RNA-dependent DNA polymerase activity in cauliflower mosaic virus-infected plant leaves

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Cauliflower mosaic virus (CaMV) is a plant DNA virus with an 8-kb circular double-stranded genome. CaMV-specific DNA and RNA molecules present in infected *Brassica* cells share some structural features with DNAs and RNAs of retroviruses and hepatitis B virus. This led to the hypothesis that CaMV replication occurs via reverse transcription of an RNA intermediate. Here we report the first characterization of a new DNA polymerase activity, specific to CaMV-infected tissues. A subcellular fraction of infected cells shows capacity to copy poly(C) and the heteropolymeric regions of natural mRNAs. Chromatographic isolation of the poly(C)-dependent activity clearly establishes that it is distinct from the classical γ -like DNA polymerases previously described in plant cells. The significant homology observed between defined regions of the Moloney murine leukemia virus (MMLV) polymerase and CaMV unassigned gene V product favors the idea that the reverse transcriptase-like DNA polymerase detected in infected cells is a virus-encoded enzyme.

Key words: RNA-dependent DNA polymerase/replication/CaMV/unassigned gene V/homology CaMV-MMLV

Introduction

Cauliflower mosaic virus (CaMV) is a DNA plant virus which is attracting interest as a model for studying the molecular biology of plant cells, and as a possible vector for genetic engineering of some higher plants (for reviews, see Shepherd, 1979; Howell, 1982; Hull and Covey, 1983a). The viral DNA is now well characterized: it is a circular double-stranded molecule, 8 kb in length, the complete sequence of which has been determined for three different strains (Franck *et al.*, 1980; Gardner *et al.*, 1981; Balazs *et al.*, 1982). The encapsidated molecule possesses three single-stranded discontinuities (Volovitch *et al.*, 1977) which are located at fixed positions, one of them ($\Delta 1$) on the transcribed strand α and the other two ($\Delta 2$ and $\Delta 3$) on the opposite strand β (Volovitch *et al.*, 1978; Hull and Howell, 1978; Howell and Hull, 1978). At the positions of these discontinuities, the 5' and 3' ends of the interrupted strand overlap each other (Franck *et al.*, 1980; Richards *et al.*, 1981). This type of molecule co-exists in infected cells with supercoiled viral DNA (Menissier *et al.*, 1982), present as minichromosomes in the nuclei (Olszewski *et al.*, 1982; Menissier *et al.*, 1983).

Recently, it was discovered that duck hepatitis B virus, a

DNA animal virus whose genome shares some structural features with the genome of CaMV, can replicate by reverse transcription of an RNA intermediate (Summers and Mason, 1982). This led to the proposal that replication of CaMV might also occur by reverse transcription (Guilley *et al.*, 1983; Hull and Covey, 1983b; Pfeiffer and Hohn, 1983). The following observations support this model: (i) the major polyadenylated virus-specific transcript (8.2-kb RNA), complementary to the α strand, spans the entire genome with a terminal redundancy of 180 nucleotides (Covey *et al.*, 1981; Guilley *et al.*, 1982); (ii) small single-stranded DNA molecules complementary to the major transcript extend counter-clockwise from the discontinuity $\Delta 1$ in CaMV DNA to the 5' end of this large viral 8.2-kb RNA. They also bear ribonucleotides at their 5' ends (Covey *et al.*, 1983; Guilley *et al.*, 1983) and other unencapsidated molecules can also be taken as evidence consistent with the reverse transcription model (Hull and Covey, 1983c); (iii) there is perfect complementarity between the 14 nucleotides just beyond $\Delta 1$ and the 3' end of wheat and bean initiator tRNA^{Met} (Guilley *et al.*, 1983; Hull and Covey, 1983b; Pfeiffer and Hohn, 1983); (iv) nucleoprotein complexes extracted from infected plants support CaMV DNA synthesis, which is partially resistant to actinomycin D and partially impaired by RNase treatment (Pfeiffer and Hohn, 1983).

Preliminary data we obtained had suggested the existence of a peculiar DNA polymerase activity in subcellular extracts from infected cells (Modjtahedi, 1982). Here, the characterization of an RNA-dependent DNA polymerase present only in CaMV-infected cabbage cells is reported. We have also examined the possibility that this enzyme could be encoded by the unassigned reading frame V of the CaMV genome.

Results

CaMV-infected cells contain a poly(C)-dependent DNA polymerase activity

We have shown that a subcellular fraction P1V, isolated from infected *Brassica* leaves and made of all the large organelles of infected cells (nuclei, chloroplasts and viroplasms) supports viral DNA synthesis (Modjtahedi *et al.*, 1984). In search of putative reverse transcriptase activity in the P1V fraction, we used poly(C)-(dG)_{12–18} which is an efficient template-primer for tumor virus reverse transcriptases (Verma, 1977) and a poor one for other DNA polymerases (Falaschi and Spadari, 1978). Under these conditions, polymerization of dGMP is obtained, without endogenous DNA synthesis, since only one dNTP is present in the incubation mixture. The reaction is strictly dependent on the addition of both primer and template (Table I). In the presence of poly(C)-(dG)_{12–18} the enzyme fails to incorporate any nucleotide other than dGTP (Table I). The rate of dGTP incorporation is constant for at least 10 min and it depends on the amount of added fraction P1V (not shown). The poly(C)-dependent activity requires the presence of a divalent cation, Mg²⁺ or Mn²⁺

(Tables I and II).

Poly(C)-dependent DNA polymerase activity is not detected in uninfected cells

When the experiments described above were performed with subcellular fraction P1H prepared from healthy plants, none of the batches examined incorporated dNTPs in the presence of poly(C)-(dG)₁₂₋₁₈ (Table I). These fractions, however, were endowed with poly(A)-directed DNA polymerase activity (Table I), and they could also use activated DNA and poly(dA)-(dT)₁₂₋₁₈ as template-primers (not shown). The fact that poly(A) copying was detected in both infected and uninfected extracts whereas poly(C) copying was restricted to fraction P1V suggests that an additional polymerase activity is present in the extracts of infected cells.

Compatible with this, the ion requirements for poly(A)- and poly(C)-dependent activities present in fraction P1V are different (Table II). When Mn²⁺ is substituted for Mg²⁺, polymerization is stimulated for poly(A) to a higher extent

than for poly(C); optimal conditions for poly(C) copying are somehow inhibitory for the poly(A)-dependent activity. In no case was it possible to detect any poly(C)-dependent DNA polymerase activity in extracts of healthy leaves, even under conditions favoring optimal detection of this activity in infected cells (Table II).

To check whether the lack of detectable poly(C)-(dG)₁₂₋₁₈ dependent activity in the P1H fraction could be explained by the presence of a diffusible inhibitor in this fraction (or by an activator in its infected counterpart) we carried out incubations in the presence of poly(C)-(dG)₁₂₋₁₈ with both extracts mixed in various proportions. The initial rate of polymerization was found to be proportional to the amount of fraction P1V, irrespective of the amount of added fraction P1H (Figure 1). This demonstrates that no diffusible inhibitor or activator can be responsible for the difference of activity observed between the P1V and P1H fractions.

CaMV-infected cells contain a DNA polymerase activity able to copy heteropolymeric regions of messenger RNAs

The capacity of the infected cell extract P1V to copy poly(C) led us to check whether this RNA-dependent DNA polymerase activity could also be observed with natural mRNAs as templates. In a complete incubation mixture containing the four deoxyribonucleotides, addition of P1V extract resulted in a limited but detectable DNA polymerase activity, even in the presence of actinomycin D (Table III). This could be attributed to an endogenous copy of either single-stranded DNA or RNA molecules present in the extract. When poly(A)⁺ mRNAs purified from animal cell cultures were added, a 4- to 8-fold stimulation of the incorporation of dNTP occurred, providing that oligo(dT)₁₂₋₁₈, which had no effect by itself, was present (Table III). We chose dATP as a label rather than dTTP, the incorporation of which could merely represent the copy of the poly(A) tails of mRNAs. The observed stimulation was completely prevented by RNase addition, and the omission of either dCTP or dGTP in the incubation mixture caused a drastic inhibition of dATP polymerization. The absolute requirement for oligo(dT)₁₂₋₁₈ primer taken together with the other results strongly suggests that heteropolymeric regions of the mRNAs are copied.

Table I. Synthetic RNA-dependent DNA polymerase activities in extracts from *Brassica* leaves

Extract	Template-primer	[³ H]Substrate	Additions	Incorporated dNMP ^a
P1V	none	dGTP	Mg ²⁺ ^b	≤0.5
P1V	poly(C)	dGTP	Mg ²⁺	≤0.5
P1V	(dG) ₁₂₋₁₈	dGTP	Mg ²⁺	≤0.5
P1V	poly(C)-(dG) ₁₂₋₁₈	dGTP	Mg ²⁺	30
P1V	poly(C)-(dG) ₁₂₋₁₈	dGTP	Mg ²⁺ + dATP + dCTP + dTTP	30
P1V	poly(C)-(dG) ₁₂₋₁₈	dTTP	Mg ²⁺	≤1.0
P1V	poly(C)-(dG) ₁₂₋₁₈	dATP	Mg ²⁺	≤0.5
P1V	poly(C)-(dG) ₁₂₋₁₈	dGTP	none	≤0.5
P1V	poly(A)-(dT) ₁₂₋₁₈	dTTP	Mg ²⁺	65
P1H	poly(C)-(dG) ₁₂₋₁₈	dGTP	Mg ²⁺	≤0.5
P1H	poly(A)-(dT) ₁₂₋₁₈	dTTP	Mg ²⁺	11

^aThe incubations were carried out as described in Materials and methods. 20 µl of the extracts (corresponding to ~20 µg of proteins) were added to the incubation mixtures. Results are expressed as pmol of dNMP incorporated in 10 min at 37°C.

^bMgCl₂ was used at a final concentration of 10 mM.

Table II. Ion requirements for homoribopolymer-dependent activities in extracts from *Brassica* leaves

Extract	Template-primer	[³ H]Substrate	Incubation mixture			Incorporated dNMP ^a
			pH	Divalent cation ^b	Monovalent cation ^c	
P1V	poly(C)-(dG) ₁₂₋₁₈	dGTP	8.0	Mg	K	30
P1V	poly(C)-(dG) ₁₂₋₁₈	dGTP	8.0	Mn	K	35
P1V	poly(C)-(dG) ₁₂₋₁₈	dGTP	8.3	Mg	none	41
P1V	poly(A)-(dT) ₁₂₋₁₈	dTTP	8.0	Mg	K	65
P1V	poly(A)-(dT) ₁₂₋₁₈	dTTP	8.0	Mn	K	135
P1V	poly(A)-(dT) ₁₂₋₁₈	dTTP	8.3	Mg	none	47
P1H	poly(C)-(dG) ₁₂₋₁₈	dGTP	8.0	Mg	K	≤0.5
P1H	poly(C)-(dG) ₁₂₋₁₈	dGTP	8.0	Mn	K	≤0.5
P1H	poly(C)-(dG) ₁₂₋₁₈	dGTP	8.3	Mg	none	≤0.5
P1H	poly(A)-(dT) ₁₂₋₁₈	dTTP	8.0	Mg	K	11
P1H	poly(A)-(dT) ₁₂₋₁₈	dTTP	8.0	Mn	K	27

^aExperimental conditions are the same as in Table I, except for modifications of the incubation mixture which are indicated here. Results are expressed as pmol of dNMP incorporated in 10 min at 37°C.

^bMgCl₂ and MnCl₂ were used at final concentration of 10 mM and 0.5 mM, respectively.

^cKCl was used at a final concentration of 50 mM.

Chromatographic isolation of the RNA-dependent DNA polymerase specific to CaMV-infected cells

To test whether the γ -like DNA polymerase previously described in plant cells (Castroviejo *et al.*, 1979; Sala *et al.*, 1980) could account for the poly(C)-directed polymerization of dGTP in infected cells, fractionation of the soluble extracts was attempted by phosphocellulose chromatography. In parallel, and under identical conditions, extracts from uninfected plants were fractionated. Elution of proteins was performed with a linear salt gradient, and the eluted fractions were tested for both poly(A)- and poly(C)-dependent DNA polymerase activities. The results, illustrated in Figure 2, show that: (i) the soluble extract prepared from fraction P1H gave only one peak, eluted at 0.4 M KCl, of poly(A)-directed activity, but no detectable poly(C)-directed activity (Figure 2A); (ii) the soluble extract prepared from fraction P1V gave two peaks of poly(A)-directed activity, eluting respectively at 0.4 and 0.57 M KCl. Only one peak of poly(C)-directed ac-

Fig. 1. Effect of the addition of fraction P1H on the poly(C)-(dG)₁₂₋₁₈ dependent activity of fraction P1V. The two fractions were mixed as follows (expressed as P1V/P1V + P1H): (a) 0; (b) 0.25, (c) 0.50; (d) 0.75, (e) 1.0; the mixtures were tested as indicated in Materials and methods.

Table III. Natural mRNA-dependent DNA polymerase activity in extracts from CaMV-infected *Brassica* cells

Template-primer	[³ H]Substrate	Other nucleotides	Incorporated dNMP ^a
None	dTTP	dATP + dGTP + dCTP	3.5
polyA ⁺ (RNA)-(dT) ₁₂₋₁₈	dTTP	dATP + dGTP + dCTP	11
None	dATP	dTTP + dGTP + dCTP	0.4
(dT) ₁₂₋₁₈	dATP	dTTP + dGTP + dCTP	0.4
polyA ⁺ (RNA)	dATP	dTTP + dGTP + dCTP	0.4
polyA ⁺ (RNA)-(dT) ₁₂₋₁₈	dATP	dTTP + dGTP + dCTP	3.1
polyA ⁺ (RNA)-(dT) ₁₂₋₁₈ + RNase	dATP	dTTP + dGTP + dCTP	0.4
(dT) ₁₂₋₁₈	dATP	dTTP + dGTP	0.3
polyA ⁺ (RNA)-(dT) ₁₂₋₁₈	dATP	dTTP + dGTP	1.1
(dT) ₁₂₋₁₈	dATP	dTTP + dCTP	0.3
polyA ⁺ (RNA)-(dT) ₁₂₋₁₈	dATP	dTTP + dCTP	1.9

^aThe incubations were carried out as described in Materials and methods. 10 μ l of the extracts (corresponding to ~10 μ g of proteins) were added to the incubation mixtures. Results are expressed as pmol of total dNMP incorporated in 10 min at 37°C.

tivity, coincident with the second peak of poly(A)-directed activity, was observed (Figure 2B). In this latter case, the magnitude of the first peak of poly(A)-dependent activity was too low to ensure that poly(C)-dependent DNA polymerase activity had not escaped detection. This possibility was excluded using another batch of infected plants, the activity of which (6 pmol/h in the 0.4 M peak) was sufficient to allow detection of a possible associated poly(C)-dependent activity; again none was detected (data not shown). It should also be noted that the poly(A)-dependent activity from uninfected cells (Figure 2A) did not show any poly(C)-(dG)₁₂₋₁₈ associated activity. These data prove that two polyribonucleotide-dependent DNA polymerases can be separated by chromatography from infected cell extracts: one, eluted with 0.57 M KCl from phosphocellulose, is able to copy both poly(A) and poly(C) templates, whereas the second, eluted with 0.4 M KCl, copies only poly(A). Only the latter is found in uninfected cells and is likely to represent the host plant γ -like polymerase. The low recovery of enzyme activity after solubilization of P1 extracts, phosphocellulose chromatography and dialysis have not yet made possible the study of the catalytic properties of the partially purified poly(C)-dependent DNA polymerase with natural mRNAs as templates.

Fig. 2. Phosphocellulose chromatography of soluble extracts prepared from fraction P1H (A) or fraction P1V (B). 5 μ l samples of the fractions were tested for DNA polymerase activity in the presence of poly(A)-(dT)₁₂₋₁₈ (●—●) or poly(C)-(dG)₁₂₋₁₈ (○—○). Conditions used were those described in Materials and methods. Vertical arrows indicate the KCl concentrations at which the different peaks of activity were eluted.

Fig. 3. Homology between attested and putative reverse transcriptase sequences. MMLV polymerase, HBV putative polymerase and products of CaMV genes IV, V and VI were compared as described in Materials and methods. For MMLV, the amino acid sequence of the entire *pol* gene product was used, because the exact limits of the polymerase itself are not known (Shinnick *et al.*, 1981); for HBV polymerase we used the assignment given by Galibert *et al.* (1979), and for CaMV strain CM1841, the data of Gardner *et al.* (1981). Arrows indicate the homologies detected: (A) between MMLV polymerase and HBV polymerase; (B) between MMLV polymerase and CaMV V protein.

Homology between amino acid sequences of CaMV open reading frame V product and Moloney murine leukemia virus (MMLV) polymerase

The presence in CaMV-infected cells of a new DNA polymerase with some properties of a reverse transcriptase suggests that the enzyme may be specified by the viral genome. Disregarding the known coding regions IV and VI, among the six long open reading frames identified in CaMV DNA (Frank *et al.*, 1980) only one is of sufficient size to accommodate a gene for a DNA polymerase; it is region V, which could encode a polypeptide of 79 kD, in the same mol. wt. range as RNA-dependent-DNA polymerases from MMLV or avian myeloblastosis virus form α (Verma, 1977). This similarity of size has also been noticed by Hull and Covey (1983b). It has already been pointed out that this putative gene of CaMV is the most strongly conserved region of the CaMV genome (Gardner *et al.*, 1981; Volovitch *et al.*, 1981). This prompted us to look for possible homology between the amino acid sequence of CaMV gene product V and those of two reverse transcriptases: the attested polymerase of MMLV and the possible polymerase of human hepatitis B virus (HBV). Figure 3 shows the matrices of homology obtained with different pairs of sequences. Figure 3A and B indicates that a clear homology exists between defined segments of HBV polymerase and MMLV polymerase, and between defined segments of CaMV V protein and MMLV polymerase. Moreover, these homologies involve the same segment in MMLV polymerase, centered around amino acids 250–450. Figure 3C indicates that, using the same level of stringency for the homology search, there is little apparent homology between HBV polymerase and CaMV V protein. In addition, no homology is detected between MMLV polymerase and two large products encoded by CaMV genome: the coat protein, corresponding to CaMV IV (Figure 3D) and the viroplasm major protein, corresponding to CaMV VI (Figure 3E). To analyze further the significance of the homology detected between MMLV polymerase and CaMV V protein, we looked at the secondary structures predicted for segment 250–450 of MMLV polymerase and segment 300–500 of CaMV V protein. This analysis points to one region of well conserved secondary structure, spanning ~100 amino acids, from positions 320 to 420 in MMLV polymerase, and from positions 380 to 480 in CaMV V protein (not shown). The best alignment between MMLV polymerase and CaMV V protein was established and is presented in Figure 4. It shows that 26 positions out of 80 amino acids are identical. The homology between the two defined segments of MMLV polymerase and CaMV V product has been assessed by statistical tests, and shown to be highly significant (the similarity value for the optimal alignment is 10.26 times the standard deviation above the mean value obtained for 50 shuffled sequences).

Discussion

Since the early 1970s reverse transcription has been recognised as a step in retrovirus RNA replication and integration in the host DNA. Lately, this process has been suggested as a more general mechanism of DNA sequence modification in normal or embryonic cells (for reviews, see Verma, 1977; Sharp, 1983; Finnegan, 1983). So far, however, demonstration of the presence of an authentic reverse transcriptase in retrovirus-free cells remains controversial. As emphasized by Varmus (1983), CaMV is an attractive system for such studies since DNA structural data and appropriate models for reverse

Fig. 4. Alignment of CaMV CM1841 putative polymerase with MMLV polymerase. CaMV V amino acids (upper line) from position 380 to position 454 have been aligned with MMLV polymerase amino acids (lower line) from position 316 to position 395, as indicated in Materials and methods. Nomenclature is as recommended by IUPAC-IUB. Gaps are indicated by blanks, and identical amino acids are boxed. Amino acids belonging to the same group (basic, acidic, polar uncharged, unpolar) are indicated by open circles.

transcription are now available. Our first approach was to look for an RNA-dependent DNA polymerase activity in infected plant tissues. Other laboratories had shown that plant cells are endowed with poly(A)-directed (or γ -like) DNA polymerase activities: namely the cytoplasmic A enzyme found in wheat embryos (Castroviejo *et al.*, 1979) and the chloroplastic enzyme from spinach (Sala *et al.*, 1980). In addition, plant mitochondrial DNA polymerase copies poly(A) poorly (Christophe *et al.*, 1981), whereas the α 1-like enzyme (or DNA polymerase B) from wheat embryos does so efficiently in the presence of Mn^{2+} (Castroviejo *et al.*, 1979). We have confirmed that such a poly(A)-directed DNA polymerase activity is present in the subcellular fractions P1 of *Brassica* cells. The comparison between fractions P1V and P1H, prepared respectively from CaMV-infected and uninfected cells, reveals that only the latter exhibits poly(C)-dependent activity. Mixing experiments indicate that this activity is specific to the virus-infected cells. This is in complete agreement with previous reports indicating that none of the known plant DNA polymerases show capacity to copy poly(C) templates (Castroviejo *et al.*, 1979; Sala *et al.*, 1980; Christophe *et al.*, 1981).

In addition, we have tested the possible presence of a soluble enzyme in the supernatant fraction S1H from healthy tissues: no RNA-dependent DNA polymerase activity was detected while, under the same conditions, a low activity was observed in the corresponding infected S1V fraction (data not shown). The ability to copy poly(C) could be indicative of a reverse transcriptase activity, because retrovirus polymerases use this template with high efficiency (Verma, 1977). This point was further investigated using natural mRNAs as templates. We have demonstrated that the P1V fraction contains a DNA polymerase activity stimulated by natural mRNAs in the presence of actinomycin D. This activity is sensitive to RNase treatment of the templates, and is dependent on the presence of the four dNTPs. That this RNA-dependent activity is a true copy, not the mere RNA-initiated copy of some single-stranded DNA molecules, is indicated by the absolute requirement for both poly(A)⁺ RNA and oligo(dT)_{12–18}. It should be noted, however, that the omission of one deoxyribonucleotide does not result in the complete abolition of the reaction. A possible explanation of this incomplete inhibition is that the copy of the heteropolymeric region of the mRNAs is limited to relatively short stretches of ribonucleotides, close to the poly(A) tails initiated with oligo(dT)_{12–18}. This could be the consequence of either unfavorable reaction conditions, or partial degradation of added mRNAs in the relatively crude extract which has been used.

The occurrence of a specific RNA-dependent DNA polymerase in virus-infected cells has been further demonstrated by chromatographic separation of this enzyme from the classical γ -like DNA polymerase. It should also be noted that the novel DNA polymerase activity, eluted at 0.57 M KCl,

also separates from the major poly(dA)-(dT)_{12–18} dependent activity present in the extract (not shown). The poor activity observed with poly(dA) templates is another common feature of most of the known reverse transcriptases (Verma, 1977). At this point of our study we do not know yet if the novel enzyme in infected cells exhibits all the typical properties of retroviral polymerases. Improvement of the solubilization procedures, which presently result only in a low recovery of activity, will be necessary before physical and catalytic characterization of the enzyme can be achieved.

At any rate this new DNA polymerase, specific to infected cells, is either virus-induced or virus-encoded. The latter is made likely by the significant homology observed between defined regions of the CaMV V protein and the MMLV polymerase. However, one cannot exclude the possibility that the reverse transcriptase-like activity in CaMV-infected cells evolves from the association and complementation of a host DNA polymerase (for instance, the γ -like polymerase) with some virus-induced or virus-coded factor. Thorough purification of the CaMV-specific RNA-dependent DNA polymerase as well as molecular cloning and expression of the CaMV V region, will help to clarify this.

Materials and methods

Materials

CaMV (Cabbage B, strain PV147 from ATCC) was maintained in *Brassica rapa* grown in green-houses for 4 weeks. Mock-infected cultures of *B. rapa* were grown under the same conditions. The homopolymers were from PL Biochemicals and the labelled nucleotides from New England Nuclear.

Subcellular fractionation of *Brassica* leaves

Infected and healthy leaves from parallel cultures were homogenized as described previously (Modjtahedi *et al.*, 1984) in MMDSG buffer (25 mM MES, pH 6.0, 5 mM MgCl₂, 1 mM dithioerythritol (DTE), 0.4 M sucrose and 50% (v/v) glycerol). The crude extract was filtered through eight layers of cheesecloth and one layer of Miracloth and centrifuged 30 min at 10 000 g in a Beckman JS13 rotor. The pellet P1 was resuspended in 10 ml of MMDSG with a Dounce homogenizer, and stored at -80°C . The subcellular fractions prepared from virus-infected and healthy tissues were termed P1V and P1H, respectively.

Soluble extract preparation

Soluble extraction was achieved as described by Sala *et al.* (1980) in a buffer containing 50 mM Tris-HCl, pH 7.5, 25 mM KCl, 5 mM MgCl₂, 0.5 mM DTE and 0.5% Triton X-100. After homogenization with a Dounce homogenizer, potassium phosphate (pH 7.5) was added to a final concentration of 0.2 M and the suspension was homogenized again. It was then centrifuged at 24 000 g for 30 min and the supernatant stored at -70°C .

Phosphocellulose chromatography

Soluble extracts from P1 pellets were dialyzed for 3 h against a solution containing 50 mM Tris-HCl, pH 7.5, 20 mM KCl, 0.2 mM EDTA, 2 mM β -mercaptoethanol and 20% (v/v) glycerol. They were then adjusted to 50 mM KCl and loaded onto 3 ml phosphocellulose columns equilibrated in the same buffer used for dialysis except for KCl concentration which was 50 mM. The columns were washed with 10 volumes of the same buffer (except that the glycerol concentration was 10%), and eluted with a 10 column volume linear gradient from 50 to 800 mM KCl. Fractions of 0.3 ml were collected, and 5 μ l aliquots were tested for DNA polymerase activity. The slope of the gradient was checked by measuring the conductivity of every second fraction.

Preparation of template-primers

The homopolymer hybrids poly(A)-(dT)₁₂₋₁₈ and poly(C)-(dG)₁₂₋₁₈ were made up to 1.5 mM in template nucleotide and 0.3 mM initiator nucleotide in 10 mM Tris-HCl pH 7.4, 10 mM KCl [for poly(A)-(dT)₁₂₋₁₈] or 1 M KCl [for poly(C)-(dG)₁₂₋₁₈]. The solutions were heated at 80°C for 10 min and cooled slowly to room temperature. In addition, the poly(C)-(dG)₁₂₋₁₈ solution was dialyzed.

Total cellular RNA was prepared from SW613 cells generously provided by Dr.C.Lavialle, as described by Wahl et al. (1979). Poly(A)⁺ RNAs were isolated by passing total RNA twice over a column of oligo dT-cellulose (T3, Collaborative Research), according to Padgett et al. (1979).

DNA polymerase assays

DNA polymerase activities were tested under the following conditions: 5–40 µl of the extract to be tested were incubated at 37°C in the presence of radioactive dNTP, in a final volume of 200 µl, and aliquots of 40 µl were withdrawn at 3, 6, 9 and 12 min; acid-insoluble radioactivity was then measured according to Bollum (1966). In the case of column fractions, 5 µl aliquots were incubated for 30 min at 37°C in a final volume of 50 µl. Unless otherwise stated, the reaction mixtures were as follows. Poly(A)-directed activity was tested in 50 mM Tris-HCl, pH 8.0, 50 mM KCl, 0.5 mM MnCl₂, 1 mM DTE, 25 µM [³H]dTTP (2500 c.p.m./pmol) and 50 µg/ml poly(A)-(dT)₁₂₋₁₈. Poly(C)-directed activity was tested in the same mixture as poly(A)-directed activity, except that 10 mM MgCl₂ was substituted for MnCl₂ and poly(C)-(dG)₁₂₋₁₈ for poly(A)-(dT)₁₂₋₁₈, and the radioactive substrate was [³H]dGTP (1000 c.p.m./pmol). Poly(A)⁺ RNA-directed activity was tested in the same mixture as poly(C)-directed activity except that template-primer, i.e., poly(A)⁺ (RNA)-(dT)₁₂₋₁₈ was used at 40 µg/ml, the unlabelled dNTPs at 50 µM, and ³H-labelled substrates at a final concentration of 5 µM with a specific activity of 10 000 c.p.m./pmol for dTTP, and 30 000 c.p.m./pmol for dATP.

When P1H and P1V fractions were tested, actinomycin D was added to a final concentration of 100 µg/ml.

Homology searches

The comparison by pairs of amino acid sequences were made with the PBASCOMP program written by B.Caudron and F.Brgre (unpublished). The predictions of secondary structure for the polypeptides were by the method of Garnier et al. (1978). The algorithm of Needleman and Wunch was used as adapted by Smith et al. (1981).

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