Electron microscopic mapping of wheat germ RNA polymerase II binding sites on cloned CaMV DNA

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ABSTRACT

The binding sites of wheat germ RNA polymerase II were mapped on the cloned CaMV genome by observation of enzyme-linear DNA complexes by electron microscopy. Twelve sites are observed. Three of them are relatively stable in the presence of heparin and are found at positions 8-9, 21-23, and 41-44 map units on the physical map of the genome. These positions correspond to AT-rich regions of the viral genome which contain potential promoter sites. These results are discussed with reference to current information on the structure and expression of the CaMV genome.

INTRODUCTION

In previous studies (1, 2), we have used the DNA of Cauliflower mosaic virus (CaMV) as a template for investigating the mode of action of wheat germ RNA polymerases. This virus, with a small double-stranded DNA genome of 5×10^6 daltons, is considered as one of the best models available for the study of gene expression in higher plants and has also been proposed as a potential plant gene vector (3, 4). Its DNA is somewhat unusual in having three specifically-located single-strand interruptions, two in one strand, and one in the other (5). In vivo, only the strand with one break is transcribed (6) but, while several transcripts have been identified (6, 7), no transcription origin has yet been localised with certainty. Recently, the complete sequence of the genome of one isolate of CaMV has been established (8).

RNA polymerase II has been implicated in the "in vivo" transcription of CaMV (9). We have previously shown that wheat germ RNA polymerase II transcribes CaMV DNA even under stringent conditions, i.e. in the presence of heparin (1,2). Similar observations have been made using various inhibitors of reinitiation, heparin, rifamycin AF/013 and polyinosinic acid (10, 11), on different templates, SV 40 and Adenovirus 2 DNA. This property of wheat germ enzyme II distinguishes it from class II enzymes from other sources. It has been suggested that complexes formed by this enzyme under restrictive conditions may resemble "rapid start" complexes formed by *Escherichia coli* holoen-

zyme under similar conditions at sites at which transcription is initiated "in vivo" (11).

In a few cases, the sites of binary complex formation by the wheat germ enzyme on various heterologous templates in the absence of inhibitors have been mapped (11, 12). SEIDMAN et al. (11) have reported that these complexes are formed at or near sites at which transcription is initiated "in vivo". However, recent evidence suggest that transcription observed in the presence of heparin does not reflect initiation at specific sites, but rather at single-strand breaks (13, 14, 15). Our previous experiments (2) support this conclusion, a region of the CaMV genome adjacent to one of the single-strand interruptions being preferentially transcribed in the presence of heparin.

These observations prompted us to investigate the ability of wheat germ RNA polymerase II to form binary complexes on a DNA molecule free of single-strand breaks by using cloned CaMV DNA. We show that the enzyme forms binary complexes at several sites on the linearised template. Those formed at three of these sites are relatively resistant to the action of heparin and are located in regions of the genome which are AT-rich and contain potential promoter sites.

MATERIALS AND METHODS.

Cloning of CaMV DNA. The DNA of CaMV isolate Cabb. B-JI (16) was cut at its unique Sal I site and was cloned in the Sal I site of plasmid pAT 153 (17). One of the hybrid plasmids, pMD 125, containing a full length genome, was selected and used in the experiments described in this paper. A detailed characterisation of this plasmid will be published in a separate report (DELSENY and HULL, manuscript in preparation). It is worth mentioning that the cloned DNA has conserved all the restriction sites for 15 different restriction enzymes and has been shown to be infectious when inoculated onto turnips. Therefore, this cloned DNA is a faithful copy of the viral DNA. It should be emphasised that the hybrid plasmid pMD 125 no longer has the single-strand breaks which are characteristic of the native viral DNA (18). Recombinant plasmid pMD 125 DNA was prepared essentially as described by BOLIVAR and BACKMAN (19).

Digestion by restriction endonucleases. The digestion of pMD 125 DNA by Sal I (Sigma) or by Sal I and Xho I (B.R.L.) was carried out for 1 hr at 37°C in 100mM Tris-HCl pH 7.5, 50mM NaCl and 10 mM MgCl₂ (Eco R I buffer) using 3-5 units of enzyme for 1 µg of DNA. Digestion was stopped by adding EDTA to

10mM. The DNA was extracted three times with phenol saturated with Eco R I buffer made 10 mM in EDTA, and then twice with ether. The aqueous phase was dialysed against 10 mM Tris-HCl pH 7.5, 1 mM EDTA for 2 hrs at 0°C, stored at -20°C and used directly in polymerase binding experiments.

It has recently been suggested that certain restriction enzymes can introduce single-strand nicks into a DNA molecule (15). We have tested the possibility that Sal I introduces such nicks at specific sites on the CaMV template, which could subsequently serve as binding sites for the wheat germ polymerase. Sal I-digested pMD 125 DNA was denatured and the fragments separated by electrophoresis on a 1,4% agarose gel. Only two bands, corresponding to pAT 153 and CaMV DNA molecules are visible. To ensure that nicks are not even introduced into a small fraction of molecules, we transferred the denatured DNA to a nitrocellulose filter (20) and hybridised this with CaMV DNA labelled to high specific activity by nick translation (21). This DNA hybridises to only one band of 2.5×10^6 daltons corresponding to linear single-stranded CaMV DNA, as determined by comparison with denatured viral DNA which had been extracted from CaMV virions and analysed on the same gel. Thus, these findings show that Sal I-digestion does not introduce artifactual nicks on our template.

Wheat germ RNA polymerase II preparation. Wheat germ enzyme II was prepared as previously described (22, 23). Its specific activity was 128 units/mg. One unit corresponds to the incorporation of 1 nmole UMP in 30 minutes at 35°C on incubation of the enzyme with 15 µg of native calf thymus DNA in a standard buffer as described elsewhere (1, 2).

RNA polymerase binding assay. The incubation medium for the RNA polymerase binding assay contained 30 mM Tris-HCl pH 7.9, 1.5 mM $MnCl_2$, 60 mM $(NH_4)_2 SO_4$, 0.5 mM dithiothreitol and 12% glycerol. The concentration of DNA (pMD 125 digested by Sal I or by Sal I and Xho I) was 50 µg/ml in each assay, and binding of enzyme was carried out at enzyme to DNA molar ratios of 2:1, 8:1 or 16:1 in a final volume of 50 µl.

Incubation was for 10 minutes at 37°C. Glutaraldehyde in 30 mM triethanolamine (TEA) pH 7.9 was then added to 0.1 % and incubation was continued for 15 minutes. The sample mixture was then diluted to 0.5 µg DNA/ml with 30mM TEA pH 7.9, 0.1 % glutaraldehyde, and spread immediately for electron microscopy.

For restrictive binding conditions, heparin (Sigma) was added after the 10 minute binding incubation, to a final concentration of 15 µg/ml. After a 5 minute or 30 minute incubation at 0°C, glutaraldehyde was added (0.1 %

final concentration) and incubation was continued for 15 minutes at 37°C. The sample mixture was then diluted as described above and spread for electron microscopy.

Electron microscopy. Positively-charged carbon-coated grids, freshly prepared according to DUBOCHET et al. (24), were deposited on 10 μ l drops of diluted sample mixture for 3 minutes. They were transferred to 10 μ l drops of uranyl acetate (2% in water) for 3 minutes and then to 20 μ l drops of water for 30 seconds. Grids were finally dried on blotting paper and rotary-shadowed with platinum. Electron micrographs were taken at 20000 x magnification with an accelerating voltage of 75 kV on a Hitachi HU 12 A electron microscope. For DNA length measurements, micrographs were enlarged 7 to 10 times and DNA molecules were measured with a map measurer.

The orientation of individual molecules and the distribution of binding sites were determined by using a written FORTRAN IV computer program based on the method of GIACOMONI et al. (25) with the modification proposed by SEIDMAN et al. (11). Storage and analysis of the data were performed by a Mitra 15 computer with a printer automatically drawing the binding histograms.

RESULTS.

Nature of the template.

Figure 1 illustrates some characteristics of the cloned DNA which was used in the polymerase binding experiments. Native circular CaMV Cabb. B-JI is cleaved at only one site by the restriction enzyme Sal I (26). This property was used to clone CaMV DNA in the plasmid pAT 153 which also contains a unique site for the enzyme. As shown in figure 1A, digestion of the recombinant plasmid pMD 125 by Sal I produces the linearised molecules of both pAT 153 and CaMV DNA. The restriction enzyme Xho I has a unique site on CaMV DNA (26) and no site on pAT 153. Double digestion of the recombinant plasmid pMD 125 with Sal I and Xho I produces the pAT genome and two CaMV DNA fragments of molecular weight 3×10^6 and 2×10^6 daltons (26, and our unpublished data). These two CaMV DNA fragments were used for the orientation of the entire CaMV DNA molecule.

The orientation of the physical map of CaMV DNA (figure 1B) is as defined by FRANCK et al. (8). The zero point is defined as gap 1, the single break in the transcribed strand of the native viral genome. The cleavage site for Sal I is located at 60 map units (in a clockwise direction) from this gap. The Xho I site is located at 20 map units.

The length of Sal I linearised CaMV DNA molecules was measured on more

Figure 1: Structure of the recombinant plasmid pMD 125 and Cabb.B-JI CaMV DNA. A-1% agarose gel electrophoresis of restriction endonuclease digest fragments of pMD 125. Lane 1: pMD 125 digested by Sal I. Lane 2: pMD 125 digested by Sal I and Xho I. Lane 3: plasmid pAT 153 linearised by Sal I digestion. Lane 4: native viral CaMV DNA linearised by Xho I digestion. The minor band is the product of digestion of molecules linearised at gap 1 during extraction of the viral genome (16) and represents 80% of the whole genome. B-Physical map of plasmid pMD 125. the thick line represents plasmid pAT 153. Sites for Sal I and Xho I are indicated.

than 400 electron micrographs, as illustrated in figure 2, using pAT 153 (3740 base pairs) as standard (17). The mean size of CaMV DNA molecules was $2.56 \mu\text{m} \pm 0.1 \mu\text{m}$ corresponding to about 8000 base pairs. In electron microscopy, the longer Xho I CaMV DNA fragment was easily distinguishable from both pAT 153 and the smaller CaMV DNA fragment. The longer fragment was $1.53 \mu\text{m} \pm 0.1 \mu\text{m}$ corresponding to about 4800 base pairs. This fragment was used for the absolute orientation of the CaMV DNA molecule in our studies of wheat germ RNA polymerase II binding sites on the linearised cloned CaMV DNA.

Electron microscopic mapping of wheat germ RNA polymerase II binding sites on linear CaMV DNA.

Wheat germ RNA polymerase II was incubated with Sal I digested pMD 125 as described in Materials and Methods. At an enzyme to DNA molar ratio of 2:1, nearly 60% of CaMV DNA molecules contained one to four bound enzymes, as

Figure 2: Electron micrograph of Sal I digested recombinant plasmid pMD 125. The sample was prepared and visualised by electron microscope as described in Materials and Methods. The bar represents 100 nm.

illustrated in figure 3 (a,b,c), some of them being bound to the sticky ends of the DNA. At a molar ratio of 8:1, up to 8 enzyme molecules were bound to 60-65% of the CaMV DNA molecules. At a higher ratio (16:1), the increase in bound enzyme molecules appeared to be due to the formation of enzyme doublets or triplets at specific sites rather than recognition of additional sites on the DNA molecule. A similar observation was reported by LESCURE et al. (27) for E.coli RNA polymerase binding to polyoma virus DNA.

The distribution of wheat germ enzyme binding sites on linear CaMV DNA was correlated with the physical map of the DNA by comparison with the distribution of binding sites on the longer Xho I restriction fragment. Figure 4 shows a binding histogram established for enzyme/DNA molar ratios of 2:1 (fig. 4A), and 8:1 (fig. 4B). The binding histogram for the Xho I restriction fragment was obtained using an enzyme/DNA molar ratio of 16:1 (fig. 4C) because fragments with more than two bound enzymes were rather unfrequently encountered on grids when lower enzyme/DNA ratios were used, and it was hazardous or impossible to build binding histograms with DNA molecules or fragments containing only one or two bound enzymes.

Due to the relative imprecision of the measurements with a map measurer, the width of peaks appears to be quite large in the binding histograms automatically drawn by the computer, leading to a loss of mapping precision. At a molar ratio of 2:1 (fig. 4A), twelve binding sites are detected on the CaMV genome, at 2-3, 8-9, 13-16, 21-23, 27-28, 34-35, 41-44, 52-54, 63-65,

Figure 3: Electron microscopic visualisation of wheat germ polymerase II bound to linear CaMV DNA. Samples were prepared and visualised as described in Materials and Methods, at an enzyme to DNA molar ratio of 2:1 without heparin (a,b,c), or at a ratio of 8:1 and after a 30 minute incubation with heparin (d,e). The bar represents 100 nm.

Figure 4: Histograms of wheat germ RNA polymerase II binding sites on linear CaMV DNA. A-Distribution of binding sites for an enzyme/DNA molar ratio of 2:1 (see Materials and Methods). The histogram was traced after analysis of 72 complexes on DNA molecules with 2 to 4 bound enzymes. The intervals represent 1% of the genome length (80 base pairs). The zero point corresponds to the position of gap 1 on the native viral genome. The origin of the DNA molecules (left end) is the Sal I site corresponding to 60 map units (see fig. 1B). B-Distribution of binding sites for an enzyme/DNA molar ratio of 8:1. The histogram was traced after analysis of 131 complexes on DNA molecules with 3 to 5 bound enzymes. C-Distribution of binding sites on the longer Xho I restriction fragment for an enzyme/DNA molar ratio of 16:1. The histogram was traced after analysis of 72 complexes on DNA fragments with 2 to 4 bound enzymes.

72-75, 80-82, and 88-89 map units. The site at 21-23 units is the most frequently encountered. At a higher enzyme to DNA molar ratio (8:1), the same sites are observed with five, at 21-23, 34-35, 41-44, 80-82 and 88-89 map units, being found at a high frequency (figure 4B). The distribution of binding sites on the longer Xho I restriction fragment coincides with the distribution of binding sites on the whole DNA molecule only in one orientation, as illustrated in figure 4C. The similarity of the computer-generated histograms suggests that the location of the peaks are qualitatively correct.

Effect of heparin on the stability of wheat germ RNA polymerase II-CaMv DNA binary complexes visualised by electron microscopy.

As described in Materials and Methods, heparin was added, after incubation of the enzyme with DNA, to a final concentration of 15 µg/ml binding medium, i.e. in large excess, since DYNAN and BURGESS (13) have shown that heparin completely inhibits free wheat germ RNA polymerase II when it is present in an amount equimolar to the enzyme. Grids observed by electron microscopy show two striking differences when heparin is present in the incubation medium. Firstly, the presence of inhibitor enhances the adsorption of free enzyme molecules to grids charged with amylamine by the method of DUBOCHET. For a given enzyme to DNA molar ratio, and using the same batch of positively charged grids, we observed up to five-fold more adsorbed free enzyme molecules than in the absence of heparin. Secondly, the number of complexes per DNA molecule decreases markedly. At an enzyme to DNA molar ratio of 8:1, no more than four bound enzyme molecules were observed per molecule of DNA after a 30 minute incubation at 0°C with heparin.

The binding histograms obtained in the presence of heparin (figure 5) show that all sites recognised in the absence of the inhibitor are present. However, comparison with figure 4 shows considerable differences in the frequency of binding at particular sites. At an enzyme to DNA molar ratio of 2:1, and with a five minute incubation at 0°C in the presence of heparin, the most stable binding site is at 21-23 map units (fig. 5A). Relatively stable complexes are also found at 8-10 (probably corresponding to the site at 10-11 map units in fig. 5B), 41-44 and 80-82 map units. Similar results were obtained at an enzyme to DNA molar ratio of 8:1 (not shown). When incubation with heparin is longer, the differences in binding stability are enhanced. As shown in figure 5C, a 30 minute incubation at 0°C with heparin at an enzyme to DNA molar ratio of 8:1 results in a higher relative binding frequency at 21-23 map units, while binary complexes at sites located between 50 and 100 map units are greatly destabilised by the inhibitor. Sites at 8-10 and 41-44 map

Figure 5: Histograms of wheat germ RNA polymerase binding sites on linear CaMV DNA in the presence of heparin. A-Distribution of binding sites for an enzyme/DNA molar ratio of 2:1 and a 5 minute incubation at 0°C with 15 µg/ml heparin(see Materials and Methods). The histogram was traced after analysis of 120 complexes on DNA molecules with 2 or 3 bound enzymes. B-Distribution of binding sites on the longer Xho I restriction fragment for an enzyme/DNA molar ratio of 16:1 and a 5 minute incubation at 0°C with 15 µg/ml heparin.The histogram was traced after analysis of 103 complexes on DNA fragments with 2 to 4 bound enzymes. C-Distribution of binding sites for an enzyme/DNA molar ratio of 8:1 and a 30 minute incubation at 0°C with 15 µg/ml heparin.Histogram was traced after analysis of 85 complexes on DNA molecules with 3 or 4 bound enzymes.

units are also occupied by relatively stable binary complexes.

Thus, in the absence of stabilising initiators in the binding medium, wheat germ RNA polymerase II is capable of recognising certain specific sites on the cloned CaMV genome where it binds strongly despite the dissociating action of heparin.

DISCUSSION

In the work presented here we have studied the distribution of wheat germ RNA polymerase II binding sites on cloned CaMV DNA by electron microscopy. Our results show that this enzyme forms binary complexes at twelve sites on the unnicked viral template. A greater relative affinity is observed for the sites at 21-23, 34-36, 41-44, 80-82 and 88-89 map units. The presence of heparin in the incubation medium markedly reduces the number of complexes formed on the template. Under these conditions, complex stability varies according to the binding site, complexes formed at sites between 50 and 100 map units being greatly destabilised by the inhibitor while complexes at 21-23 map units and, to a lesser degree, at 41-44 and 8-9 map units are much more resistant to the action of heparin.

Current data on the "in vivo" transcription of the CaMV genome is scarce and it is difficult to compare our results with the little information available. HULL et al. (28) have proposed the existence of an "in vivo" promoter immediately clockwise from gap 1, a region in which we detect a binding site at which complexes are relatively stable in the presence of heparin. In the absence of further data, a certain amount of information can nonetheless be obtained by comparison of the location of polymerase binding sites with the nucleotide sequence of Cabb.B-S isolate of CaMV published by FRANCK et al. (8). Cabb.B-S DNA contains six possible protein coding sequences with AUG initiation codons in the correct polarity, beginning at 4.12 (region I), 16.55 (II), 22.58 (III), 27.02 (IV), 44.75 (V) and 71.20 (VI) map units (Table I of their article). We observe complex formation by wheat germ RNA polymerase II at sites which coincide with or are very close to these six sites (figure 4A of this paper).

In the presence of heparin, stable binding sites are located in regions which, in Cabb.B-S, are richest in A+T. In the vicinity of two of these sites, at 21-23 and 41-44 map units, are found sequences resembling the "Hogness box", considered by certain authors to be promoter sequences analogous to the procaryotic "Pribnow box". It is of interest to note that both of these sites are located within fragments which have been shown to

contain promoter sequences active in *Escherichia coli* when cloned into plasmid pBR 322 lacking the tetracycline resistance gene promoter (29). Fragments containing the region 21-23 map units are particularly efficient in this respect. Our observations are similar to those described by HALE et al. (30), who have recently shown that the *Escherichia coli* holoenzyme is capable of binding to SV 40 DNA upstream from all but one of the viral structural genes. On this matrix the procaryotic enzyme is also capable of initiating transcription of two of these genes in the correct polarity. Thus at least some sites recognised by the procaryotic enzyme appear to share similar features with eucaryotic polymerase binding sites, (to the extent that, in some instances, the *E.coli* enzyme is capable of initiating transcription). A correspondance between the majority of binding sites for the two enzymes has also been reported by LESCURE et al. (27) using polyoma DNA as the template.

The significance of the presence of polymerase binding sites upstream from structural genes at sites which are not necessarily used for transcription initiation "in vivo" is uncertain. The binding sites which we observe for wheat germ RNA polymerase II on CaMV DNA do not apparently serve as sites for transcription initiation "in vitro". The RNA synthesis which we have observed on the viral template "in vitro" (2) is probably initiated at the level of the single-strand breaks, while a preliminary study has shown that the wheat germ enzyme initiates RNA synthesis rarely, if at all, on a cloned CaMV template lacking these breaks. Purified eucaryotic RNA polymerases thus appear to be incapable of initiation at true promoter sites, but probably form complexes in AT-rich regions at promoter-like sequences. SARAGOSTI et al. (12) have drawn similar conclusions from observations of the interaction of procaryotic and eucaryotic RNA polymerases with SV 40 DNA. LESCURE et al. (31) have recently demonstrated a certain selectivity of initiation by yeast RNA polymerase B on a cloned template in the presence of one specific dinucleotide triphosphate. We are currently investigating this possibility in our system.

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