ASYMMETRIC TRANSCRIPTION OF CAULIFLOWER MOSAIC VIRUS GENOME BY THE *ESCHERICHIA COLI* RNA POLYMERASE IN VITRO

M. VOLOVITCH, Y. CHOUIKH, H. KONDO* and P. YOT
Section de Biologie, Institut Curie, 75231 Paris Cedex 05, France

Received 9 April 1980
Revised version received 3 June 1980

1. Introduction

Cauliflower mosaic virus (CaMV) is a DNA virus [1], whose genome is a circular double-stranded DNA molecule of 5×10^6 daltons [2,3]. The virion-extracted CaMV DNA possesses 3 single-stranded interruptions, at fixed positions (break Δ1, at map position 0.705 in strand 1, and breaks Δ2 and Δ3 at map positions 0.225 and 0.907, respectively, in strand 2) and bearing free hydroxyl-3′-ends [4–6] and -5′-ends [7]. So far it is not established whether the DNA within the virion has these discontinuities but as their existence has been confirmed in 32 out of 33 isolates tested, CM4-184 having only two breaks [8,9], they must be of biological significance. Up to now, nothing is known about the molecular genetics of this virus. A possible approach to functional mapping of such a small DNA molecule is to use linked transcription–translation in a cell-free system, in the presence of *E. coli* RNA polymerase [10]. The relative absence of specificity generally observed in heterologous systems is not an obligatory obstacle for this kind of experiment, as illustrated by the data obtained with SV40 DNA [10]. In view of further mapping of CaMV gene(s), we first investigated in vitro transcription of CaMV DNA by *E. coli* RNA polymerase. These results show that the enzyme transcribed CaMV DNA through all the *Hin*dIII fragments, starting at preferential sites and copying mostly one strand in a direction which has been determined with regard to the restriction map.

* Present address: Faculty of Pharmaceutical Sciences, University of Hokkaido, 060 Sapporo, Japan

2. Materials and methods

Virus (Cabbage B, PV147 from ATCC) and DNA were prepared as in [1,11]. *Eco*RI and *Hin*dIII were purified and incubated as in [12,13]. Radioactive labelling of the 3′-ends at the level of the interruptions, using chicken thymus terminal deoxynucleotidyl transferase (kindly provided by M. Dorizzi) and d[α-^{32}P]TTP was according to [14]. RNA polymerase of *E. coli* (holoenzyme) was either prepared according to [15], a gift from P. Giacomoni, or purchased from New England Biolabs. Gel electrophoresis, ethidium bromide staining, photograph, scanning and autoradiography were performed as in [5], and gel blotting according to [16]. Elution of DNA from gels was achieved following [17]. Transcription was done at 30°C in 0.02 M Hepes (pH 7.9), 0.15 M KCl, 0.01 M $MgCl_2$, 1 mM DTE, 0.1 mM ATP, GTP and CTP, 0.035 mM [α-^{32}P]- or [^{3}H]UTP (100–150 Ci/mmol or 40 Ci/mmol, respectively), with 1 μg DNA and 1.5 unit RNA polymerase, in 50 μl final vol. After DNase I treatment and phenol extraction, free nucleotides were removed by Sephadex G-50 filtration, and RNA recovered by precipitation in the presence of ethanol. The transcripts (cRNA) were hybridized to cellulose nitrate-bound DNA according to [16]. For experiments of self-annealing, cRNA solutions in 2 × SSC were heated at 65°C for 6 h and subsequently left until room temperature was reached. Then, digestion with pancreatic RNase (5 μg/ml) was performed either in 2 × SSC or in 0.2 × SSC, in the presence of a control corresponding to double-stranded RNA (dsRNA; 1.5×10^6 daltons) from rod-shaped virus particles of *Agaricus bisporus* (Lapierre, P. Y., unpub-

lished). Kinetics of hydrolysis was determined by trichloroacetic acid precipitation of radioactive cRNA and by scanning the negatives of photographs after gel electrophoresis of dsRNA.

3. Results

3.1. *Polarity of the single-stranded breaks relative to the restriction map*

From hybridization of RNAs to the separated strands of DNA the direction of transcription can be deduced if the orientation 5′ → 3′ of DNA is known. Consequently, the determination of the polarity of the single-stranded breaks relative to the restriction map was a prerequisite for further characterization of transcripts derived from CaMV DNA.

After ^{32}P-labelling of the 3′-ends of the breaks, CaMV DNA was cleaved by *Eco*RI, and isolated fragments A and B (containing, respectively, two and one break) were denatured and analyzed by gel electrophoresis and by autoradiography (fig.1d,e). As control, unfractionated *Eco*RI digest was denatured: three major radioactive bands appear (fig.1b), migrating at the positions observed for the radioactive denaturation products of *Eco*RI-A and -B. According to the restriction map [6] and depending on the polarity of the discontinuities, the sizes (expressed in kilobases, kb) of these three radioactive products are either 1.9, 0.72 and 1.35 or 3.5, 2.3 and 1.15 (fig.2). Comparison with the experimental values (3.5, 2.5 and 1.1 kb, fig.1) makes it possible to determine the polarity of the breaks, as presented in fig.2. Minor radioactive bands (fig.1) that can not be reconciled with either of the the two possible orientations, can be related to strain heterogeneity with regard to the *Eco*RI sites or to the presence of a low proportion of linear molecules [5]. In any case, the existence of these faint bands does not modify our conclusion. The different intensities of labelling in the *Eco*RI denatured fragments of 3.5, 2.5 and 1.1 kb (fig.1d,e) are in agreement with those generally observed at the level of the discontinuities (radioactivity of $\Delta 2 > \Delta 1 > \Delta 3$) when total DNA is similarly labelled and denatured [5].

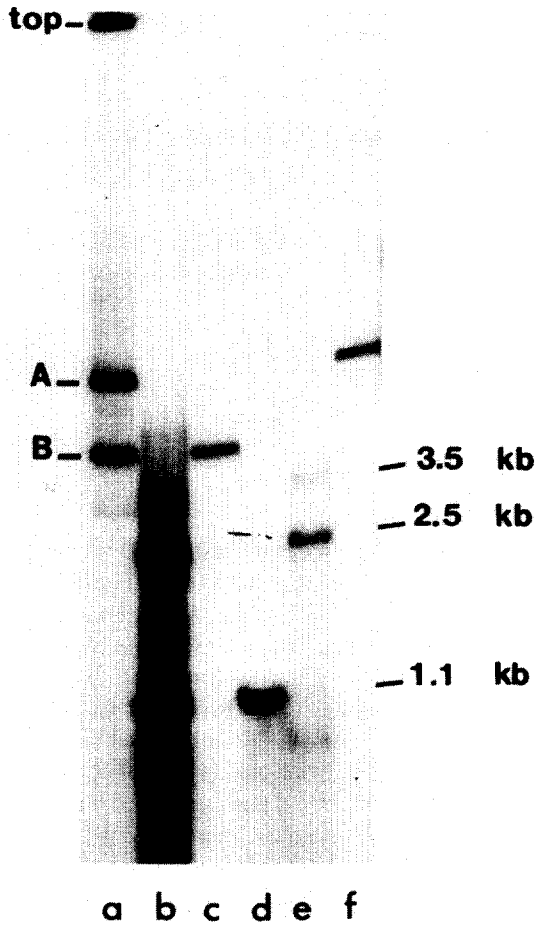

Fig.1. Denaturation of *Eco*RI fragments ^{32}P-labelled at the level of the discontinuities. Autoradiogram of a 2.6% acrylamide–0.5% agarose gel (40 cm long). *Eco*RI digest: (a) native; (b) denatured. *Eco*RI-A fragment: (f) native; (e) denatured. *Eco*RI-B fragment: (c) native; (d) denatured. The gel was calibrated using denatured *Eco*RI fragments of CaMV DNA [6] after ethidium bromide treatment. Lengths are in kilobases (kb).

3.2. *In vitro transcription by RNA polymerase of* E. coli

In order to determine what region of the CaMV genome is transcribed, RNA produced by 1 h incubation was hybridized to *Hin*dIII fragments of CaMV DNA transferred to cellulose nitrate sheets. The autoradiographic pattern obtained shows that RNA is complementary to all *Hin*dIII fragments A–I (fig.3A2). With short incubation periods, transcription products hybridize mostly to *Hin*dIII fragments A, D and G, to a greater extent to fragment A, as illustrated for a 30 s incubation period (fig.3A3).

To identify the DNA strand(s) which is (are) transcribed, RNA samples were hybridized also to the separated strands (as defined in fig.2) of CaMV

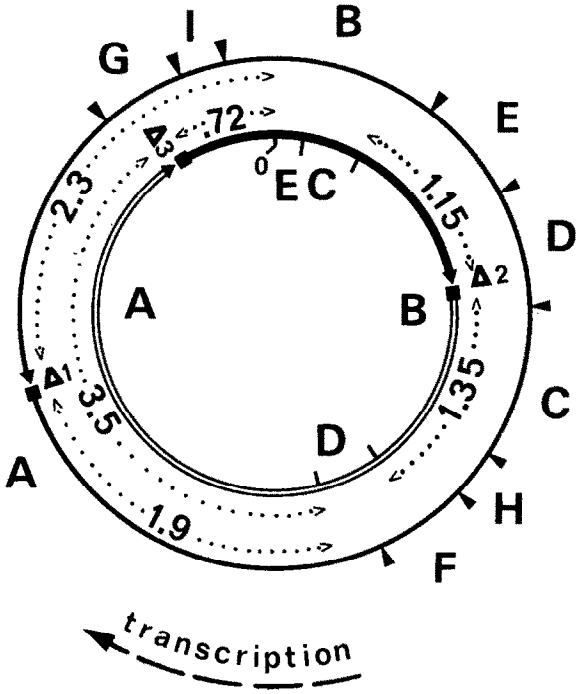

Fig.2. Orientation of the breaks of CaMV DNA and direction of transcription by *E. coli* RNA polymerase in vitro. External circle represents strand 1 with break Δ1, corresponding to the single-stranded fragment 'a'; internal circle represents strand 2 with breaks Δ2 and Δ3, composed of the two single-stranded fragments 'b' (open line) and c (full line). *Hin*dIII sites and fragments (A–I) are indicated outside and *Eco*RI sites and fragments (A–E) are mentioned inside (O: map origin). The numeric values between the two circles are the distances expressed in kilobases, from one end of a break to the proximal *Eco*RI site, and deduced from the data in [6]. (▶) and (■) correspond, respectively, to the 3'- and 5'-ends at the level of the breaks. Dashed arrow (outer part) indicates the direction of transcription by *E. coli* RNA polymerase in vitro.

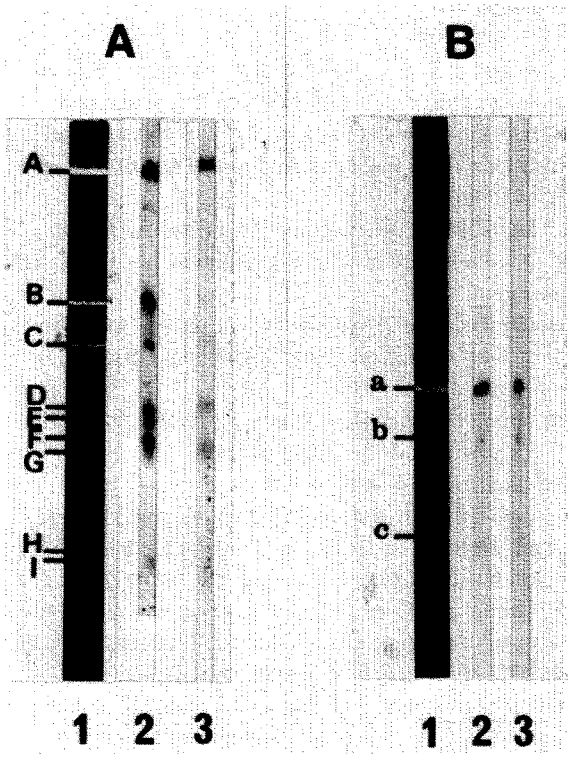

Fig.3. Hybridization of ^{32}P-labelled transcripts from CaMV DNA produced by *E. coli* RNA polymerase in vitro. [^{32}P]-RNA synthesized for various times was hybridized to restriction fragments or to denatured DNA transferred to cellulose nitrate after electrophoretic separation. (A) *Hin*dIII digest of CaMV DNA electrophoresed in a 2% agarose gel. (1) Ethidium bromide–fluorescent pattern. (2,3) Autoradiographic patterns resulting from hybridization with RNA obtained, respectively, for 120 min and 30 s. (B) Denatured CaMV DNA electrophoresed in a 1% agarose gel. (1) Ethidium bromide–fluorescent pattern. (2,3) Autoradiographic patterns resulting from hybridization with RNA produced, respectively, for 2 min and 15 min.

DNA. Two such patterns are presented in fig.3B (2,3). Whatever the duration of transcription, ~90% of the RNA produced is complementary to the strand which has only one break (Δ1). However, this percentage could be either underestimated because the largest fragment 'a' is less efficiently transferred (as checked from scans of gels after transfer) or overestimated because retention is lowered for smaller fragments (although the smallest fragment 'c' which is 2840 nucleotides long, is far larger than the limit of 500 nucleotides mentioned for poor retention in [16].

In order to confirm this asymmetry, the amount of self-annealed cRNA was measured by checking its resistance to RNase hydrolysis in 2 × SSC. A mixture of c[^{3}H]RNA, which has been allowed to self hybridize at 65°C in 2 × SSC, and of dsRNA, added as a control, was incubated in the presence of pancreatic RNase for different times, and the resistant material was estimated as in section 2. At the plateau level, only 9% of the cRNA is RNase-resistant while dsRNA is totally resistant. If hydrolysis is done in 0.2 × SSC, both RNAs are completely digested.

4. Discussion

Whilst this work was in progress, the polarity of another strain of CaMV (Cabbage B-JI) was established by a different approach [7]. Our result confirms that the polarity is the same in the two strains PV147 and JI.

The CaMV DNA regions transcribed by *E. coli* RNA polymerase in vitro are composed of sequences present:

(i) In the *Hin*dIII fragments A, D and G for short times of transcription, fragment A being transcribed to a greater extent;
(ii) In all the *Hin*dIII fragments when transcription is performed for long periods;
(iii) In strand 1 in either case.

Each of the fragments A, D and G contains one single-stranded interruption. The corresponding region binds preferentially *E. coli* RNA polymerase (F. Grellet, R. Cooke and P. Penon, personal communication) and transcription could be initiated at their level. There would be a discrepancy between initiation at the level of discontinuities present in both strands, and obtention of RNA complementary to one strand. However, it should be kept in mind that binding and initiation are two distinct events, and in particular that, although the holoenzyme tightly binds to single-strand breaks, most of these breaks cannot serve as initiation sites [18,19].

Seeking potential plant viral promoters with CaMV DNA cloned in pHB1, a viral DNA sequence was shown to function as a promoter in *E. coli* cells (restoring tetracycline resistance) [20]. This viral sequence has been tested in vitro [20] for binding with RNA polymerase: both *E. coli* and wheat germ class II enzymes bind to the same site, suggesting that this sequence could act also as a promoter in the eukaryotic cell.

It is clear that *E. coli* RNA polymerase discriminates between the two strands of CaMV DNA, in our experimental conditions. It has to be pointed out that the strand transcribed in vitro is that copied for the main virus-coded RNA species found in infected protoplasts [21].

Acknowledgements

H. K. was recipient of a fellowship from Ministère des Affaires Etrangères. Part of this work was supported by grant no. 3529 from the Centre National de la Recherche Scientifique.

References

[1] Shepherd, R. J., Bruening, G. E. and Wakeman, R. J. (1970) Virology 41, 339–347.
[2] Shepherd, R. J. and Wakeman, R. J. (1971) Phytopathology 61, 188–193.
[3] Russell, G. J., Follett, E. A. C., Subak-Sharpe, J. H. and Harrison, B. D. (1971) J. Gen. Virol. 11, 129–138.
[4] Volovitch, M., Drugeon, G., Dumas, J. P. and Yot, P. (1976) in: Nucleic acids and protein synthesis in plants (Bogorad, L. and Weil, J. H. eds) colloq. no. 261, pp. 635–641, CNRS, Paris.
[5] Volovitch, M., Drugeon, G. and Yot, P. (1978) Nucleic Acids Res. 5, 2913–2925.
[6] Volovitch, M., Drugeon, G., Dumas, J. P., Haenni, A. L. and Yot, P. (1979) Eur. J. Biochem. 100, 245–255.
[7] Hull, R., Covey, S. N., Stanley, J. and Davies, J. W. (1979) Nucleic Acids Res. 7, 669–677.
[8] Hull, R. and Howell, S. H. (1978) Virology 86, 482–493.
[9] Hull, R. (1980) Virology 100, 76–90.
[10] Roberts, B. E., Gorecki, M., Mulligan, R. C., Danna, K. J., Rozenblatt, S. and Rich, A. (1975) Proc. Natl. Acad. Sci. USA 72, 1922–1926.
[11] Hull, R., Shepherd, R. J. and Harvey, J. D. (1976) J. Gen. Virol. 31, 93–100.
[12] Greene, P. J., Betlach, M. D., Goodman, H. M. and Boyer, H. W. (1974) in: Methods Mol. Biol. 7, 87–111.
[13] Old, R., Murray, K. and Roizes, G. (1975) J. Mol. Biol. 92, 331–339.
[14] Roychoudhury, R., Jay, E. and Wu. R. (1976) Nucleic Acids Res. 3, 101–116.
[15] Burgess, R. R. (1969) J. Biol. Chem. 244, 6160–6167.
[16] Southern, E. M. (1975) J. Mol. Biol. 98, 503–517.
[17] Chouikh, Y., Volovitch, M. and Yot, P. (1979) Mol. Biol. Rep. 5, 237–239.
[18] Hinckle, D. C., Ring, J. and Chamberlin, M. J. (1972) J. Mol. Biol. 70, 197–207.
[19] Dausse, J. P., Sentenac, A. and Fromageot, P. (1972) Eur. J. Biochem. 31, 394–404.
[20] Meagher, R. B. and Mc Knight, T. D. (1980) in: Genome organization and expression in plants (Leaver, C. J. ed) Plenum, New York, in press.
[21] Howell, S. H. and Hull, R. (1978) Virology 86, 468–481.