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Cleavage of DNA.RNA hybrids by Type II restriction enzymes

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ABSTRACT

The action of a number of restriction enzymes on DNA.RNA hybrids has been examined using hybrids synthesised with RNAs of cucumber mosaic virus as templates. The enzymes EcoRI, HindII, SalI, MspI, HhaI, AluI, TaqI and HaeIII cleaved the DNA strand of the hybrids (and possibly also the RNA strand) into specific fragments. For four of these enzymes, HhaI, AluI, TaqI and HaeIII, comparison of the restriction fragments produced with the known sequences of the viral RNAs confirmed that they were recognising and cleaving the DNA strand of the hybrids at their correct recognition sequences. It is likely that the ability to utilise DNA.RNA hybrids as substrates is a general property of Type II restriction enzymes.

INTRODUCTION

The specific cleavage of duplex DNA by Type II restriction enzymes isolated from a wide range of bacterial sources and the properties of these enzymes have been extensively studied (1,2). The action of these enzymes involves an initial recognition of a specific sequence in a double-stranded DNA substrate, followed by cleavage of a phosphodiester bond in each strand, with this cleavage occurring within the recognition site for most enzymes.

Single-stranded DNA has also been found to act as a substrate for a limited number of restriction enzymes, e.g., HaeIII (and its isoschitzomer BspI), HhaI, MboI, HinfI and HpaII (3,4,5). Of these, the enzyme HaeIII has been most widely used for the cleavage of single-stranded DNA. Analysis of the temperature dependence of the reaction led Blakesley *et al.* (6) to conclude that cleavage required the formation of duplex regions within single-stranded molecules and that the reaction was not, therefore, essentially different to that on a double-stranded substrate.

The cleavage of one strand only of a duplex DNA at a modified EcoRI site has also been reported (7) indicating that neither the recognition nor cleavage step requires the full double-stranded recognition site. In this

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case the EcoRI site contained a single base substitution adjacent to the cleavage site in one strand; only the strand containing the unmodified sequence at the cleavage site was cut.

In this paper we report on the ability of a number of restriction enzymes to utilise DNA.RNA hybrid duplexes as substrates. The enzymes retain their sequence specificity in cutting the DNA strand of the duplex and possibly also specifically cleave the RNA strand.

MATERIALS AND METHODS

RNA. Cucumber mosaic virus (CMV) RNA 4 and its satellite RNA (SAT RNA) were prepared by a two-step polyacrylamide slab gel procedure (8) followed by sucrose gradient centrifugation to remove contaminating acrylamide. Polyadenylation of the RNAs was as described by Gould *et al.* (9).

Enzymes. Restriction enzymes were obtained from New England Biolabs, with the exception of EcoRI, SalI and HindII which were prepared in the Department and kindly provided by S.J. Clark, Prof. G.E. Rogers and R.P. Harvey, respectively. Digestions with restriction enzymes were done in the buffers recommended by N.E. Biolabs. Nuclease SI was prepared by the method of Vogt (10) up to and including the DEAE-cellulose step. Reverse transcriptase from avian myeloblastosis virus was generously provided by the Office of Program Resources and Logistics, National Cancer Institute, Bethesda, Maryland.

Synthesis of complementary DNA. cDNA synthesis was done in a buffer containing 20 mM Tris.HCl, pH 8.3, 70 mM KCl, 10 mM $MgCl_2$, 4 mM $Na_4P_2O_7$, 20 mM dithiothreitol, 500 μM each of dATP, dTTP, dGTP and dCTP in the presence of 2 to 3 μCi/μl of α-^{32}P-dCTP (11). Reaction mixtures of 50 μl also contained 2 to 5 μg of polyadenylated RNA, a tenfold molar excess of d(pT_8G)(P/L Biochemicals) and 10 units of reverse transcriptase. After 45 min at 37°C reactions were terminated by the addition of EDTA to 15 mM and SDS to 0.5%, extracted with phenol and the cDNA.RNA hybrid recovered by gel filtration over Sephadex G50 followed by ethanol precipitation. When added to reaction mixes, Actinomycin D was present at 100 μg/ml.

Gel electrophoresis and autoradiography. Polyacrylamide gels (4% or 5%) were essentially as described by Sanger and Coulson (12); urea was present at 7 M in denaturing gels and absent in non-denaturing gels. Autoradiography was done at 4°C or -70°C using pre-flashed Fuji-RX medical X-ray film and Ilford fast tungstate intensifying screens (13).

Nuclease SI digestions. The determination of the proportion of single-stranded nucleic acid by nuclease SI digestion was as described by Gould and

Symons (14).

RESULTS

Synthesis of cDNA.RNA Hybrids

Synthesis of cDNA using reverse transcriptase has been observed to give varying amounts of single- or double-stranded DNA as well as cDNA still bound to its RNA template under some synthetic conditions (15,16). This has been ascribed to the action of RNAse H which degrades the RNA strand of a DNA.RNA hybrid, thus producing regions of single-stranded DNA and releasing small RNA fragments which can act as primers for synthesis of anticomplementary DNA on the cDNA strand (16). The addition of sodium pyrophosphate (4 mM) or high levels of deoxynucleotides was found to totally inhibit the action of RNAse H and under these conditions the RNA and product cDNA remained stably complexed (16). Synthesis of cDNA was, therefore, done in the presence of 4 mM sodium pyrophosphate and 500 μM of each dNTP.

The RNAs used for the synthesis of DNA.RNA hybrids were SAT RNA and RNA 4 of CMV which are, respectively, 336 and approximately 1000 nucleotides in length. Most of SAT RNA has been sequenced (unpublished results) and it has been found to be very similar to another satellite RNA of CMV, CARNA 5, sequenced by Richards *et al.* (17); the sequence of the first 270 residues from the 3'-end of CMV RNA 4 has been determined (18). As SAT RNA and CMV RNA 4 terminate at the 3'-end in the bases -CC and -CCA, respectively (18,19 and unpublished results), it is possible following polyadenylation to specifically prime cDNA synthesis using d(pT_8G), generating cDNA.RNA hybrids having specific 5'-termini of the cDNA. A number of restriction enzyme sites can be predicted from the known sequences and the hybrids are therefore well suited for use in the analysis of the action of restriction enzymes on DNA.RNA hybrids.

DNA.RNA hybrids of both CMV RNA 4 and SAT RNA were synthesised in the presence of α-^{32}P-dCTP and their integrity examined by digestion with nuclease SI. The cDNA synthesised was 100% and 92% resistant to nuclease SI digestion, respectively, for the two RNAs; following removal of the RNA strands by treatment with 0.3 M NaOH for 3 h at 37°C the residual cDNA resistant to digestion with nuclease SI was 19% and 11%, respectively.

Survey of Action of a Number of Restriction Enyzmes on cDNA.RNA Hybrids

An initial survey of restriction enzymes was done by digesting aliquots of a cDNA.RNA 4 hybrid preparation in the appropriate buffers. Each 20 μl reaction contained 12 ng of hybrid and 0.1 to 0.2 units of enzyme and was incubated for 2 h; this 20 to 50 fold excess of enzyme over that used with

double-strand DNA is similar to that used for digestion of single-stranded DNA with HaeIII (4) and has been found necessary with most enzymes to completely digest the DNA.RNA hybrids. The digested hybrids were denatured by boiling in 70% formamide for 5 min and the radiolabelled cDNA-strand products analysed by electrophoresis on a denaturing 4% polyacrylamide-urea gel (Fig. 1). Complete or near complete digestion of the cDNA strand to discrete fragments was observed for the enzymes EcoRI, SalI, HindII, AluI, HhaI, TaqI, MspI and Hae-

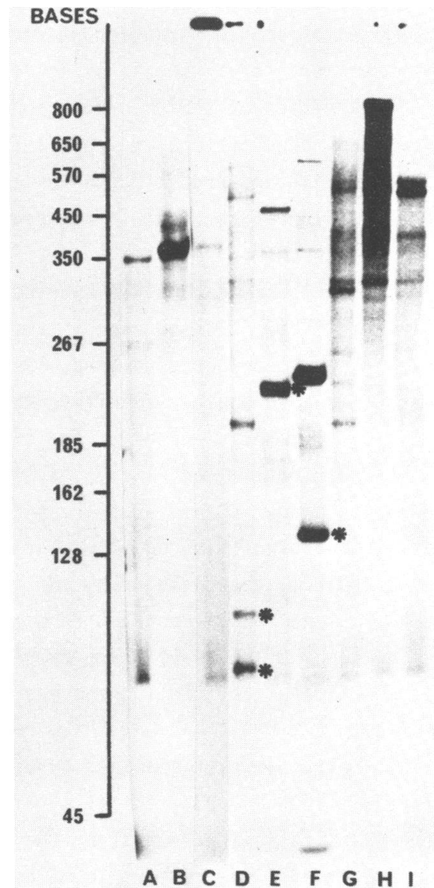

FIGURE 1. Restriction enzyme digests of cDNA.CMV RNA 4 hybrids analysed by electrophoresis on a denaturing 4% acrylamide, 7 M urea gel which was run at 20 mA for 90 min (12). End-labelled fragments derived from an MspI digest of the replicative form of bacteriophage M13 were used as size markers (24). Track A, EcoRI digest; B, SalI; C, HindII; D, AluI; E, HhaI; F, TaqI; G, MspI; H, no enzyme; I, HaeIII. Bands marked with an asterisk are those predicted from the known 3'-terminal sequence of CMV RNA 4 (18).

III. A number of enzymes which were used in the survey did not digest the hybrid at all (MboI, BamHI, PstI, HindIII, SacI, XbaI and HpaI). From the known 3'-terminal sequence of 270 residues of CMV RNA 4 (about 25% of total), no sites for these enzymes would be predicted so it is not known whether they are capable of cleaving a hybrid molecule if the correct sequences were present.

The production of specific fragments by a number of enzymes suggests that the cDNA was cut at sequence specific sites, presumably the recognition sites of the different enzymes. The appearance of the same size fragment on digestion with HindII (recognition sequence GTPyPuAC) and SalI (GTCGAC) supports this. From the known sequence of the 3'-end of CMV RNA 4 (18) sites for three restriction enzymes were predicted in the first 270 residues. Fragments predicted from the sequence are those marked with an asterisk in Fig. 1; TaqI (142 bases), HhaI (221 bases) and AluI (89 and 108 bases). This specificity of cleavage was further examined using cDNA.SAT RNA hybrids.

Digestion of cDNA.SAT RNA Hybrids

Most of the sequence of SAT RNA has been determined (Symons, unpublished data) and from this sequence the restriction sites for HaeIII and TaqI, which are depicted in Fig. 2A, were expected. Hybrid cDNA.SAT RNA was digested with both enzymes, the products boiled in 70% formamide and subjected to electrophoresis on a 4% polyacrylamide-urea gel (Fig. 2B). Digestion with TaqI yielded the expected cDNA fragments of 119 and 225 bases (including the d(pT_8G) primer); digestion with HaeIII also gave the expected products and in addition the possible partial digestion products of 197 and 221 bases could normally be seen. It is clear that both enzymes specifically recognised the restriction sites in the cDNA.RNA hybrid and specifically cleaved the cDNA strand of the hybrid in the normal positions.

To examine whether the RNA strand of the hybrid was also cleaved by the restriction enzymes the digestion products were also analysed by electrophoresis on a non-denaturing 5% polyacrylamide gel (tracks D and E, Fig. 2B). Discrete bands were again observed for the digestion products of both enzymes, with the sizes of the double-stranded fragments being as expected from the known sequence.

From this result it is possible that the RNA strand of the hybrid was also being specifically cleaved. However, it is also possible that the nicking of the cDNA strand permits cleavage of the RNA at this site by non-specific RNases or that the RNA strand was already extensively nicked leading to double-strand separation on nicking of the cDNA strand. Further experi-

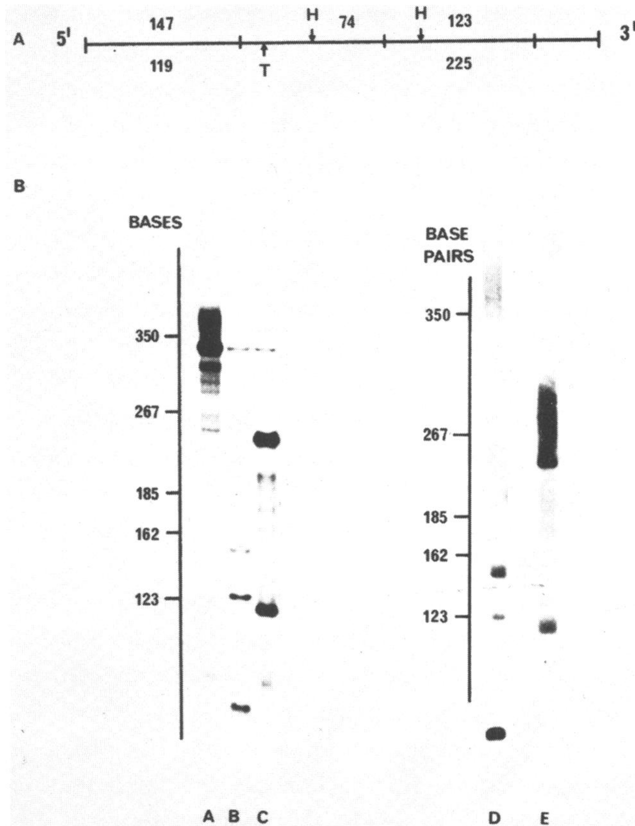

FIGURE 2. A: Map of cDNA to CMV SAT RNA. Cross bars are drawn at each 100 residues from the 5'-end; the total length including the d(pT8G) primer is 344 nucleotides. The sites of HaeIII and TaqI restriction sites are indicated by the arrows labelled H and T, respectively. The numbers above the line represent the sizes of the three fragments produced by cleavage with HaeIII and those below the line the sizes of the two fragments produced by digestion with TaqI.

B: Products of digestion of cDNA.SAT RNA hybrid. Tracks A to C, denaturing gel as in Figure 1; A, no enzyme; B, HaeIII digest; C, TaqI. Tracks D and E; products of digestion of cDNA.SAT RNA with HaeIII and TaqI, respectively, analysed by electrophoresis on a non-denaturing 5% acrylamide gel. Electrophoresis was at 15 mA for 2 h (12).

ments are required to establish the specificity of cleavage of the RNA strand.

DISCUSSION

The above results demonstrate that a number of restriction enzymes are

able to recognise their specific base sequences in DNA.RNA hybrid molecules and to faithfully cut at least the DNA strand of the hybrid, albeit at concentrations of enzyme 20 to 50 times that required for the cleavage of duplex DNA. The range of enzymes which cut the hybrid duplexes is wider than those shown to utilise single-stranded DNA as a substrate and so far no enzyme has failed to cleave a hybrid molecule at an expected recognition site. The ability to cut DNA.RNA hybrids may therefore be a general property of restriction enzymes. This is very interesting with respect to the interaction of restriction enzymes with their substrates and also raises the possibility that *in vivo* they may be able to catalyse cleavage of newly transcribed RNA still associated with its DNA template.

Of immediate technical importance is that the methodology used for the study of DNA with restriction enzymes can now be applied to the study of RNA molecules. By analysing radiolabelled cDNA.RNA hybrids, restriction maps of individual RNAs can be constructed and different RNAs or populations of RNAs compared. This could prove particularly useful for following the induction of individual messenger RNAs in a population. The isolation of specific cDNA restriction fragments provides a means of obtaining pure probes complementary to specific regions of an RNA molecule or to individual RNAs in a mixed population. Specific fragments can also be isolated and used as primers on RNA for sequencing by the dideoxynucleotide chain-termination technique (18,20,21, 22) or directly sequenced by chemical methods (23). Initial experiments using cDNA primers have been encouraging; the method offers the advantage that the template RNAs used with the specific primers need not be rigorously purified from other RNA species, and that the range of enzymes which can be used to generate fragments is wider than if single-stranded cDNA is used.

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