Signal structure for transcriptional activation in the upstream regions of virulence genes on the hairy-root-inducing plasmid A4

Takashi Aoyama*, Mituru Takanami and Atsuhiko Oka

Laboratory of Molecular Biology, Institute for Chemical Research, Kyoto University, Uji, Kyoto 611, Japan

Received September 4, 1989; Accepted October 6, 1989

EMBL accession nos X15909, 15914

ABSTRACT

The inducibility of the vir genes (virA, -B, -C, -D, -E, and -G) on pRiA4 was examined at the transcriptional level, and the RNA-starting sites were determined by S1-nuclease mapping and primer-extension experiments. All of these genes were inducible, while virA, -E, and -G were transcribed even under noninducing conditions. Each transcription of virB, -C, -D, and -E was initiated at one particular site, but that of virA and -G occurred at two and three sites, respectively, depending on the conditions used. In the DNA region upstream from each inducible transcript, one or more blocks of six base-pairs, 5'TC^AAA^C3' (vir box), were found to be placed characteristically. These blocks were phasing with an interval of 11 base-pairs, and the most upstream one in each upstream region was preceded by an additional block in the inverted orientation. Although the distance between the block(s) and the promoter varied with the vir gene, every block was placed in a phase nearly opposite to the -35 and -10 regions of the promoter.

INTRODUCTION

The soil bacteria Agrobacterium rhizogenes and A. tumefaciens confer hairy roots and crown gall, respectively, on most dicotyledonous plants. These diseases are caused by the transfer of a DNA segment (T-DNA) on Ri or Ti plasmid (pRi or pTi) into the plant nuclear genome and the consequent synthesis of plant phytohormones directed by the T-DNA. The trans-acting functions essential for the T-DNA transfer are governed by the virulence (vir) region lying outside the T-DNA (reviewed in refs. 1, 2). The vir loci, encompassing about 35 kilobase-pairs (kb) of DNA, contain several transcriptional units, virA to virG (3), although virE, virF, or both are missing in certain plasmids (4). The nucleotide sequences in the coding regions of vir loci are generally well conserved among the agropine-type pRiA4, the octopine-type pTiA6NC, and the nopaline-type pTiC58, while the sequences in noncoding regions are not (5-8).

The expression of virB, -C, -D, and -E is stimulated by plant phenolic compounds such as acetosyringone (3, 9, 10), but the inducibility of virA

and -G is different among the plasmids. pTiC58 virG is activated slightly by acetosyringone (11). On the other hand, the expression of virG on pTiA6NC is induced strongly by this compound (12) and its inducibility has partly been attributed to a change in pH from 7.0 to 5.6 during the experimental induction (13). pTiC58 virA but not pTiA6NC virA is inducible by acetosyringone when their translations are examined (3, 11). These results may indicate that the expression mode of some vir genes varies with the plasmid because of the divergence in their promoter sequences.

From the phenotypes of vir mutants together with sequence similarities to the regulatory proteins in the two-component regulatory systems (e.g., phoR-phoB and envZ-ompR), it seems that the virA gene product is a sensor to the plant factor (14, 15) and that the virG gene product, VirG, is a transcriptional positive regulator for the vir genes (8, 12, 16). Because the sequence 5'TNCAATTGAAAPy3' or a homologue is in the upstream regions of inducible pTi vir genes, it has been thought that these sequences contribute to the interaction with VirG (17, 18). However, they are located in different distances upstream from the transcriptional starting site for each vir gene, and their significance has not been proven.

In order to refine on the DNA signal that should be recognized by VirG, we here tested the inducibility by acetosyringone of vir transcription on pRiA4 and determined the transcriptional starting sites by S1-nuclease mapping and primer-extension experiments. It was found that all of the known vir genes are induced by acetosyringone and that virA, -E, and -G are transcribed even under noninducing conditions. One or more significant blocks of six base-pairs (bp) (5'TG_T^AAA^C_T3') were identified in the DNA regions upstream from the starting sites of the inducible mRNA. On the basis of the characteristic positioning of these blocks, their significance for interaction with VirG is discussed.

MATERIALS AND METHODS

General procedures

Standard procedures for recombinant DNA experiments (plasmid isolation, molecular cloning, DNA cleavage with restriction enzymes, ligation, kination, repair synthesis, DNA sequencing, etc.) were as previously described (19, 20).

Induction of Agrobacterium cells

Cells of Agrobacterium strain AR1007 (7) carrying pRiA4 were grown to 3×10^8 cells/ml in YEB medium (19), collected by centrifugation, and then suspended

in one-half the original volume of MSMES medium, which was MS medium (7) supplemented with 20 mM MES-Good's buffer (pH 5.5). The cell suspension was divided into two portions. To one portion, acetosyringone (Aldrich) was added to 0.2 mM (MSMES-inducing condition); the other portion was used as a control (MSMES condition). Cells under both conditions were cultured with aeration at 28°C for 7 hr, and 20 ml of each culture was harvested by centrifugation. Total RNA was prepared from induced and noninduced cells by the method described below. Similarly, RNA was also prepared from bacterial cells just before the change in medium from YEB to MSMES (YEB condition).

Preparation of RNA from Agrobacterium cells

The method for RNA preparation is essentially the same as reported elsewhere (21). The cell pellet was suspended in 1 ml of a solution of 20 mM sodium acetate (pH 5.5), 0.5% SDS, and 1 mM EDTA. After addition of 1 ml of phenol equilibrated in 20 mM sodium acetate (pH 5.5), the mixture was incubated at 60°C for 5 min with gentle shaking. The aqueous phase separated by centrifugation was re-extracted with phenol. The RNA was precipitated by ethanol, and then dissolved in 1 ml of the same buffer. The ethanol precipitation was repeated two more times, and the final precipitate was dissolved in 0.1 ml of distilled water. The concentration of RNA was calculated from the optical density at 260 nm.

Preparations of primers and probes

The primers used for cDNA synthesis and for construction of probes in S1-nuclease mapping were 17 bases long. They were synthesized with a Beckman System 1 DNA synthesizer except for virE, and labeled at the 5'-end using (γ -³²P)ATP (Amersham) and polynucleotide kinase (Takara Shuzo). The primer for virE was the 84-bp HindIII-PstI fragment labeled at the 5'-end of the HindIII site (see Fig. 2a).

The probes used for S1-nuclease mapping were prepared as follows. Each of the above synthetic primers was hybridized to the single-stranded DNA of M13mp18 (22) carrying the coding strand of the corresponding vir gene together with its upstream region. The primer was elongated by DNA polymerase I Klenow fragment (Takara Shuzo), and the resulting partly double-stranded DNA was then cleaved within a newly synthesized DNA region by the appropriate restriction enzyme. The 5'-end-labeled single-stranded DNA with a defined length was separated by polyacrylamide-urea gel electrophoresis and used as a probe. The probe for virE was the 282-bp HindIII-SalI fragment labeled at the 5'-end of the HindIII site (see Fig. 2a).

(b) bacterial promoter
5'-TTGaca-17+1bp-TAtatat^{3'}
(-35)

(c) vir box
5'-TGTAATC^{3'}

S1-nuclease mapping and primer extension

S1-nuclease mapping was done as described previously (21). RNA (10 µg) and about 30,000 cpm of probe DNA (less than 0.025 pmol) were coprecipitated with ethanol. The precipitate was dried and dissolved in 10 µl of hybridization buffer (80% formamide, 0.4 M NaCl, and 20 mM PIPES-Good's buffer, pH 6.4). The mixture was heated at 75°C for 10 min for denaturation and the temperature was gradually lowered from 75°C to 45°C in more than 2 hr, and then kept at 45°C for 12 hr. Then 90 µl of S1-nuclease buffer (0.1 M NaCl, 4 mM ZnCl₂, and 40 mM sodium acetate, pH 4.5) containing 200 units of S1 nuclease (Takara Shuzo) was added to the annealing solution. The mixture was incubated at 20°C for 10 min, and the reaction was stopped by ethanol precipitation.

Primer extension for cDNA synthesis was carried out as described before (23). RNA (10 µg) and labeled primer DNA (0.05 pmol, about 100,000 cpm) were coprecipitated with ethanol. The precipitate was dried and dissolved in 10 µl of the hybridization buffer and kept at 30°C for 12 hr. After ethanol precipitation, cDNA was synthesized at 42°C for 1 hr in 50 µl of reaction mixture (50 mM Tris-HCl, pH 8.2, 50 mM KCl, 10 mM MgCl₂, 1 mM dithiothreitol, and 2 mM each of the four dNTPs) containing 20 units of RAV2 reverse transcriptase (Takara Shuzo). The reaction was stopped by ethanol precipitation.

Each precipitate was dissolved in 20 µl of 80% formamide containing marker dyes and electrophoresed on an 8% polyacrylamide-urea gel. A sequence ladder for identification of RNA starting sites was made by the chain termination method (24) with the same primer as was used in the primer extension and single-stranded M13mp18 DNA carrying the corresponding vir gene with its upstream region. The autoradiograms were generated by a Fujix BA100 Bio-image analyzer (Fuji Photo Film).

Figure 1

Nucleotide sequence of the upstream regions of the vir genes. (a) The sequences are aligned with the -10 region of the promoters. The -35 and -10 regions of the promoters are underlined. The consensus sequence of vir box and its single-base variants located at significant positions are indicated by open boxes, and those appeared in the inverted orientation are indicated by hatched boxes. The starting sites of the transcripts are shown by asterisks, and the overlapping starting sites in the cases of virGII and virGIII are also indicated. (b) and (c) are the consensus sequences of the -35/-10 regions with the spacer lengths and the vir box, respectively. Upper-case letters represent the three most highly conserved bases in each region.

RESULTS

Nucleotide sequence of the promoter regions of pRiA4 *vir*

The *vir* loci of pRiA4 have been localized precisely on the basis of sequence similarity between the *vir* regions of pRiA4 and pTiA6NC or other pTi's. pRiA4 has been found to carry *virA*, *-B*, *-C*, *-D*, and *-G* (4, 7). Although the *virE* gene itself is missing on pRiA4 (4), its promoter region remained (unpublished). The nucleotide sequences of the upstream regions of *virC*, *-D*, and *-G* are already known (7, 8). The appropriate restriction fragments containing the upstream regions of *virA*, *-B*, and *-E* were cloned and sequenced. The sequences thus determined are shown in Fig. 1a.

Inducibility of *vir* genes by acetosyringone

The inducibility of each *vir* gene was examined at the transcriptional level by S1-nuclease mapping with the probe shown in Fig. 2a. RNA was prepared from *Agrobacterium* cells under the three conditions, namely the YEB

condition, the MSMES condition and the MSMES-inducing condition (see the materials and methods). The probes that had been protected by RNA from S1-nuclease digestion were subjected to polyacrylamide gel electrophoresis, and the relative amounts of transcripts and their approximate starting sites were determined from the intensities and positions, respectively, of the DNA bands that appeared (Fig. 2b).

The transcripts from virB, -C, and -D were detected only with the RNA prepared from cells grown under the inducing condition (indicated by "B", "C", and "D" in Fig. 2b). On the other hand, virA, -E, and -G were transcribed even under the noninducing conditions, and the amounts of the transcripts were greatly increased under the inducing condition. Two distinct transcripts from virA were detected, the starting sites of which were separated by 140 bp ("AI" and "AII" in Fig. 2b). Both transcripts were detected with all RNA preparations, but only the transcript "AII" was increased under the inducing condition. For virE, only one transcript (indicated by "E" in Fig. 2b) was detected, which was at a low level with the noninducing condition and at a much increased level with the inducing condition. The transcription of virG was started at three different sites within the 50-bp region ("GI", "GII", and "GIII" in Fig. 2b). "GII" transcription occurred at a constant level under the three conditions. In contrast, transcription of both "GI" and "GIII" was inducible, although "GI" was the majority of the inducible transcripts. The "GIII" transcript was detected even under noninducing conditions, though at a very low level. The uppermost band that appeared with all conditions (indicated by "P" in Fig. 2b) were probes with intact lengths, which might be produced by complete protection by the corresponding DNA included in the RNA preparations. All of

Figure 2

SI-nuclease mapping and primer-extension experiments. (a) Bars represent DNAs of vir-coding region (shaded bar) and its upstream region (open bar) with the relevant restriction sites. The probes and primers used are shown by thin and thick lines, respectively, under the bars. The locations of the transcripts deduced from the data of Fig. 2b and c are indicated by arrows in the 5' to 3' direction. (b) Autoradiograms of gel electrophoresis for SI-nuclease mapping. The RNA used was prepared from cells in the YEB condition (lane 1), MSMEs condition (lane 2), or MSMEs-inducing condition (lane 3). Lane M contained the HapII restriction fragments derived from pUC19 (22) for size markers. Their sizes in bases are 501/489, 404, 331, 242, 190, 147, and 111/110, respectively (22). "AI" to "GIII" correspond to the transcripts shown in (a), and "P" indicates the intact probe. (c) Autoradiograms of high-resolution SI-nuclease mapping (lane 1) and primer extension (lane 2) with the transcripts from virA ("AII"), virC, and virD. The RNA used was prepared from cells grown under the MSMEs-inducing condition. Lanes A, G, C, and T are the sequence ladder for identification of the RNA starting site. The sequences of both strands in the relevant regions are shown on the left side of each autoradiogram. The 5'-end of each transcript deduced from the primer-extension experiment is indicated by an arrow.

the DNA bands indicated by "AI" to "GIII", but not the other faint bands in Fig. 2b, appeared also with primer-extension experiments (data not shown). From these results, we concluded that the pRiA4 vir genes carry at least nine promoters with different inducibilities. The promoters for "AI" and "GII" are constitutive and not responsive to acetosyringone; those for "AII", "E", and "GIII" are active both constitutively and inducibly with acetosyringone; and those for "B", "C", "D", and "GI" are inducible, without basal activity.

Mapping of transcriptional starting sites

To characterize the promoter for each transcript, we accurately determined the RNA starting sites by S1-nuclease mapping and primer-extension experiments. The probe that had been protected by RNA and the cDNA that had been elongated from the primer were electrophoresed together with the corresponding sequence ladder. Some of the electropherograms obtained are shown in Fig. 2c. Each primer-extension experiment gave one or two bands, from which we could pinpoint the RNA starting site. However, each S1-nuclease mapping experiment produced several consecutive bands involving the band(s) corresponding to that detected by the primer-extension experiment. This was presumably due to imprecise and incomplete cutting at the boundary between double- and single-stranded DNA regions (25, 26). Therefore, we considered that the 3'-end of the cDNA corresponded to the 5'-end of transcript, and assigned the 5'-end of each transcript on to the DNA sequence (indicated by asterisks in Fig. 1a).

DISCUSSION

Promoter sequences for the vir genes

We tested the inducibility of six vir genes on pRiA4 at the transcriptional level, and found that the transcription of all of the vir genes including virA and -G was inducible and that virA, -E, and -G were expressed even under noninducing conditions. Each transcription of virB, -C, -D, and -E was initiated at one particular site, but that of virA and -G occurred at two and three different sites, respectively, depending on the conditions used. The results indicated that for the pRiA4 vir genes, there were at least nine promoters with different expression modes.

It is generally accepted that the promoters of eubacteria recognized by RNA polymerase with a major σ factor share the same sequence structure as has been found in Escherichia coli (27, 28). Therefore, the promoters of Agrobacterium are thought to be similar to those of E. coli. In fact, the

transcription of Tn5 kan was initiated at the identical position in Agrobacterium and E. coli (unpublished). The consensus structure (the -35 and -10 sequences and their spacer lengths) of the bacterial promoter (27, 28) is shown in Fig. 1b. In most of the promoters, transcriptional initiation occurs 5-9 bases downstream from the -10 region (28). We searched each upstream region for a homologue of the consensus promoter structure. The -35 and -10 regions of each vir promoter thus assigned are underlined in Fig. 1a. Generally, the consensus structure is well conserved in the constitutive promoters ("AI", "AII", "E", "GII", and "GIII"), but less so in the promoters without basal activity ("B", "C", and "GI"), particularly at their -35 regions. Among the six most highly conserved bases indicated by upper-case letters in Fig. 1b, four or five are conserved in the former group and two or three in the latter group. Thus the constitutive activity seems to result from high scores of the similarity to the consensus promoter, and the inducible activities are likely to appear by compensation or enhancement by VirG of defective or weak promoters. The virD promoter, which was inactive unless acetosyringone was present, exceptionally carried the well-conserved promoter sequences. This promoter might be negatively controlled under noninducing conditions by the chromosomal ros gene product, as reported for the virD gene of pTiA6NC and pTiC58 (29, 30).

Identification of characteristic sequences in the upstream regions

In order to find DNA signals required for induction by acetosyringone, we searched for common sequences in the upstream regions of the inducible transcripts ("AII", "B", "C", "D", "E", "GI", and "GIII"). As a result, a 6-bp block of 5'TG^AAA^CT^T3' was found in all the cases within an 80-bp length. We also found that sequences similar to that of the 6-bp block appeared at a high frequency in the regions and that most of these blocks were placed in the identical phase with an 11-bp interval, as shown by the open boxes in Fig. 1a. The distance between the 6-bp block(s) and the promoter varied with the vir gene, but the -35 and -10 regions of the promoters were located in a phase nearly opposite to that of the 6-bp blocks. In addition, the block present at the most upstream position was always preceded by an additional block in an inverted orientation (hatched boxes in Fig. 1a). We call the 6-bp block "vir box" here. The virD promoter did not satisfied the opposite phasing rule exceptionally. Since the regulation of this promoter seems to be complicated as described above, the vir box for virD might be able to function in a different manner from those for other vir genes.

One or more 12-bp sequences similar to 5'TNCAATTGAAAPy3' have been found

in the upstream regions of inducible pTiA6NC and pTiC58 vir genes (17,18). This sequence is involved in the inverted repeats of the vir box (5'^ATT^ACANNTG^AAA^C3'). The 3'-half of the 12-bp sequence exactly corresponds to the vir box found in this study, but its 5'-half is a part of the inverted vir box and not always conserved in the pRiA4 vir promoter regions. However, almost all of the vir boxes present in pRiA4 were conserved at the identical positions on pTiA6NC (see below). Thus the vir box sequence of 5'TG^AAA^C3' is likely to be the minimum unit of the DNA signal recognized by the trans-acting factor VirG.

Comparison of the sequence characteristics of pRiA4 and pTiA6NC

We compared the upstream sequences of the pRiA4 vir genes with those of pTiA6NC (12, 31). The sequences in the noncoding regions were divergent in the two plasmids, but the promoter sequences identified in this study were well conserved except for a few cases. The promoters for the transcripts "AI" and "GII" of pRiA4 were missing in the corresponding regions of pTiA6NC, which was consistent with the absence of the corresponding transcript from pTiA6NC (31). The promoter sequence for "GIII" was also not clear in the relevant region of pTiA6NC. Nevertheless, a constitutive transcript starting at a similar position has been detected (12). There might be a promoter recognized by RNA polymerase with an alternative σ factor in the region, since the -10 consensus sequence of heat-shock promoter (5'CCCATTTA3') in *E. coli* (32) was found in the relevant position on pTiA6NC.

All the vir boxes present on pRiA4 except for the proximal one in the "AII" upstream region were conserved on pTiA6NC. The relative position of the vir box and the promoter was also conserved between the two plasmids, except for virD. The distance between the vir box and the virD promoter was 2 bp longer in pTiA6NC than in pRiA4. Consequently, the opposite phasing rule is adopted also in pTiA6NC vir genes including virD.

The inducibility of pRiA4 virA coincides with that of pTiC58 virA (11) but not with that of pTiA6NC virA (3, 12). However, during this study, a paper reporting pTiA6NC virA to be inducible was published (33). Therefore, all of the virA genes examined so far are now considered to be inducible by acetosyringone. The increase in pRiA4 virG expression under the inducing condition agrees with the inducibility of pTiA6NC virG (3, 12) but not with that of pTiC58 virG (11). Base substitutions in the pTiC58 virG region at several positions within the vir boxes that were conserved in the pRiA4 and

pTia6NC virG regions (5) appear to be responsible for the low inducibility of pTic58 virG.

Possibility of the interaction of VirG protein with the vir boxes

As described above, the vir boxes are characteristically located in the upstream regions of all inducible vir transcripts on both pRiA4 and pTia6NC. Probably, this is closely related with the interaction between the cis-acting DNA region and VirG. VirG is most likely to bind to the inverted repeats of the vir box, because this structure exists in all of the upstream regions of the inducible promoters. Very recently, this region for pTia6NC virB has been found to be required for induction by acetosyringone in vivo (34). The inverted repeats of the vir box are accompanied by one or more phasing vir boxes in a considerable number of the inducible promoters. Therefore, we speculate that the phasing vir boxes also contribute to the interaction with VirG. The phasing interval is 11 bp long, which almost coincides with the pitch of the DNA helix (10.5 bp). This fact suggests that VirG proteins cooperatively bind to the phasing vir boxes along one side of the DNA helix. Purified VirG protein(s) did bind to the major grooves of all the vir boxes present in the pRiA4 virC upstream region (unpublished). Though some of the vir boxes are present inside the promoter, even in such a case, VirG proteins and RNA polymerase can bind to DNA simultaneously without steric distortion if they approach from different sides (35, 36).

Little is known about the mechanism by which VirG stimulates transcription of the vir genes. However, it seems that VirG interacts with the promoter region (and RNA polymerase) in a phase-specific manner because the relative phase between the vir boxes and the promoter is nearly constant. Thus, we offer a working model of the interaction between the vir boxes and VirG: VirG molecules first bind to the inverted repeats of the vir box as a dimer, and then additional VirG molecules cooperatively bind to the downstream promoter region along one side of the DNA helix, where the presence of additional vir boxes stimulate cooperative binding, leading to activation of the defective or weak promoter to allow binding of RNA polymerase from the other side of the DNA helix.

ACKNOWLEDGEMENTS

We thank Mr. T. Hirayama for assistance in the experimental work. This work was supported in part by Grants-in-Aid for Scientific Research from the Ministry of Education, Science, and Culture of Japan and from the Yamada Science Foundation.

REFERENCES

1. Nester,E.W., Gordon,M.P., Amasino,R.M. and Yanofsky,M.F. (1984) *Annu. Rev. Plant Physiol.*, 35, 387-413.
2. Hooykaas,P.J.J. and Schilperoort,R.A. (1984) *Adv. Genet.*, 22, 209-283.
3. Stachel,S.E. and Nester,E.W. (1986) *EMBO J.*, 5, 1445-1454.
4. Birot,A.-M. and Casse-Delbart,F. (1988) *Plasmid*, 19, 189-202.
5. Powell,B.S., Powell,G.K., Morris,R.O., Rogowsky,P.M. and Kado,C.I. (1987) *Mol. Microbiol.*, 1, 309-316.
6. Close,T.J., Tait,R.C., Rempel,H.C., Hirooka,T., Kim,L. and Kado,C.I. (1987) *J. Bacteriol.*, 169, 2336-2344.
7. Hirayama,T., Muranaka,T., Ohkawa,H. and Oka,A. (1988) *Mol. Gen. Genet.*, 213, 229-237.
8. Aoyama,T., Hirayama,T., Tamamoto,S. and Oka,A. (1989) *Gene*, 78, 173-178.
9. Stachel,S.E., Nester,E.W. and Zambryski,P.C. (1986) *Proc. Natl. Acad. Sci. USA*, 83, 379-383.
10. Stachel,S.E., Messens,E., Van Montagu,M. and Zambryski,P. (1985) *Nature*, 318, 624-629.
11. Rogowsky,P.M., Close,T.J., Chimera,J.A., Shaw,J.J. and Kado,C.I. (1987) *J. Bacteriol.*, 169, 5101-5112.
12. Stachel,S.E. and Zambryski,P.C. (1986) *Cell*, 46, 325-333.
13. Veluthambi,K., Jayaswal,R.K. and Gelvin,S.B. (1987) *Proc. Natl. Acad. Sci. USA*, 84, 1881-1885.
14. Leroux,B., Yanofsky,M.F., Winans,S.C., Ward,J.E., Ziegler,S.F. and Nester,E.W. (1987) *EMBO J.*, 6, 849-856.
15. Melchers,L.S., Thompson,D.V., Idler,K.B., Neuteboom,S.T.C., De Maagd,R.A., Schilperoort,R.A. and Hooykaas,P.J.J. (1987) *Plant Mol. Biol.*, 11, 227-237.
16. Winans,S.C., Ebert,P.R., Stachel,S.E., Gordon,M.P. and Nester,E.W. (1986) *Proc. Natl. Acad. Sci. USA*, 83, 8278-8282.
17. Winans,S.C., Jin,S., Komari,T., Leroux,B. and Nester,E.W. (1987) In Welstein,D.V. and Chua,N.H. (eds.), *Plant Molecular Biology*, Plenum Press, pp. 573-582.
18. Steck,T.R., Morel,P. and Kado,C.I. (1988) *Nucleic Acids Res.*, 16, 8736.
19. Nishiguchi,R., Takanami,M. and Oka,A. (1987) *Mol. Gen. Genet.*, 206, 1-8.
20. Maniatis,T., Fritsch,E.F. and Sambrook,J. (1982) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor.
21. Aiba,H., Adhya,S. and De Crombrughe,B. (1981) *J. Biol. Chem.*, 256, 11905-11910.
22. Yanisch-Perron,C., Vieira,J. and Messing,J. (1985) *Gene*, 33, 103-119.
23. Treisman,R., Proudfoot,N.J., Shander,M. and Maniatis,T. (1982) *Cell*, 29, 903-911.
24. Sanger,F. (1981) *Science*, 214, 1205-1210.
25. Grosschedl,R. and Birnstiel,M.L. (1980) *Proc. Natl. Acad. Sci. USA*, 77, 1432-1436.
26. Shenk,T.E., Rhodes,C., Rigby,P.W.J. and Berg,P. (1975) *Proc. Natl. Acad. Sci. USA*, 72, 989-993.
27. McClure,W.R. (1985) *Annu. Rev. Biochem.*, 54, 171-204.
28. Harley,C.B. and Reynolds,R.P. (1987) *Nucleic Acids Res.*, 15, 2343-2361.
29. Close,T.J., Rogowsky,P.M., Kado,C.I., Winans,S.C., Yanofsky,M.F. and Nester,E.W. (1987) *J. Bacteriol.*, 169, 5113-5118.
30. Tait,R.C. and Kado,C.I. (1988) *Mol. Microbiol.*, 2, 385-392.
31. Das,A., Stachel,S., Ebert,P., Allenza,P., Montoya,A. and Nester,E. (1986) *Nucleic Acids Res.*, 14, 1355-1364.
32. Cowing,D.W., Bardwell,J.C.A., Craig,E.A., Woolford,C., Hendrix,R.W. and Gross,C.A. (1985) *Proc. Natl. Acad. Sci. USA*, 82, 2679-2683.

33. Winans, S.C., Kerstetter, R.A. and Nester, E.W. (1988) J. Bacteriol., 170, 4047-4054.
34. Das, A. and Pazour, G.J. (1989) Nucleic Acids Res., 17, 4541-4550.
35. Siebenlist, U., Simpson, R.B. and Gilbert, W. (1980) Cell, 20, 269-281.
36. O'Halloran, T.V., Frantz, B., Shin, M.K., Ralston, D.M. and Wright, J.G. (1989) Cell, 56, 119-129.