→T	–	+
D: 10350–10563	219	G→A	+	+
E: 17620–17850	236	T→A	+	+
		G→C	–	+
		C→T	+	–
F: 20317–20611	300	C→G	+	+
		C→T	+	+
G: 29982–30219	243	G→A	–	+
H1: 30747–31022	281	G→T	–	+
		C→T	+	–
		C→T	–	–
H2: 30883–31189	312	G→A	–	+
		C→T	+	+
		T→C	–	+
		C→T	–	–
H3: 31135–31429	300	C→T	–	+
		T→C	–	–
Average size:	285	% detected:	35	70

[a]The region names correspond to the exons in the factor IX gene, and the numbers represent the bases amplified. The numbering system from Yoshitake et al. 1985; PCR primers from Koeberl et al. 1990.

[b]The number of base pairs of factor IX amplified. In each region, SSCP and rSSCP were performed from the same amplification reaction in which one of the primers contained a 29 bp T7 promoter sequence (see Koeberl et al. 1990). The size of the analyzed RNA includes the nucleotides from the factor IX gene and six transcribed nucleotides from the T7 promoter sequence. The size of the analyzed DNA is 23 bp larger because the complete T7 promoter sequence is present, thereby giving SSCP a slight disadvantage in this comparison. However, in the previous experiments, different sets of PCR primers were utilized so that identical DNA and RNA segments were compared.

[c]Sequence change.

[d] '+' = migration change detected by SSCP or rSSCP.
'–' = migration change not detected.

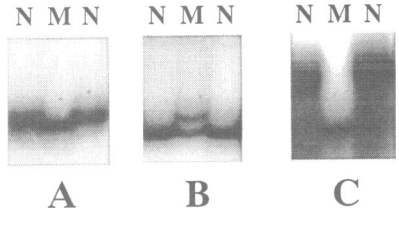

Figure 6: Sample of mutations detected by rSSCP in the blinded study. rSSCP (and SSCP) was performed at room temperature with a fan in the presence of 10% glycerol and .5×TBE buffer. To generate the figure, three autoradiograms were scanned with the HP Scan Jet Plus and the panels shown were juxtaposed with a Canvas Graphics Program. N = segment of normal sequence. M = mutant segment. **Panel A:** example of a small but clearly discernable change in migration; Region D from HB232, G→A. **Panel B:** pattern in a female heterozygous for a mutation; Region H3 from HB220, C→T. **Panel C:** example of a dramatic change in migration in a segment of 497 nucleotides; Region B+C from HB231, T→C.

is not an issue and transcription produces a great abundance of RNA. When 1.5 μl of the transcription product was used (see Methods), mutations readily could be detected by ethidium bromide staining of the gel (Fig. 5). In this experiment, SP6 transcripts were examined for the 12 mutations in the 183 nucleotide segment. The resolution was not as good as with autoradiography (Fig. 2); the two samples in which migration was closest to controls were not resolved by ethidium bromide staining. As expected, an analogous experiment with PCR amplified DNA showed only renatured duplex DNA (data not shown).

Blinded Comparison of SSCP, rSSCP, and Direct Genomic Sequencing in Patients with Hemophilia B

In a prospective analysis, DNA from 28 patients with hemophilia B was screened with SSCP and rSSCP for the presence of mutations. Segments of likely functional significance of the factor IX gene (2.2$^+$ kb/patient; see Koeberl et al. 1990) were independently sequenced by others in the laboratory. In total, only 20 different sequence changes were found since some patients had recurrent mutations and others did not have a sequence change in these regions. SSCP and rSSCP analyses were performed without knowledge of the sequencing results. The samples analyzed with SSCP and rSSCP were given different numbers so no correspondence between the two sets of analyses could be determined prior to decoding the samples. Nine PCR amplified segments were screened for each of the 28 patients. To control for position effects in the gel, a normal control was loaded after every five samples. The migration of each sample was scored relative to a nearby normal control. Sample rSSCP data are shown (Fig. 6).

On completion of the analysis, the results of SSCP and rSSCP were compared with the sequence data (Table 4). There was a remarkably low incidence of false positive signals, i.e., altered migration that was not confirmed by sequence analysis (1% and 0%, respectively, of 226 true negatives). Neither screening method detected all mutations, but rSSCP detected a substantially larger fraction than SSCP (70% versus 35%). Had smaller segments been analyzed, both methods presumably would have detected a higher fraction of mutations. However, substantially more effort would have been required.

The data indicate that sequence as well as size affect the rate at which mutations are detected. For example, the three mutations in region B + C (497 + 6 = 503 nucleotides of RNA transcript) were detected as dramatic shifts in migration with rSSCP (e.g., Fig. 5). In contrast, only a minority of mutations were detected in the previously described 520 nucleotide segments of exon h and those migration shifts seen were generally small.

DISCUSSION

The simplicity and ease of execution renders SSCP an attractive method for mutation detection. We principally sought to develop a method that would be effective at the 200–400 bp range, because many exons are of this size. The success of SSCP depends directly on altered conformation resulting from single base changes. As RNA conformations based on intrachain complementarity are expected to be more stable and the 2′ sugar hydroxyl group facilitates sugar-base and sugar-sugar hydrogen bonding, a method based on single-stranded RNA might be expected to be more informative than a DNA-based method. In addition, rSSCP routinely produces more bands than SSCP. The multiple bands may represent alternate RNA conformations, or partial termination products of the RNA polymerase. The multiple bands contribute to the informativeness of rSSCP because a mutation may change the migration of one band and not another.

The generation of an ssRNA transcript corresponding to a DNA-target requires the inclusion of a phage promoter sequence (T7 or SP6 or T3) in one (or both) of the PCR primers (13), but no purification step is involved at any stage. The results with the 183 bp targets indicate that rSSCP consistently was more informative than SSCP for various conditions tested. In addition, rSSCP was significantly better than SSCP for the detection of mutations with the 307 bp segment. In cases in which both SSCP and rSSCP were informative, it was often easier to score the RNA bands because of a greater migration difference relative to control bands. Since rSSCP uses much less radioactive precursor than SSCP, rSSCP is not more expensive than SSCP, although it does require the extra step of transcription. Although addition of 23–29 base promoter sequences to one or both primers is an added initial expense, this should constitute only a negligible fraction of the total supply and labor costs of most screening projects.

The abundance of transcript generated by rSSCP can compensate for suboptimal PCRs. Moreover, the samples can be detected directly by ethidium bromide staining of the gel, thereby eliminating the need for radioactivity and the effort involved in gel drying and autoradiography. When a blinded comparison of rSSCP and SSCP was performed in 28 patients with hemophilia B (9 different segments averaging 285 bp per sample for a total of 2.6 kb of genomic sequence per patient), 70% of the sequence changes were detected with rSSCP while only 35% were detected with SSCP. In addition, the rate of false positive was low (1% for rSSCP versus 0% for SSCP). Thus, the aggregate data indicate that rSSCP is an advantageous screen for the presence of single base changes.

ACKNOWLEDGEMENTS

The work is partially supported by NIMH MH44276. We thank Mary Johnson for secretarial help.

REFERENCES

1. Myers, R.M., Lumelski, N., Lerman, L.S. and Maniatis, T. (1985) *Nature (London)*, **313**, 495–498.
2. Fischer, S.G. and Lerman, L.S. (1983) *Proc. Natl. Acad. Sci. USA*, **80**, 1579–1783.
3. Ganguly, A., Rooney, J.E., Hosomi, S., Zeiger, A.R. and Prockop, D.J. (1989) *Genomics*, **4**, 530–538.
4. Ganguly, A. and Prockop, D.J. (1990) *Nucleic Acids Res.*, **18**, 3933–3939.
5. Orita, M., Suzuki, Y., Sekiya, T. and Hayashi, K. (1989) *Genomics*, **5**, 874–879.
6. Orita, M., Sekiya, T. and Hayashi, K. (1990) *Genomics*, **8**, 271–278.
7. Bolos, A.M., Dean, M., Lucas-Derse, S., Ramsburg, M., Brown, G.L. and Goldman, D. (1990) *JAMA*, **264**, 3156–3160.
8. Anisworth, P.J., Surh, L.C. and Coulter-Mackie, M.B. (1991) *Nucleic Acids Res.*, **19**, 405–406.
9. Murakami, Y., Hayashi, K. and Sekiya, T. (1991) *Cancer Res.*, **51**, 3356–3361.
10. Poduslo, S.E., Dean, M., Kolch, U. and O'Brien, S.J. (1991) *Am. J. Hum. Genet.*, **49**, 106–111.
11. Mullis, K.B. and Faloona, F.A. (1987) *Methods Enzymol.*, **155**, 335–350.
12. Sommer, S.S., Sarkar, G., Koeberl, D.D., Bottema, C.D.K., Buerstedde, J-M., Schowalter, D.B. and Cassady, J.D. (1990) In *PCR Protocols: A Guide to Methods and Applications*, (Innis, M.A., Gelfand, D.H., Sninsky, J.J. and White, T.J., eds.) pp. 197.

13. Koeberl, D.D., Bottema, C.D.K., Buerstedde, J-M. and Sommer, S.S. (1989) *Am. J. Hum. Genet.*, **45**, 448–457.
14. Koeberl, D.D., Bottema, C.D.K., Ketterling, R.P., Bridge, P.J., Lillicrap, D.P. and Sommer, S.S. (1990) *Am. J. Hum. Genet.*, **47**, 202–217.
15. Bottema, C.D.K., Koeberl, D.D. and Sommer, S.S. (1989) *Lancet*, **ii**, 526–529.
16. Bottema, C.D.K., Koeberl, D.D., Ketterling, R.P., Bowie, E.J.W., Taylor, S.A.M., Lillicrap, D., Shapiro, A., Gilchrist, G. and Sommer, S.S. (1990) *Br. J. Haematol.*, **75**, 212–216.
17. Bottema, C.D.K., Bottema, M.J., Ketterling, R.P., Yoon, H.S., Janco, R.L., Phillips J.A., Sommer, S.S. (1991) *Am. J. Hum. Genet.*, in press.
18. Sarkar, G., Koeberl, D.D., and Sommer, S.S. (1990) *Genomics*, **6**:133–143.
19. Sarkar, G., and Sommer, S.S. (1989) *Science*, **244**:331–334.
20. Yoshitake, S., Schach, B.G., Foster, D.C., Davie, E.W., and Kurachi, K. (1985) *Biochemistry*, **24**:3736–3750.