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SYNTHESIS OF A DOUBLE-STRANDED cDNA TRANSCRIPT OF THE KILLER TOXIN-CODING REGION OF THE YEAST M1 DOUBLE-STRANDED RNA

Nigel Skipper

Molecular Genetics Group, Division of Biological Sciences
National Research Council of Canada Ottawa, Ontario K1A 0R6
Canada

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Reverse transcription of methylmercuryhydroxide-treated M1 double-stranded RNA of yeast produces several discrete single-stranded and double-stranded cDNA's from oligo$(dT)_{12-18}$ primer. S1 nuclease analysis shows that the longest transcript of the 1.8 kb template, a 2.2 kb molecule, is a 1.1 kb duplex terminated at one end by a hairpin-like structure. The 1.1 kb ds cDNA contains a complete copy of the M1-1, toxin-coding, sequence of M1 double-stranded RNA, and its synthesis is primed from oligo$(dT)_{12-18}$ that anneals to a sequence within the internal, AU-rich, region of the template.

M1 double-stranded RNA (M1 dsRNA) of the yeast Saccharomyces cerevisiae is a linear molecule of 1.8 kb that exists encapsidated in a virus-like particle. The particles are maintained by an interaction with nuclear genes and a second, separately-encapsidated, molecule, L double-stranded RNA (L dsRNA), (1-4). M1 dsRNA codes in vitro for a precursor of the secreted K1 yeast killer toxin (5); it also encodes a component of toxin-immunity, which remains to be identified (6). M1 dsRNA contains a 0.19 kb internal AU-rich sequence. This sequence can be eliminated by nuclease S1 or by heat, leaving the 0.98 kb M1-1 double-stranded RNA (M1-1 dsRNA) and the 0.66 kb M1-2 double-stranded RNA (M1-2 dsRNA) fragments (7,8). In vitro, M1-1 dsRNA can be translated into a polypeptide which contains the toxin (7). We are interested in the sequence requirements for synthesis and intracellular processing of the toxin precursor in vivo. To obtain DNA copies of the M1 dsRNA suitable for cloning and sequence analysis we have attempted to reverse transcribe the dsRNA, and report here that transcription of methylmercuryhydroxide-treated M1

Abbreviations: dsRNA, double-stranded RNA; M1-1, the large S1-resistant dsRNA fragment from M1 dsRNA; M1-2, the small S1-resistant fragment from M1 dsRNA; 5 x SSPE, 0.9 M sodium chloride, 50 mM sodium phosphate, 5 mM disodium EDTA, pH 6.5 to 7.0; EDTA, ethylenediaminetetraacetic acid.

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dsRNA produces a number of discrete products, including a 1.1 kb hairpin-terminated duplex which contains a complete copy of the toxin-coding sequence.

MATERIALS AND METHODS

Total dsRNA (L dsRNA, M1 dsRNA) was isolated from cells of S. cerevisiae T158c/S14a (9) by the procedure of Fried and Fink (10) then the M1 dsRNA purified by electrophoresis of the dsRNA in agarose and electroelution from the gel. Elimination of the internal AU-rich sequence from M1 dsRNA was done by S1 nuclease digestion as described (7). To prepare cDNA, M1 dsRNA in water was added to 1 vol. 40 mM methylmercuryhydroxide (Alfa Products, Danvers, Massachussetts) and after 10 min. added to 22 vol. reaction mix to contain, in 15 µl, 50 mM Tris.HCl, pH 8.3, 10 mM $MgCl_2$, 50 mM KCl, 5 mM 2-mercaptoethanol, 12.5 mM dithiothreitol, 100 µM each of dATP, dTTP, dCTP, and dGTP (usually including one labeled dNTP, at 50 µCi [α^{32}P] dNTP per reaction), 100 µg/ml oligo$(dT)_{12-18}$ (P.L. Biochemicals, Milwaukee, Wisconsin), 5 µg/ml dsRNA, and 100 units/ml AMV reverse transcriptase (Life Sciences, St. Petersberg, Florida). After 40 min. at 46°C Na_2.EDTA was added to 10 mM, sodium dodecylsulphate to 0.1 percent, NaCl to 0.1 M, and the cDNA purified by passage over G-75. When used, actinomycin D was 50 µg/ml in transcription reactions. S1 nuclease digestion of cDNA was done for 30 min. at room-temperature in 30 mM Na acetate, pH 4.5, 0.3 M NaCl, and 4.5 mM $ZnCl_2$, at 180 units enzyme per ml, then the reaction stopped by addition of pH 8.5 Tris.HCl to 0.1 M and carrier tRNA to 50 µg/ml.

For use as hybridization probe, [^{32}P]-labeled cDNA was denatured 10 min. in 50 mM NaOH, diluted 10-fold in 150 µg/ml carrier DNA, adjusted with HCl to pH 7, and added to a stock solution to give 0.9 NaCl, 50 mM Na phosphate, pH 6.5-7.0, 5 mM Na_2.EDTA, (5 x SSPE), and 1 percent sarkosyl. To transfer dsRNA from agarose gels to nitrocellulose paper, the gels were soaked 60 min. in 50 mM NaOH, washed twice in 0.2 M Tris.borate buffer, pH 8, twice in 0.05 M Tris.borate buffer, pH 8, and blotted against the paper (BA 85, Schleicher and Schuell, Keene, New Hampshire) in 3 M NaCl, 0.3 M Na acetate, pH 6.5-7.0. The blots were then baked 120 min. at 80°C, pre-hybridised 20 hours at 65°C in 5 x SSPE, 1 percent sarkosyl, containing 10 µg/ml denatured carrier DNA, then hybridised 20 hours at 65°C to radioactive probe, washed once at 65°C with 5 x SSPE, 1 percent sarkosyl, 4 times at room-temperature with 1 x SSPE, 1 percent sarkosyl, and autoradiographed at -80°C with intensifying screens. Non-denaturing agarose gels, polyacrylamide gels, and electroelutions were run in 90 mM Tris. 90 mM borate, 2.5 mM Na_2.EDTA, pH 8.3 (11); denaturing agarose gels were run in 30 mM NaOH, 2 mM Na_2.EDTA (12). Restriction enzymes were used as recommended by their suppliers (New England Biolabs, Beverely, Massachusetts, and BRL, Gaithersberg, Maryland).

RESULTS

Figure 1 shows autoradiographs of typical [^{32}P]-labeled reaction products of reverse-transcribed M1 dsRNA. Three prominent cDNA's were seen in samples electrophoresed under non-denaturing conditions (Figure 1A, Lane 1), with apparent lengths of 1.10, 0.86, and 0.63 kb; under denaturing conditions an additional band was resolved, with an apparent length of 2.20 kb (Figure 1B, Lane 1). In some reactions (Figure 2, Lane 1), a 1.65 kb cDNA was seen in denaturing gels. The absence of the 2.20 kb cDNA in non-denaturing gels and

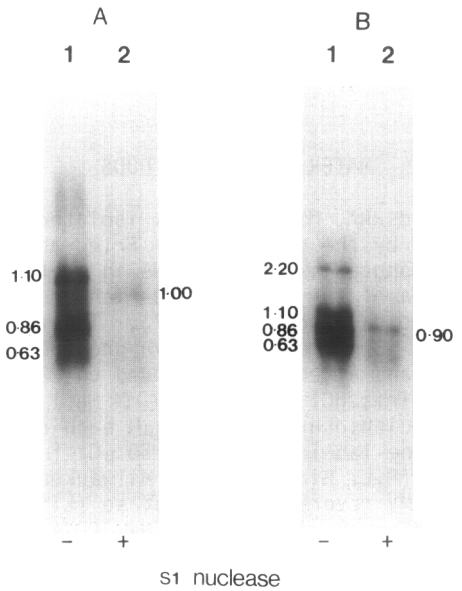

Fig. 1. Single-stranded and double-stranded (hairpin-terminated) reverse transcripts of M1 dsRNA. [^{32}P]-labeled cDNA was incubated with or without S1 nuclease, electrophoresed in non-denaturing (A) or denaturing (B) 2 percent agarose, and autoradiographed. cDNA length, in kb, was derived by comparison to the migration of the Hind III DNA fragments of lambda (λ PAPA$_{c1857}$, 23) which were electrophoresed in the gels, stained with ethidium bromide, and photographed under uv light.

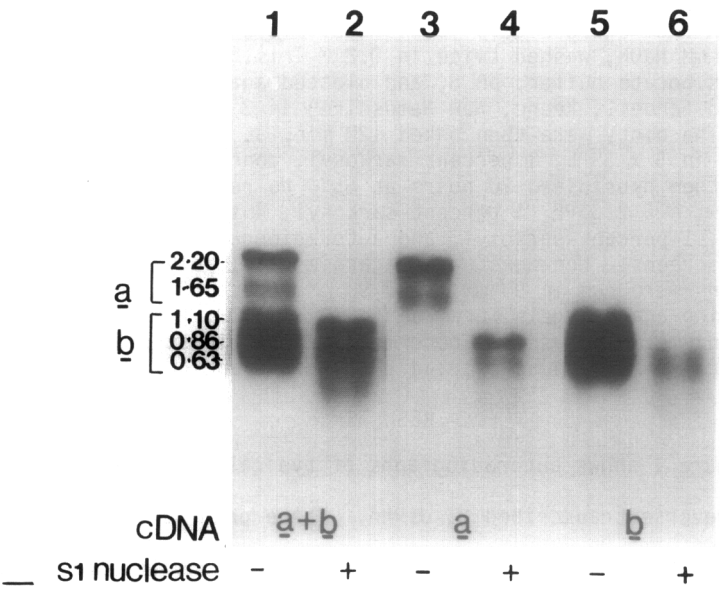

Fig. 2. S1 nuclease digestion of fractionated cDNA. [^{32}P]-labeled cDNA from M1 dsRNA was electrophoresed in denaturing 2 percent agarose, autoradiographed, and the regions of the gel corresponding to cDNA's a and b (Lane 1) removed and the cDNA's purified by electroelution. cDNA's a and b, either separately or together, were incubated with S1 nuclease, electrophoresed in denaturing 2 percent agarose, and autoradiographed, as shown. The length in kb of prominent cDNA's is indicated.

the fact that it exceeded the length (1.8 kb) of the template dsRNA implied that this species was the denatured form of a 1.10 kb ds cDNA in which one end was closed in a hairpin-like structure; such molecules have often been described in reverse transcription products of 3'-polyadenylated RNA's (13). Digestion of the cDNA with nuclease S1, expected to eliminate single-stranded sequences, including hairpin termini (14), eliminated all the components except one, of apparent length 1.00 kb in non-denaturing gels and 0.90 kb in the denaturing gels (Figure 1A, Lane 2; Figure 1B, Lane 2). This S1-resistant transcript was presumably the product of digestion of the 1.1 kb, hairpin-terminated, ds cDNA species. To further define the structure of the cDNAs produced from M1 dsRNA, they were fractionated and then digested with S1 nuclease. The result (Figure 2) confirmed the result of the experiment shown in Figure 1, by demonstrating that the 2.20 kb species was converted by S1 into a species of about 1.0 kb; the 1.65 kb species produced in this particular reaction was converted to a molecule of 0.8 kb (Lane 5), implying that, like the 2.20 kb molecule, it was a hairpin-terminated duplex transcript.

To define the RNA sequences represented by the cDNAs, the M1 dsRNA, and the S1-resistant M1-1 and M1-2 fragments obtainable from it, were transferred to nitrocellulose paper and hybridised to radioactive, unfractionated cDNA. Figure 3 shows that the cDNA was complementary only to sequences contained in M1-1 dsRNA. Since the longest component of the probe, the 1.10 kb duplex, had not hybridised to M1-2 dsRNA, it contained most of the M1-1 sequence. Figure 4A shows a model for oligo(dT) primed reverse transcription of M1 dsRNA, similar to the scheme proposed by Brizzard and De Kloet (15). To provide evidence for this model, and to define the extent to which the M1-1 region of M1 dsRNA had been copied into duplex DNA, the transcripts were examined for some of the unique restriction sites that can be predicted (Figure 4A) from examination of the known 175 kb of the M1-1 dsRNA terminus (16). Pst I, Bgl II, and Ava II each restricted the 1.1 kb duplex cDNA to produce a large DNA fragment of about 0.92, 0.88, and 1.00 kb,

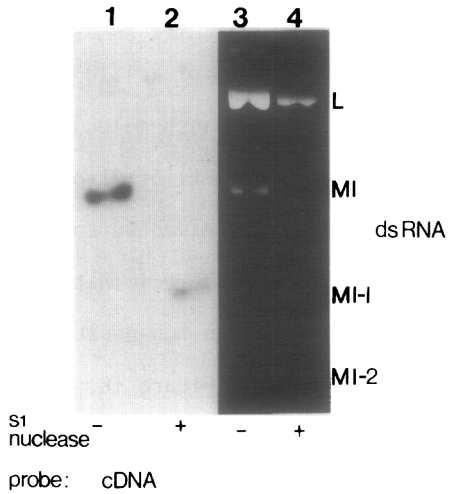

Fig. 3. Hybridisation of cDNA to M1-1 dsRNA. Total dsRNA (L dsRNA, M1 dsRNA), incubated with or without S1 nuclease at 65°C (7) was electrophoresed in several lanes of a non-denaturing 1.8 percent agarose gel. Separate lanes of the gel were either stained with ethidium bromide and photographed (Lanes 3 and 4) or blotted to nitrocellulose paper and hybridised to [^{32}P]-labeled cDNA (Lanes 1 and 2).

respectively (Figure 4B, Lanes 2, 3 and 5), consistent with the model in Figure 4A. Sau 961 digestion produced 2 fragments (Figure 4B, Lane 6), also consistent with the model if it is assumed that an additional site exists in the 1.10 kb cDNA, at some distance from the region corresponding to the M1-1 terminus. The short DNA fragments predicted for Pst I and Ava II restrictions of a full-length duplex transcript of the M1-1 region are shown in Figure 4C (Lanes 1 and 2). Hae III produced several fragments from the 1.1 kb duplex cDNA (Figure 4C, Lane 3); the 104 b DNA is probably the terminal fragment predicted from the RNA sequence (Figure 4A).

DISCUSSION

I have shown that a duplex, apparently full-length, cDNA transcript of the M1-1, toxin coding, sequence from yeast can be synthesised from methyl-mercuryhydroxide-treated M1 dsRNA and that the transcription primer anneals to an internal sequence in the template. Others (15) have shown recently that single-stranded 1.1 kb cDNA is produced from heat-denatured M1 dsRNA, in a reaction which included the inhibitor of DNA-dependent DNA synthesis (17), actinomycin D. The inclusion of 50 µg/ml actinomycin D in the protocol

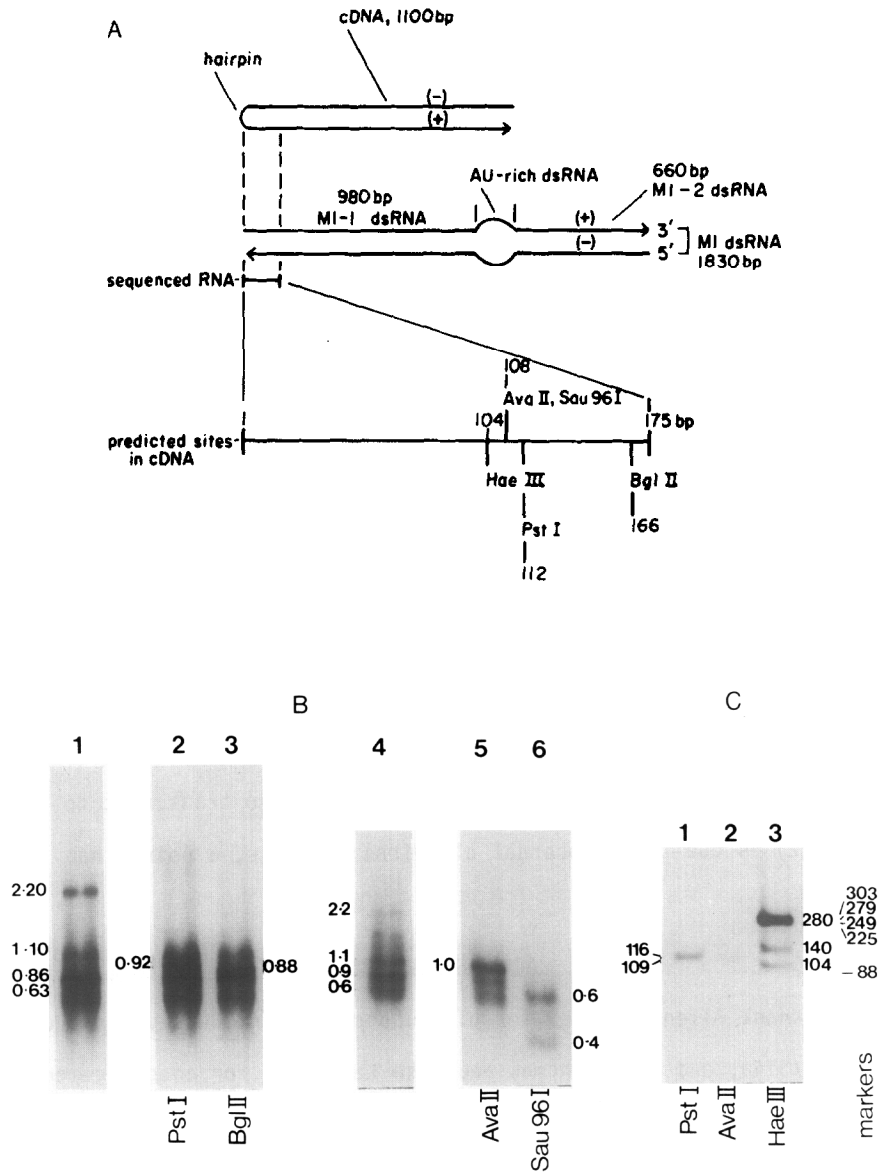

Fig. 4. Model for reverse transcription of M1 dsRNA. (A) The oligo(dT)$_{12-18}$ primer anneals to an oligo(rA) sequence present on one strand (+) of the methylmercuryhydroxide-treated dsRNA. In the absence of actinomycin D, reverse transcripts include a duplex 1.1 kb species in which the second-strand is primed by a hairpin-loop at the 3' end of the first, RNA-dependent, strand. The presence of actinomycin D blocks synthesis of the second strand (result not shown). Only the 1.1 kb cDNA is indicated; it includes a complete transcript of the M1-1 region. The restriction map of the M1-1 terminus is based on the RNA sequence of this region (15). (B) Autoradiograph of restriction digests of unfractionated, [^{32}P]-labeled, cDNA that were electrophoresed in denaturing 2 percent agarose. Lanes 1 and 4 contained undigested cDNA, from separate preparations. DNA lengths are in kb. (C) Autoradiographs of restriction digests of purified 1.1 kb (hairpin-terminated) cDNA that were electrophoresed in 10 percent polyacrylamide. The DNA length markers were the fragments produced from plasmid pBR322 by Ava II (24).

described here prevents synthesis of the second-strands of the hairpin-terminated duplex cDNA's; the longest transcript under these conditions, a 1.1 kb cDNA, is probably identical to the product described in (15), (data not shown). Duplex cDNA's have been synthesised from dsRNA's of bacteriophage Ø6, rotavirus, and reovirus (18,19,20,21) by the strategy of enzymatically adding primer-annealing sites to the dsRNA termini prior to reverse transcriptions; the approach reported here, while not allowing transcription of the complete M1 sequence, may prove to be useful for partial-length cDNA synthesis from other dsRNA's that contain internal primer-annealing sites.

There is no evidence for reverse transcription of both strands of M1 dsRNA, since the cDNA anneals to only the M1-1 dsRNA. The strand of M1 dsRNA that contains the internal oligo(rA) sequence able to bind oligo$(dT)_{12-18}$ is (+) polarity, since the 1.1 kb cDNA produced in the presence of actinomycin D anneals to the full-length in vivo transcript of M1 dsRNA (unpublished results) which is messenger-sense or (+) (22). This raises the possibility that the binding of the full-length in vivo transcript to poly(U)-Sepharose (22) is due to an internal oligo(rA) sequence, a point that is being examined.

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