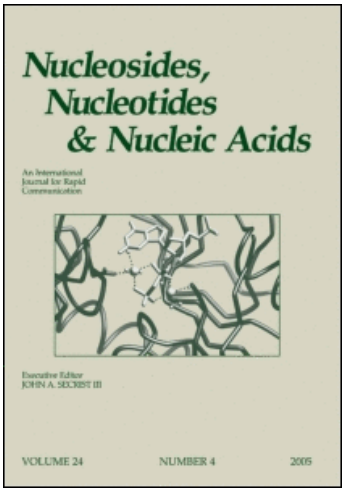

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THE USE OF SYNTHETIC OLIGO-RNA AND -DNA AS DEFINED TEMPLATES FOR THE DETERMINATION OF CATALYTIC PROPERTIES OF RNA-DIRECTED RNA POLYMERASE FROM TOMATO LEAF TISSUE

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Abstract. The in vitro transcription of nucleic acids by RNA-directed RNA polymerase, purified from tomato leaf tissue and obtained as a single polypeptide of electrophoretic homogeneity, was studied using defined oligo-RNA and -DNA as template. From the analysis of transcription products certain novel catalytic properties of RdRP became apparent.

RNA-directed RNA polymerase (RdRP, E.C. 2.7.7.48) is known since 1971 (1) and has, so far, only been found in higher plants. But until now the physiological function of this enzyme is still enigmatic. It had been assumed that in plants infected with RNA viruses this host-encoded enzyme is utilized as a replicase for the viral RNA (2). This assumption became untenable when virus-encoded RNA replicases were found (3). Since the replication process of viroids, which are the smallest infectious agents presently known, is fully dependent on enzymes of the RNA synthesizing machinery of the viroid host plant, RdRP has become one of the host RNA polymerases possibly involved in viroid RNA-RNA transcription (4).

The lack of detailed knowledge on the catalytic properties of RdRP prompted us to purify this enzyme from tomato leaf tissue. We obtained a single polypeptide of about 128 kD which was electrophoretically homogeneous in polyacrylamide (PAA)-SDS gels as detected by silver-staining. To study the transcription potentials of RdRP in vitro, we used as templates synthetic oligo-RNA and -DNA molecules of defined sequence. RNA-templates were synthesized enzymatically (5,6) whereas DNA-templates were obtained by chemical solid-phase synthesis according to the phosphoramidite-triester method (7). The reaction steps and the enzymes used for the enzymatic synthesis of two oligoribonucleotide templates are summarizied in FIG. 1 and demonstrate the strategy applied for oligonucleotide-building. Oligonucleotides which exhibit the general sequence XYZ_n or XY_np were synthesized with the aid of polynucleotide phosphorylase (PNPase) from M. luteus. In detail, oligonucleotides of the type XYZ_n were obtained by adding identical mononucleotides to dinucleoside-monophosphate-primers XY (FIG. 1A) mainly with primer-dependent PNPase ($PNPase^P$). Purified PNPase was hereto digested with trypsin under limited conditions. The presence of $PNPase^P$ and RNase T1 in the reaction mixture allowed the addition of pGp at the 3'-end of primer molecules

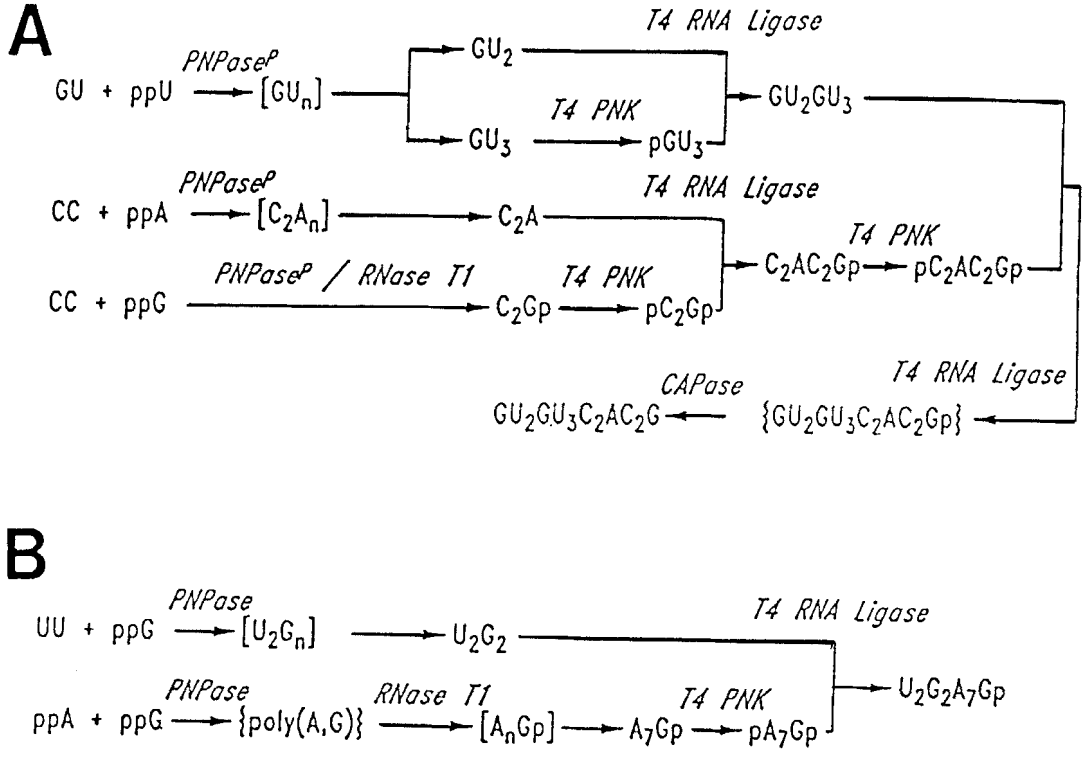

FIG. 1. Flow diagram of the enzymatic synthesis of a 13mer (A) and a 12mer (B) oligoribonucleotide of defined sequence. [..] indicates a series of homologous oligonucleotides from which representative members were isolated for further reactions. {..} denotes RNAs which were used after their synthesis for the next reaction without purification.

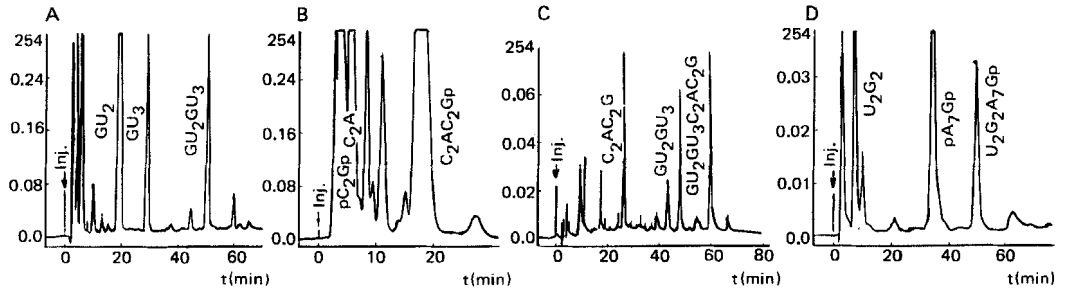

FIG. 2. Reverse phase HPLC elution profiles of reaction mixtures in which RNA molecules are joined by T4 RNA ligase to obtain the 13mer (A-C) and 12mer (D) ribooligonucleotide. Separations were performed on a 3.9 x 300 mm μBondapak C18 column (10 μm) or on a 4.6 x 250 mm ZORBAX ODS column (5 μm), using a linear gradient of an increasing CH_3CN concentration in 0.1 M TEAA in the range of pH 7.8-8.

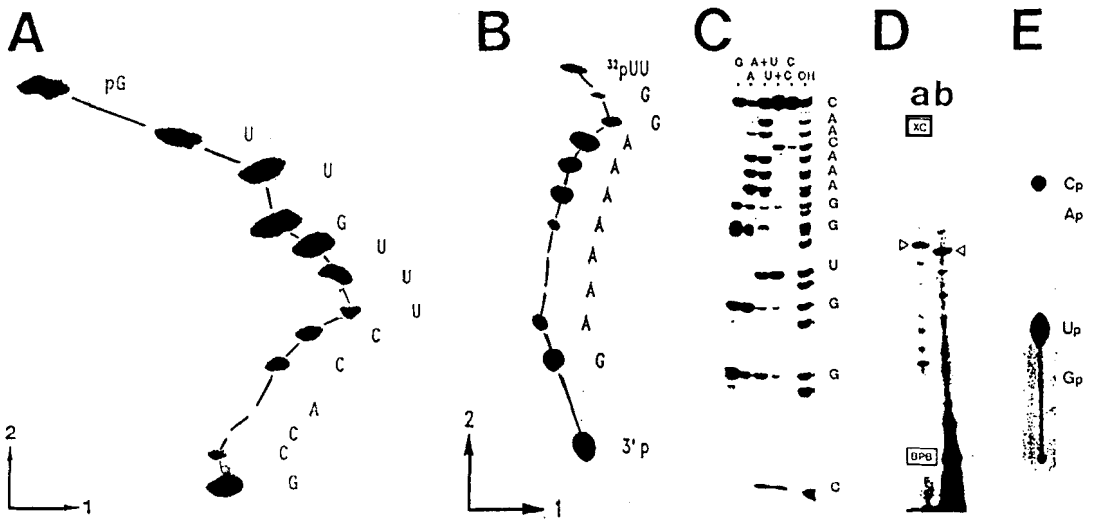

FIG. 3. Sequence analysis of RNA templates and of their transcription products obtained by RdRP. (A) "Mobility shift" analysis of the 13mer and (B) 12mer oligoribonucleotide. (C) Sequence determination by the method of Donis-Keller of the runoff transcript obtained from the [5'-^{32}P]pCpG-primer initiated transcription of the 13mer oligoribonucleotide. (D) PAA-urea gel electrophoresis of transcripts from the 12mer oligoribonucleotide, which were 3'-end-labelled by the addition of [5'-^{32}P]pG as the non-template directed nucleotide (lane a) and of the same reaction products treated with CAPase (lane b). (E) "Nearest neighbor" analysis of a full-length transcript obtained from the 12mer oligoribonucleotide but [5'-^{32}P]UMP-labelled and treated with CAPase. RNase T2-digestion of this transcript yielded Cp and Up in the expected molar ratio of 1:6 as determined after their separation on a PEI-cellulose thin layer plate.

(FIG. 1A). Oligonucleotides of the type XY_np were produced from copolymers which were degraded with RNase T1 or RNase A. The copolymers had been synthesized from appropriate mononucleotides by unmodified PNPase (FIG. 1B). Joining of these short RNA molecules with T4 RNA ligase (6) requires oligonucleotides with distinct functional end groups. Donor molecules must posses a 5'-phosphate group for forming the internucleotide bond between the donor and the acceptor molecule. To prevent self-polymerization of donor molecules they should have, in addition, a 3'-phosphate group as a blocking group. Acceptor molecules must carry a 3'-hydroxyl group, but a 5'-phosphate group should be absent. Therefore, oligonucleotides which served as donors in such reactions were 5'-phosphorylated by T4 polynucleotide kinase (T4 PNK) before ligation. Furthermore oligonucleotides designated as acceptor molecules but which carried a 3'-terminal phosphate group were dephosphorylated with alkaline phosphatase from calf intestine (CAPase). Depending on their complexity, the oligonucleotide mixtures obtained in the different enzymatic reactions were separated either by chromatography on DEAE-cellulose-, DEAE-Sephadex- and BioGel-columns or by reverse-phase high pressure liquid chromatography (FIG. 2). The correct sequence and purity of the oligonucleotides was determined by "mobility shift" analysis (8) as shown in FIG. 3A,B.

In order to demonstrate the sequence complementarity of transcription products obtained from oligonucleotide templates by RdRP, transcription was carried out in the presence of [5'-^{32}P]-labelled diribonucleotides as primers and unlabelled ribonucleoside 5'-triphosphates as substrates. After separation of the reaction mixtures on thin PAA-urea gels under denaturing conditions, the sequence of certain primer-elongated transcripts isolated from the gel was determined by the method of Donis-Keller (9) and by "mobilty shift" analysis (FIG. 3C). The initiation site of non-primed transcription was determined by "nearest neighbor" analysis of RNA-products which were labelled by means of distinct [α-^{32}P]rNTPs during transcription (FIG. 3D,E).

It was found that after runoff transcription of the template, RdRP terminates RNA synthesis quite frequently by adding one non-template-directed nucleotide to the transcript in which case pA is prefered to pG. Non-primed transcription is initiated at the 3'-terminal region of the template (FIG. 3D,E). At this site, transcription can be effectively started by RNA and DNA primers consisting of two or three nucleotides. Moreover it was found that RdRP is able to transcribe not only RNA templates but also DNA templates into complementary RNA products. Thus, the possible functions of RdRP in plant cells have become even more enigmatic.

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