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35. We thank Irene Fecycz and Gunter Blobel for supplying the *E. coli* S135 extract and Pablo Garcia and Peter Walter for providing the *lpp* clone. We thank John Atkins, Bob Weiss, and Ray Gesteland for helpful discussions and materials and Janine Marinos for assistance in preparing the manuscript. Supported by a UCSF Earle C. Anthony/Dean's Fellowship (T.E.J.), American Cancer Society Research Professorship (H.E.V.), and grants from the National Institutes of Health and the American Chemical Society.

28 August 1985; accepted 30 October 1985

RESEARCH ARTICLE

Detection of Single Base Substitutions by Ribonuclease Cleavage at Mismatches in RNA:DNA Duplexes

Richard M. Myers, Zoia Larin, Tom Maniatis

Physical methods for detecting single base substitutions have provided powerful tools for the analysis of human genetic diseases (*1-4*) and the establishment of human genetic linkage maps (*5–7*). These techniques could also be of considerable value in the detection and analysis of single base mutations in regulatory or protein-coding sequences. Procedures available for detecting base substitutions rely on differences in restriction endonuclease cleavage sites (*8–12*), or on differences in the melting behavior of wild-type and mutant DNA duplexes (*13–21*). For example, some single base substitutions result in the loss or gain of a restriction endonuclease cleavage site, and can therefore be detected in Southern blotting experiments (*8–12*). However, it is usually necessary to use a large number of different restriction enzymes before a change is detected. In addition, many substitutions cannot be detected by this procedure because they do not alter a restriction site. Another approach involves the use of synthetic oligodeoxyribonucleotides as differential hybridization probes (*13–16*). In this method, a labeled synthetic oligonucleotide homologous to the mutant or wild-type DNA is hybridized to blotted genomic DNA. Hybridization or washing conditions are then adjusted to allow the differential melting of the mismatched and perfectly paired duplexes. This method is useful for scoring substitutions at specific locations, but is not practical for screening large regions of DNA for new mutations or polymorphisms.

Differential DNA melting is also the basis for detecting single base substitutions by denaturing gradient gel electrophoresis (*17–21*). In this method, wild-type and mutant DNA molecules are separated by electrophoresis in polyacrylamide gels containing a gradient of formamide and urea. Duplex DNA fragments move through these gels with a constant mobility determined by molecular weight until they migrate into a portion of the gel containing a denaturant concentration sufficient to melt the DNA. When the DNA undergoes melting, its electrophoretic mobility abruptly decreases. Thus, the final position of a DNA fragment in the gel is determined by its melting temperature. The difference in melting temperature between two fragments that differ by a single base change is sufficient to allow separation on the gel. Even greater separation is achieved with DNA duplexes containing a single base mismatch (*18*). With specially designed plasmid vectors, virtually all possible single base substitutions can be detected in cloned DNA fragments (*19, 20*). However, for technical reasons (*18–21*), only 25 to 40 percent of all possible substitutions can be detected directly in total genomic DNA.

Because of the limitations in the procedures discussed above, we developed an alternative method for detecting single base substitutions in cloned and genomic DNA. This method involves the enzymatic cleavage of RNA at a single base mismatch in an RNA:DNA hybrid. The strategy used is based on the development of methods for synthesizing RNA probes (*22–24*), and on the observation that many ribonucleases are specific for single-stranded RNA under appropriate reaction conditions (*25*). A similar strategy had been developed earlier to detect mutations in duplex DNA containing single base mismatches (*26,*

Richard M. Myers is a postdoctoral fellow, Zoia Larin is a research assistant, and Tom Maniatis is a professor in the Department of Biochemistry and Molecular Biology, Harvard University, 7 Divinity Avenue, Cambridge, Massachusetts 02138. After 1 January the address for R.M.M. is Department of Physiology, University of California, San Francisco 94143.

Abstract. *Single base substitutions can be detected and localized by a simple and rapid method that involves ribonuclease cleavage of single base mismatches in RNA:DNA heteroduplexes. A ^{32}P-labeled RNA probe complementary to wild-type DNA is synthesized in vitro and annealed to a test DNA containing a single base substitution. The resulting single base mismatch is cleaved by ribonuclease A, and the location of the mismatch is then determined by analyzing the sizes of the cleavage products by gel electrophoresis. Analysis of every type of mismatch in many different sequence contexts indicates that more than 50 percent of all single base substitutions can be detected. The feasibility of this method for localizing base substitutions directly in genomic DNA samples is demonstrated by the detection of single base mutations in DNA obtained from individuals with β-thalassemia, a genetic disorder in β-globin gene expression.*

27). In this case, attempts were made to cleave DNA:DNA mismatches with the single-strand specific nuclease S1. However, only a small amount of cleavage occurs at a few mismatches while most mismatches are not cleaved at all (*26, 27*). In this article, we demonstrate that many single base mismatches in RNA:DNA hybrids are cleaved specifically by ribonuclease A (RNase A).

The steps in the RNase cleavage procedure are outlined in Fig. 1. A ^{32}P-labeled RNA probe is synthesized from a wild-type DNA template with the SP6 transcription system (*22–24, 28*). The RNA probe is hybridized to denatured test DNA (*29*) in solution, and the resulting RNA:DNA hybrid is treated with RNase A (*30*). The RNA products are then analyzed by electrophoresis in a denaturing gel. If the test DNA is identical to wild-type DNA, a single band is observed in the autoradiogram of the gel, since the RNA:DNA hybrid is not cleaved by RNase. However, if the test DNA contains a single base substitution that results in a mismatch recognized by RNase A, two new RNA fragments will be detected. The total size of these fragments should equal the size of the single RNA fragment observed with wild-type DNA. Thus, the mutation can be localized relative to the ends of the RNA probe by determining the sizes of the cleavage products. The end of the RNA probe mapping nearest to the substitution can be determined when the experiment is performed with DNA digested by an additional restriction enzyme (*29*), thus localizing the substitution unambiguously.

For convenience, single base mismatches in the RNA:DNA hybrids are presented as X:Y, where X and Y designate the mismatched RNA and DNA bases, respectively. For example, "C:A" refers to a mismatch in which cytosine appears in the RNA strand opposite adenine in the DNA strand.

Detection of single base substitutions in cloned DNA fragments. To establish optimal conditions for recognizing single base mismatches, and to determine which types of mismatches can be cleaved by RNase, we examined a large number of single base substitutions in the mouse β-major globin promoter region (*21, 31*). With this collection, it was possible to examine all 12 types of mismatches possible in RNA:DNA hybrids in several different sequence contexts. The results of several RNase cleavage reactions are shown in Fig. 2. The RNA probe used in these reactions is complementary to the sense strand of the β-globin promoter, and therefore is designated an "antisense" probe. When this probe was annealed to the wild-type promoter fragment and then digested with RNase A, a single, full-length RNA fragment of 186 nucleotides (nt) was observed (Fig. 2, lane 1). In some experiments, faint background bands were visible in the wild-type lane, indicating that a low level of cleavage occurs at bases that are not mismatched. In contrast, when an RNA:DNA duplex containing a C:A mismatch at position −40 in the promoter was analyzed, three bands were observed (Fig. 2, lane 2). One of these bands, representing about 50 percent of the total radioactivity in the lane, corresponds to the full-length RNA probe. The lengths of the other two RNA fragments correspond to the sizes expected for cleavage at the mismatch at position −40 in the promoter (66 and 120 nt). In this and other mismatches examined, one of the RNA fragments (the 66-nt fragment) appears as a doublet on the autoradiogram, which is probably the result of further reaction of RNase at pyrimidines near the ends of the cleaved RNA product.

Similar results were obtained with another C:A mismatch located at position −60 in the promoter (Fig. 2, lane 4). In contrast, in the case of a third C:A mismatch, occurring at −56 in the promoter, all of the radioactivity is present in the two cleavage products, indicating that 100 percent of the mismatches were cleaved under the same conditions (Fig. 2, lane 3). Altogether, 21 different C:A mismatches in the promoter were tested, and more than 50 percent of each mismatch was cleaved by RNase A in every case (Table 1). Similar results were obtained with C:C and C:T mismatches (Fig. 2, lanes 5 to 7, and Table 1). In contrast, only six of ten U:G mismatches in the promoter were cleaved by RNase, and the efficiency of cleavage varied from 10 to 90 percent (Fig. 2, lanes 8, 9, and 11, and Table 1). Three U:C mismatches were tested, and in each case cleavage was very inefficient (only 5 to 10 percent; lane 10 and Table 1). Three U:T mismatches in the promoter were cleaved at a level of 25 percent (Table 1).

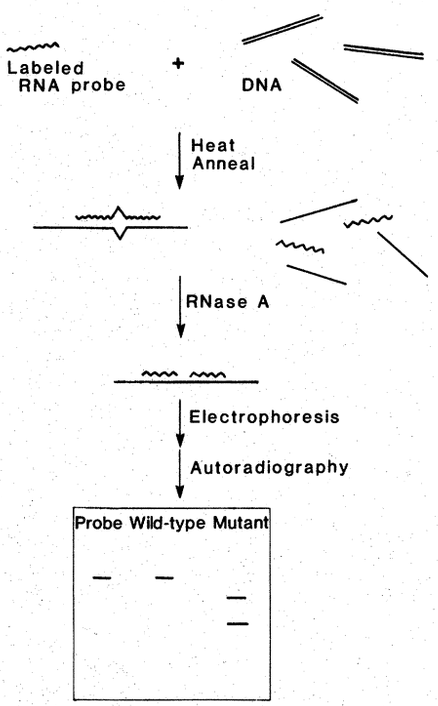

Fig. 1. Detection and localization of single base substitutions by the RNase cleavage procedure. A labeled RNA probe is synthesized with the use of the SP6 transcription system. Double-stranded DNA is digested with restriction enzymes that cleave outside the region covered by the probe and then denatured and annealed to a large molar excess of the RNA probe. Digestion of the hybridization mixture with RNase removes all of the unhybridized RNA probe and cleaves the specific RNA:DNA duplex at the position of the mismatched base. The RNase resistant products are then size-fractionated by gel electrophoresis and detected by autoradiography. In the absence of a mutation, the full-length probe fragment is observed. If the test DNA contains a single base mutation, cleavage at the resulting mismatch generates two RNA fragments whose total lengths are equal to that of the probe.

Several G:G, G:A, G:T, A:A, A:C, and A:G mismatches were tested and no cleavage by RNase A was observed in most cases (for example, see Fig. 2, lane 15). However, a small amount (10 to 20 percent) of cleavage occurred at two A:A mismatches and one G:T mismatch, and efficient cleavage occurred at three A:C and two A:G mismatches (Table 1). It is surprising that cleavage occurred at these mismatches since RNase A cleaves after pyrimidines (*25*). However, it is possible that destabilization of the mismatched RNA:DNA duplex leads to cleavage at nearby pyrimidine bases.

To determine whether this procedure can be used to detect small deletions, we analyzed several promoter fragments containing different single base deletions. In each case, nearly complete cleavage of the probe was observed at the resulting single base "loop-out," or at nearby pyrimidines (Fig. 2, lane 12, and Table 1). Similarly, RNA:DNA duplexes containing two mismatches in close proximity were efficiently cleaved in the assay (Fig. 2, lane 13, and Table 1).

Detection of β-thalassemia mutations in cloned and genomic DNA. To establish the feasibility of detecting single base mutations associated with human genetic diseases, we analyzed a number of different cloned and genomic DNA's bearing β-thalassemia or sickle cell anemia mutations. In these experiments, the RNA probes used were about 615 nt in length, spanning the region of the gene and 5′ flanking sequences from −128 to +485 (*32*). Two RNA probes were synthesized to test both the sense and antisense strand of the region. With this set of substitutions and probes, 10 of the 12 types of RNA:DNA mismatches could be formed, and 7 out of the 10 types were cleaved to some extent by RNase (Table 1).

To determine whether the RNase cleavage procedure could be used to detect single base substitutions in total genomic DNA, we analyzed DNA samples from two individuals with β-thalassemia. One individual carried a C to T transition at codon 39 of the β-globin gene in both chromosomes. The second individual was homozygous for the hemoglobin β^E (HbE) allele, which contains a G to A transition at codon 26 in the gene. The codon 39 ($\beta^0$39) DNA was tested with the sense strand RNA probe, whereas the HbE DNA was tested with the antisense RNA probe. Both of these hybrids result in C:A mismatches with their corresponding probes. When a control experiment was performed with the sense probe and genomic DNA from an individual with wild-type β-globin genes, a single band appearing at the full-length position resulted (Fig. 3A, lane 1). When DNA from the individual homozygous for the $\beta^0$39 mutation was analyzed, RNA fragments 430 and 185 nt in length were observed (Fig. 3A, lane 2), indicating that cleavage at the C:A mismatch occurred at a high efficiency. Similar results were obtained with the analogous cloned DNA samples (Fig. 3A, lanes 3 and 4). In another experiment with the antisense RNA probe, genomic DNA from an individual with normal β-globin genes also resulted in a single band appearing at the full-length probe position (Fig. 3B, lane 1). Genomic DNA from a patient homozygous for the HbE allele resulted in two RNA fragments of the expected sizes of 355 and 260 nt (Fig. 3B, lane 2), again indicating complete cleavage of the mismatch by RNase A. These results were obtained with 3 μg of total genomic DNA, and RNA probes with an [α-^{32}P]GTP specific activity of 400 Ci/mmol. A signal could be clearly detected after a 24-hour autoradiographic exposure. These experiments therefore establish the feasibility of detecting single base mutations and linked polymorphisms in genomic DNA with this method, at a level of sensitivity at least comparable with existing techniques.

Analysis of mismatch recognition. We find that 4 (C:A, C:C, C:T, and U:T) out of the 12 possible types of mismatches are recognized efficiently by RNase A in all sequence contexts tested. Thus, approximately one-third of all possible single base substitutions can be detected with an RNA probe homologous to one strand of the test DNA. This number can be doubled with the use of a second RNA probe, homologous to the opposite strand of the test DNA. For example, a

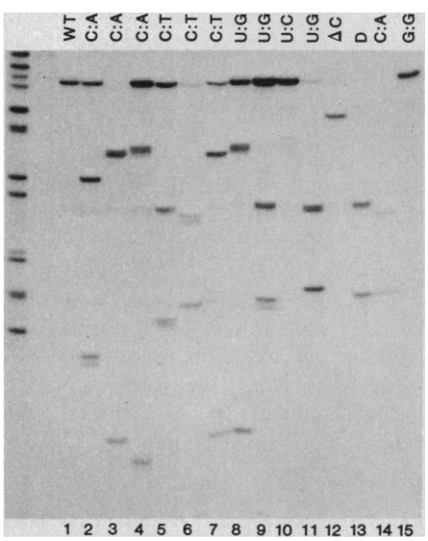

Fig. 2. RNase cleavage analysis of singe base mutations in a cloned mouse β-major globin promoter fragment. A 186-nt antisense RNA probe was annealed to wild-type and mutant promoter fragments and the resulting RNA:DNA duplexes treated with RNase A. The digestion products were analyzed by polyacrylamide gel electrophoresis and autoradiography. The DNA sample analyzed in each lane was: wild-type (lane 1); mutant −40A (lane 2); −56A (lane 3); −60A (lane 4); −33T (lane 5); −25T (lane 6); −54T (lane 7); −57G (lane 8); −31G (lane 9); −62C (lane 10); −26G (lane 11); −76 deletion (lane 12); −28G/−26G (lane 13); −25A (lane 14); −49G (lane 15). The type of mismatch produced by annealing the wild-type antisense RNA probe to each mutant DNA fragment is indicated at the top of each lane.

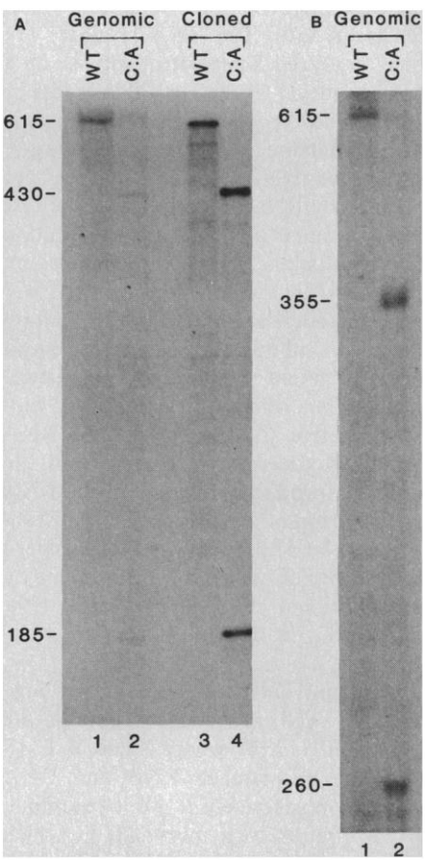

Fig. 3. RNase cleavage analysis of human genomic DNA samples from individuals with β-thalassemia. (A) Analysis of the $\beta^0$39 thalassemia mutation. Genomic and cloned DNA from an individual with wild-type β-globin genes and an individual homozygous for a nonsense mutation in codon 39 were analyzed by the RNase cleavage procedure with a sense strand RNA probe. An autoradiogram of the RNase digestion products is shown. Genomic wild-type β-globin DNA (lane 1); genomic $\beta^0$39 DNA (lane 2); cloned wild-type β-globin DNA (lane 3); cloned $\beta^0$39 DNA (lane 4). The sizes of the RNase digestion products are indicated. (B) Analysis of the HbE thalassemia mutation. Genomic DNA from an individual with wild-type β-globin genes and an individual homozygous for HbE were analyzed by the RNase cleavage procedure an antisense RNA probe. Genomic wild-type β-globin DNA (lane 1); genomic HbE DNA (lane 2).

G:T mismatch formed between one strand of the test DNA and the homologous RNA probe may not be cleaved by RNase A. However, when the other DNA strand is hybridized to its homologous RNA probe, the C:A mismatch at that same position will be cleaved by RNase. Thus, approximately two-thirds of all possible single base substitutions should be detected. This is clearly a minimum estimate, since we have observed cleavage at seven of the remaining eight possible types of mismatches in some sequence contexts.

We do not understand why some mismatches can be cleaved in some sequence contexts but not in others. It seems likely that differences in accessibility to cleavage are the result of differences in the overall structure of the mismatched duplex. However, we have not been able to discern a sequence pattern surrounding a mismatch that can be correlated with the observed efficiency of RNase cleavage.

As indicated in Fig. 2 and Table 1, some mismatches are only partially cleaved in the assay. Our data were obtained by performing the RNase reactions for a fixed length of time (30 minutes). In a time-course experiment, we found that many mismatches that are only partially cleaved in 30 minutes can be cleaved almost to completion in 90 minutes under the same conditions and with only a slight increase in background. However, mismatches not cleaved in 30 minutes are also not affected by longer incubation times. Thus, it may be desirable to perform the RNase reactions for various lengths of time in cases where partial cleavage occurs.

The temperature and ionic strength of the solution in which the RNase reaction is performed also contribute to the degree of cleavage and the apparent effects of sequence context. In fact, altering the reaction conditions to higher temperature and lower ionic strength results in cleavage at some mismatches that are not normally cleaved, and more complete cleavage of mismatches that are normally only partially cleaved. These reaction conditions may be desirable in some cases, but are not ideal since internal cleavage at perfectly matched positions also increases significantly.

The fact that some mismatches are never or rarely cleaved by RNase A and that partial cleavage sometimes occurs led us to test the ability of other ribonucleases to cleave at mismatches. We have not detected any cleavage with RNase T1 and RNase T2 under various reaction conditions.

The lack of complete cleavage of some mismatches may pose a difficulty when the RNase cleavage procedure is used for determining the genotype of a diploid genome. In cases where 50 percent or less of the RNA probe is cleaved, low efficiency of cleavage could be an intrinsic property of the mismatch in question, or the individual may be heterozygous for the mutant allele. This ambiguity may often be eliminated by performing a time-course of RNase treatment. Alternatively, as with oligonucleotide probes (*3, 16, 33*), the genotype can be unambiguously determined if probes are available for both wild-type and mutant alleles. Thus, it should be possible to use this method for prenatal diagnosis of genetic diseases. Partial cleavage at mismatches is not a problem when mapping mutations in cloned DNA samples, genomic DNA from haploid organisms, or genomic DNA sequences within the X chromosome of human males.

We have learned that a similar approach was independently developed to detect single base substitutions in messenger RNA (*34*). In that case ^{32}P-labeled antisense SP6-RNA was annealed to total cellular RNA to generate an RNA:RNA duplex containing a single base mismatch. As in the case of the RNA:DNA mismatches analyzed here,

Table 1. Tabulation of the results of an analysis of single base substitutions in the mouse β-major globin promoter region and in the human β-globin gene.

Mismatch*	Cleaved (%)†	Mutant‡	Probe§	Context‖
C:A	50	M -40A	AS	CCCUG
	100	M -56A	AS	CUCUA
	50	M -51A	AS	GCCCU
	100	M -22A	AS	CUCAC
	100	M -19A	AS	UACCU
	100	M +19A	AS	AACUA
	50	M -33A	AS	UGCUC
	50	M -50A	AS	UGCCC
	100	M -52A	AS	CCCUC
	100	M -65T	S	CACAC
	100	M -63T	S	CACAG
	75	M -49T	S	GGCAG
	100	M -42T	S	GCCAG
	50	M -37T	S	GGCAG
	75	M -32T	S	AGCAU
	50	M -4T	S	CUCCU
	100	M -1T	S	CUCAC
	100	M +2T	S	CACAU
	60	M -77T	S	GGCCA
	100	H HbE	AS	CUCAC
	50	H IVS11	AS	AACCU
	100	H C39	S	CCCAG
C:C	100	M -42G	S	GCCAG
	100	M -32G	S	AGCAU
	100	M -76G	S	GCCAA
	100	H IVS15C	AS	UACCU
C:T	50	M -33T	AS	GACCA
	75	M -25T	AS	AACGU
	75	M -54T	AS	GACAG
	100	M -65A	S	CACAC
	100	M -63A	S	CACAG
	50	M -43A	S	AGCCA
	100	M -32A	S	AGCAU
	100	M -67A	S	CUCAC
	90	H IVS15T	AS	UACCU
U:G	50	M -57G	AS	UCUAU
	30	M -31G	AS	UAUGC
	90	M -26G	AS	CCUUA
	0	M -48G	AS	CCUGC
	0	M -45G	AS	GCUCC
	40	M -59G	AS	UAUCC
	30	M -34G	AS	CGUCU
	0	M -23C	S	GGUGA
	10	M -18C	S	GGUAG
	0	M +12C	S	UCUGA
	25	H IVS16	S	GGUAU
U:C	5	M -62C	AS	CCUGU
	5	M -66C	AS	UGUGA
	5	M -45C	AS	GCUCC
U:T	25	M -13A	S	GAUCA
	25	M -2A	S	CCUCA
	25	M +6A	S	UUUGC
	75	H HbS	AS	CCUCU
G:A	0	M -49A	AS	CUGCC
	0	M -42A	AS	CUGGC
	0	M -54T	S	GAGAG
	0	M -35T	S	CAGAG
	0	M -33T	S	GAGCA
	0	M -25T	S	AAGGU
	75	H IVS15T	S	UGGUA
G:T	15	M -49T	AS	CUGCC
	0	M -42T	AS	CUGGC
	0	M -50A	S	GGGCA
	0	M -51A	S	AGGGC
	0	M -40A	S	CAGGG
	0	M -25A	S	AAGGU
	0	M -22A	S	GUGAG
	0	M -19A	S	AGGUA
	0	M -61A	S	CAGGA
	0	M -60A	S	AGGAU
	0	M -56A	S	UAGAG
	0	H Hb39	AS	CUGGG
	0	H IVS11	S	AGGUU
	0	H HbE	S	GUGAG
G:G	0	M -49G	AS	CUGCC
	0	M -42G	AS	CUGGC
	0	M -76G	AS	UUGGC
	0	M -77G	AS	UGGCC
	0	M +13C	S	CUGAC
	0	H IVS15C	S	UGGUA
A:C	0	M -23C	AS	UCACC
	0	M -58C	AS	CUAUC
	75	M -57G	S	AUAGA
	0	M -48G	S	GCAGG
	0	M -74G	S	CAAUC
	0	M -55G	S	AGAGA
	0	M -45G	S	GGAGC
	0	M -34G	S	AGAGC
	100	M -31G	S	GCAUA
	100	M -29G	S	AUAUA
	0	M +14G	S	UGACA
	50	H IVS16	AS	AUACC
A:A	10	M -13A	AS	UGAUC
	0	M -2A	AS	UGAGG
	0	M -73A	AS	UGAUU
	0	M -30A	AS	UAAUG
	20	M -48T	S	GCAGG
	0	H HbS	S	UGAGG
A:G	100	M -66C	S	UCACA
	50	M -62C	S	UCAGG
	0	M -45C	S	GGAGC
Deletion	100	M +2CD	AS	AUGUG
	100	M -76CD	AS	UUGGC
	100	M +10TD	AS	CUUCU
	100	M -18TD	AS	GGUAG
Double	100	-28G/-26G	AS	
	100	-28C/-23C	AS	

*The type of mismatch formed in each case. †The fraction of the total protected RNA probe that is present in cleaved RNA fragments. ‡The mouse promoter mutants are indicated by M followed by a number designating their position relative to the cap site of β-globin transcription (*32*). The human β-thalassemia mutations are indicated by H followed by name of the mutation. §The probe used in each case is designated either as sense (S) or antisense (AS). ‖The nucleotides surrounding each mismatch in the RNA strand are indicated in a 5′ to 3′ direction. The underlined nucleotide in each case occurs at the position of the mismatch.

RNA:RNA mismatches are also cleaved by RNase A.

Applications. The RNase cleavage procedure described provides a sensitive, rapid, and simple means of detecting single base substitutions in cloned or genomic DNA. The ^{32}P-labeled RNA probes are easily prepared with well-characterized SP6-plasmid vectors, the required enzymes are commercially available, and the electrophoresis involves the use of standard DNA sequencing gels. In addition, analysis of the sizes of the RNase cleavage products of the RNA:DNA heteroduplexes not only provides evidence for the presence of a single base mismatch in the test DNA but also makes it possible to localize the mismatch to within a few nucleotides.

The RNase cleavage procedure should be applicable to problems where the detection and localization of single base substitutions is important. For example, the procedure can be applied to the analysis of human genetic diseases. By establishing sets of SP6-plasmids containing DNA fragments that span an entire gene, it should be possible to survey rapidly even the largest genes for single base mutations. Similarly, this method should be valuable for detecting neutral polymorphisms in genetic linkage studies. The ability to detect a large fraction of all possible single base substitutions in a DNA fragment with a single RNA probe represents a significant advance over current methods that involve the detection of restriction fragment length polymorphisms. Another application of this procedure is the localization of mutations that are genetically selected.

References and Notes

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28. Uniformly labeled single-stranded RNA probes were made as described (*24*) with [α-^{32}P]GTP as the only labeled nucleotide. Bacteriophage SP6 RNA polymerase and [α-^{32}P]GTP were purchased from New England Nuclear. RNasin was from Promega Biotec and nucleoside triphosphates were from PL Biochemicals. Probes used with cloned DNA samples were synthesized with [α-^{32}P]GTP (40 Ci/mmol, 100 μM) in the transcription reaction, and could be used for a period of 2 weeks with little evidence of degradation. Probes used with genomic DNA samples contained [α-^{32}P]GTP (400 Ci/mmol) and were used within 3 days. After synthesis, the probe made from 1 μg of template DNA was resuspended in 150 μl of 10 mM tris-HCl (*p*H 7.5), 1 mM EDTA, and 0.1 percent SDS. Approximately 0.5 μl of probe, which contained 5×10^4 to 1×10^5 cpm or 5×10^5 to 1×10^6 cpm for probes made for cloned or genomic DNA samples, respectively, was used in each hybridization reaction. RNA probes used in this procedure must be full-length to obtain unambiguous cleavage results. With some DNA sequences it is difficult to achieve 100 percent full-length probe synthesis in the runoff transcription reaction, particularly when synthesizing genomic probes where the GTP concentration is low. In these cases, the full-length RNA probe should be purified by gel electrophoresis after synthesis.
29. To prepare a cloned or genomic DNA sample for RNase cleavage analysis, the DNA was digested with restriction enzymes that do not cleave within the sequence homologous to the RNA probe. By performing the annealing and cleavage reactions with this DNA and analyzing the RNA products by gel electrophoresis, the position of a substitution can be mapped accurately relative to one or the other of the two ends of the probe. To determine unambiguously which end of the probe is nearest to the substitution, a second experiment can be performed as follows. The DNA sample is digested with a restriction enzyme that cleaves once within the sequence homologous to the probe, generating an additional DNA fragment. After the hybridization reaction, each RNA:DNA duplex will contain a single-stranded "overhang" of RNA probe that will be digested to oligonucleotides in the RNase reaction. Analysis of the RNA products by electrophoresis will result in the replacement of one of the RNA's with two species whose total size equals that of the missing fragments, revealing the exact position of the substitution relative to the restriction site. The hybridization is carried out with the RNA probe in molar excess to avoid rejoining the two DNA fragments released by restriction digestion by hybridization to the same RNA molecule.
30. Ribonuclease A (Sigma, R-5125) was dissolved in distilled water to a concentration of 2 mg/ml and then placed in a boiling water bath for 10 minutes. After cooling to room temperature, the solution could be stored at +4°C for as long as 1 year. Formamide (MCB, Inc.) was deionized by stirring with Dowex Mixed Bed Resin AG501X8D (Bio-Rad) at room temperature for 30 minutes and was stored at +4°C. Hybridizations were performed by suspending 20 to 50 ng of cloned plasmid DNA or 3 to 6 μg of total genomic DNA in 30 μl of hybridization buffer [80 percent formamide, 40 mM Pipes (*p*H 6.4), 0.4M NaCl, and 1 mM EDTA], adding 0.5 μl of labeled RNA probe (*28*) and treating the mixture at 90°C for 10 minutes. The samples were then incubated at 45°C for 30 minutes (cloned DNA) or 10 hours (genomic DNA). After the annealing, 350 μl of a solution containing RNase A (40 μg/ml) in 10 mM tris-HCl (*p*H 7.5), 1 mM EDTA, 200 mM NaCl, and 100 mM LiCl was added to the hybridization mixture. The sample was then mixed by vortexing and incubated at 25°C for 30 minutes. The RNase reaction was stopped by the addition of 10 μl of 20 percent SDS and 10 μl of proteinase K (10 mg/ml) (Boehringer Mannheim) followed by incubation at 37°C for 15 to 30 minutes. Protein was then removed by extraction with an equal volume of phenol-chloroform (1:1) containing 4 percent isoamyl alcohol and 0.01 percent hydroxyquinoline. The aqueous supernatant (300 μl) was removed without disturbing the interface to ensure that no traces of RNase remained. After the addition of 10 to 20 μg of carrier transfer RNA, the samples were precipitated with ethanol and resuspended in 5 μl (genomic DNA) or 25 μl (cloned DNA) of loading buffer and analyzed by denaturing polyacrylamide gel electrophoresis [T. Maniatis, E. F. Fritsch, J. Sambrook, *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., 1982), p. 184]. A time-course of annealing the RNA probe to genomic DNA indicated that the hybridization was complete after 6 hours with the quantities of probe and genomic DNA (3 to 6 μg) used here. Because there may be variations in probe and genomic DNA concentration in different experiments, 10-hour hybridizations were routinely performed to ensure maximum signals. Reaction conditions for RNase cleavage were chosen empirically. We found that a buffer containing 100 mM LiCl and 200 mM NaCl suppressed the background to a greater extent than did 300 mM NaCl alone. In addition, a slightly higher temperature (30°C) of RNase treatment resulted in more complete cleavage at partially cleaved mismatches, but also caused some increase in background. Results very similar to our previous result with RNase treatment at 16°C in a buffer containing 100 mM LiCl and 100 mM NaCl.
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35. We thank H. Kazazian for providing us with genomic DNA samples of defined genotype; S. Goodbourn and B. Seed for helpful discussions and suggestions for reaction conditions; P. Krieg and A. Krainer for comments on the manuscript, and J. Ma and M. Green for contributing to the early stages of this work. Supported by grants from the National Institutes of Health (T.M.), The Wills Foundation, and a Special Fellowship of the Leukemia Society of America (R.M.M.).

6 September 1985; accepted 28 October 1985