Binding of the Global Response Regulator Protein CovR to the sag Promoter of Streptococcus pyogenes Reveals a New Mode of CovR-DNA Interaction*

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CovR (CsrR) is a response regulator of gene expression in Streptococcus pyogenes. It regulates ~15% of the genome, including the genes encoding several streptococcal virulence factors, and acts primarily as a repressor rather than an activator of transcription. We showed that in vitro, CovR is sufficient to repress transcription from the sag promoter, which directs the expression of streptolysin S, a hemolysin that can damage the membranes of eukaryotic cells and subcellular organelles. Repression was stimulated 8–32-fold by phosphorylation of CovR with acetyl phosphate. In contrast to binding at the has and cov promoters, which direct the expression of genes involved in capsule biosynthesis and of CovR itself, binding of CovR to Psag was highly cooperative. CovR bound to two extended regions of Psag, an upstream region overlapping the −35 and −10 promoter elements and a downstream region overlapping the translation initiation signals of the sagA gene. Each of these regions contains only a single consensus CovR binding sequence, ATTARA, which at the has promoter defines individual sites to which CovR binds non-cooperatively. At Phas and Pcov the T residues in the sequence ATTARA are important for CovR binding. However, using uracil interference experiments we find that although the ATTARA sequence in the Psag upstream region contains thymine residues important for CovR binding, important thymine residues in the Psag downstream region are located outside this sequence. Furthermore, again in contrast to its behavior at the has and cov promoters where phosphorylation of CovR leads to a 2–3-fold increase in DNA binding affinity, binding of CovR to the sag promoter was stimulated 8–32-fold by phosphorylation. We suggest that these differences in CovR binding mean that individual promoters will be repressed at different intracellular levels of phosphorylated CovR, permitting differences in the response of members of the CovR regulon to environmental and internal metabolic signals.

Two component regulatory systems and the related phosphorelay systems allow bacteria to sense changes in their internal or external environment and to respond to these changes by alterations in gene expression (1–3). In a typical two-component system, a sensor kinase (membrane bound in the case of those systems that sense the external environment) autophosphorylates in response to a signal. Usually a histidine residue in the sensor kinase receives a phosphoryl group from the environment (autophosphorylates) and transfers it to an aspartate residue on a second protein, the response regulator. Phosphorylation of the response regulator alters the ability of the response regulator to interact with either DNA or with DNA and RNA polymerase to activate or repress transcription in response to the signal received by the sensor kinase. Some sensor kinases are bifunctional (4). They not only act as kinases but can modulate the activity of their cognate response regulator proteins by acting as phosphatases to remove the phosphoryl group from the response regulator.

CovR (CsrR) is a response regulator present in Streptococcus pyogenes (Group A streptococci or GAS3) (5, 6) that acts as a global regulator influencing the transcription of ~15% of the genome (7). It is related to the group of response regulators exemplified by the OmpR and PhoB proteins of Escherichia coli (8). Unlike the majority of response regulators, CovR acts primarily to repress transcription of genes under its regulation (7). It is encoded as part of an operon that includes CovS (CsrS) (5), a protein that shows homology to histidine kinases, and so CovS and CovR are thought to act as a two-component regulatory system in GAS. CovS has been shown to be involved in Mg2+-stimulated repression of the has operon (responsible for capsule synthesis) by CovR (9). The CovR/S system is also responsible for mediating, at least in part, the general stress response of GAS to changes in temperature, pH, and osmolarity (10).

GAS is a major human pathogen that causes a wide variety of diseases ranging from self-limiting pharyngitis and superficial skin infections to severe invasive diseases such as necrotizing fasciitis and systemic infections that can result in toxic shock syndrome (11). That a single strain of GAS is able to cause such a variety of infections suggests that the bacterium can adapt to many different environmental conditions within the human host. Based upon its global influence on gene expression and stress resistance, it seems probable that the activity of the CovR/S two-component regulatory system is central to these adaptations and thus is an important factor determining the course of different GAS infections.

The interaction between CovR and some of the promoters that it represses has been examined (12–15). CovR binds to the has, sag, speB, ska, and speMF/sda promoters, which direct the transcription of genes involved in the synthesis of capsule, streptolysin S, pyrogenic exotoxin B, streptokinase, and DNase, respectively. CovR also binds to the promoter of its own gene, cov, and thus regulates its own transcription (12). At all these promoters, CovR interacts with sequences within regions of DNA of 100 base pairs or more. Phosphorylation of CovR by incubation with acetyl phosphate results in moderate increases in affinity (2–3-fold) of CovR for DNA at the has and cov promoters (12, 13) and in extensions of the promoter segments protected from nuclease digestion (12, 13) or attack by hydroxyl radicals (14). It has also been

3 The abbreviations used are: GAS, Group A streptococci; CovR-P, phosphorylated CovR.
reported that phosphorylation causes CovR to form a higher molecular weight complex in solution (14).

The interaction between CovR and the has promoter has been studied in some detail (13). Uracil interference (16) and site-directed mutagenesis have been used to demonstrate the requirement for the sequence ATTARA for CovR binding (13). Binding of CovR to the has promoter region does not involve cooperative interactions between CovR molecules, because mutation at one site abolishes binding to that site without affecting binding to other sites in vitro (13). However, mutations that abolish binding to individual sites relieve repression and increase has gene expression in vivo, although the other unmutated sites can presumably still bind CovR (13). It has been suggested that the presence of multiple binding sites, all of which must be occupied to repress has gene expression, is a mechanism to ensure that repression of has expression only occurs at relatively high intracellular levels of CovR (13).

The interactions of CovR with the promoter of its own gene, Pcov, are similar to the interactions observed at Phas (12). However, there is evidence of some cooperativity in the interactions between CovR molecules bound at different sites at Pcov because mutations that abolish binding at some sites also affect binding to other sites in vitro (12). The affinities of unphosphorylated and phosphorylated CovR for Pcov are similar to those observed at Phas (12, 13).

We have examined the interaction between CovR and another promoter whose activity it represses, Psag. We show that the interactions between CovR and the sag promoter are very different from those observed at Phas and Pcov. Binding of both unphosphorylated and phosphorylated CovR to Psag is highly cooperative. Furthermore, phosphorylation stimulates binding of CovR by 8–32-fold so that Psag is bound by phosphorylated CovR at much lower concentrations than Phas or Pcov. These results imply that CovR has more than one mode of DNA binding, which may allow greater differences in the expression of individual genes in the CovR regulon in response to environmental changes than previously suspected.

EXPERIMENTAL PROCEDURES

Purification and Phosphorylation of CovR—CovR was purified from E. coli BL21(DE3) (pLysS) (pEU7561) by chromatography on HiTrap Q HP, HiTrap SP HP, and Superdex 200 (columns from Amersham Biosciences) as described (12, 13, 15), with one modification. Prior to chromatography on HiTrap SP HP, the partially purified CovR was passed through a HiTrap Heparin HP column. CovR does not bind to heparin in the buffer conditions used (20 mM Tris-HCl, pH 7.5, 50 mM NaCl, 2 mM β-mercaptoethanol). This step removed a nuclease activity that occasionally contaminated the CovR preparations. To phosphorylate CovR, the protein was incubated for 2 h in 50 mM Tris-HCl, pH 8.0, 10 mM MgCl₂, 3 mM dithiothreitol, and 32 mM acetyl phosphate. Under these conditions, 100% of the CovR is phosphorylated and the half-life of CovR-P is ~45 min.

In Vitro Transcription Assays—To construct the Psag template used in the in vitro transcription reactions, an 845-bp region including the sag promoter (from −669 to +176 with respect to the start of transcription) was amplified by PCR from the chromosome of the GAS strain JRS4 (17) using primers PsagA Bam (ggcgcctagctCAATACAAAGGTAT-CCAACT) and PsagA Xho (ggccggctagctAGTAGCTAAAAATTT- GAAG) (uppercase letters indicate chromosomal sequence, underlined letters indicate added BamHI and Xhol restriction sites, respectively). The resulting PCR product was cloned into the BamHI/Xhol sites of pBluescript II to construct pEU7501. In vitro transcription reactions were performed as described previously (12) at 37 °C. The reaction buffer contained 33 mM Tris acetate (pH 7.9), 10 mM magnesium acetate, 0.5 mM dithiothreitol, 0.15 mg of bovine serum albumin, and ribonucleotides (500 μM final concentration of ATP, CTP, and GTP and 2 μCi of [α−32P]UTP, 400 Ci/mmole). Each 25-μl reaction contained 1.5 μg of DNA template and varying amounts of CovR. After a 15-min incubation, Gas RNA polymerase (0.05 μM final concentration) was added to each reaction and incubation was continued for 5 min. Unlabeled UTP (500 μM final concentration) was added for an additional 5-min incubation.

Gel Mobility Shift Assay—A 210-bp fragment containing the downstream region (Fig. 1, labeled B) was amplified from the plasmid pEU7501 using as primers SagadsFW (ATTTGAGCTCTATGAGTAG) and SagadsRV (TGAATCTTAGATCAC). PCR products were end-labeled, and gel mobility shift assays were carried out as described previously (12, 13). The binding reactions contained 20 ng of DNA template in 10 mM Tris-Cl, pH 7.4, 10 mM MgCl₂, and 2 mM dithiothreitol. Bound and unbound DNA were quantitated using a Molecular Dynamics Storm Phospholmager. A rectangle sufficiently large to include the whole of a single DNA band in the image was copied over all the bands and also immediately below the bands to estimate the background. The pixel volume of each band was calculated by subtracting the background pixel volume that corresponded to an identical area of the gel in close proximity to each DNA band.

Uracil Interference Experiments—Probe fragments were the 210-bp fragments used in gel mobility shift assays described above and a 243-bp fragment containing the upstream region (Fig. 1, labeled A), amplified from the plasmid pEU7501 using as primers SagadsFW (GATGAAG- TAAAAGATTTAGCTAGAGTT) and SagadsRV (AAGGCTAGCT- CAATTGCTACTG). A dUTP:TTP ratio of 1:20 was used in the amplifi-
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RESULTS

CovR Is Sufficient to Repress Transcription from the sag Promoter—
CovR has previously been shown to bind to a long segment of DNA (~150 bp) that overlaps the promoter and transcription initiation site of the sag operon. To determine whether binding of CovR to DNA directly inhibits transcription from the sag promoter, we carried out in vitro transcription reactions using purified GAS RNA polymerase (12) in the presence of increasing concentrations of both untreated CovR and

**FIGURE 2.** Effect of CovR and CovR-P on transcription from the sag promoter. The top panel shows the effect of CovR and CovR-P on transcription from the aphA-3 promoter (transcript labeled K) and from the sag promoter (transcript labeled S). Lane 1, no CovR; lanes 2–8, 0.44–28 \( \mu \text{M} \) unphosphorylated CovR increasing in 2-fold increments; lane 9, no CovR; lanes 10–16, 0.13–8.6 \( \mu \text{M} \) phosphorylated CovR increasing in 2-fold increments; lane M, molecular weight markers. The bottom panel shows a densitometric analysis of the data shown in the top panel. The graph shows the ratio of the sagA transcript to the aph-3 transcript for each lane in the top panel plotted against CovR concentration. The data have been normalized to a value of 1.0 for samples with no CovR. Open circles, phosphorylated CovR; closed circles, unphosphorylated CovR.

**FIGURE 3.** Determination of the Hill coefficient for binding of unphosphorylated CovR to a 210-base pair fragment containing the downstream region from the sag promoter. The top part of the figure shows the gel mobility shift assay. Lane 1, probe alone; lanes 2–18, increasing amounts of unphosphorylated CovR from 4 to 64 \( \mu \text{M} \) in 2.5 \( \mu \text{M} \) increments. The line was fitted by linear regression analysis, and the results of four separate determinations are presented.

Open circles, phosphorylated CovR; closed circles, unphosphorylated CovR.
CovR that had been phosphorylated (CovR-P) by incubation with acetyl phosphate. Prior to addition of purified GAS RNA polymerase, CovR or CovR-P was incubated with linear plasmid DNA that included the sag promoter, untranslated leader, and 5'-end of the sagA gene as well as the promoter of the aphA-3 gene that is not regulated by CovR. The linear plasmid template was expected to produce two run-off transcripts, a 185-bp transcript from the sag promoter and a 510-bp transcript from the aphA-3 promoter. We anticipated that transcription from Psag would be inhibited by CovR whereas transcription from PaphA-3 would be unaffected. As shown in Fig. 2, in the absence of CovR, transcripts of the expected sizes were the most abundant products of the in vitro transcription reactions. Increasing concentrations of both CovR and CovR-P added prior to RNA polymerase inhibited synthesis of the 185-bp sag transcript. Synthesis of the 510-bp aphA-3 transcript was unaffected except at the highest concentrations of CovR and CovR-P.

The ratio of the sag-specific transcript to the aphA-3-specific transcript versus the concentration of CovR showed that both CovR and CovR-P inhibited synthesis of the sag transcript (Fig. 2). In both cases, 90% inhibition of transcription occurred over a narrow range of CovR concentration (4-fold for CovR compared with 2-fold for CovR-P). A comparison of the amount of protein required to cause 50% inhibition of transcription showed that 10-fold less CovR-P was required than CovR. Thus, although both CovR and CovR-P alone was found to repress transcription from Psag, CovR-P was more effective.

CovR Binds Cooperatively to sag DNA—In preliminary nuclease protection experiments we found that in addition to the region of DNA overlapping the promoter and transcription initiation site of the sag operon (the upstream region, Fig. 1, labeled A), CovR also bound with similar affinity to a second, downstream segment of DNA that included much of the untranslated leader region of the sagA gene (the downstream region, Fig. 1, labeled B). Because this downstream protected segment of DNA was shorter (80 bp) than the upstream protected region overlapping the sag promoter (150 bp), we first examined binding of CovR to the downstream region using gel mobility shift assays. DNA-binding response regulator proteins related to CovR, such as PhoB, recognize and bind short sequences (10 bp) arrayed along the DNA (19). Thus, the length of the DNA segment bound by CovR (80 bp) would predict binding of more than a single molecule of CovR. Using a 210-base pair DNA fragment containing this downstream region, we found that increasing concentrations of CovR gave rise to only a single radiolabeled species of reduced electrophoretic mobility under our experimental conditions (Fig. 3). The failure to observe multiple DNA-
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protein complexes suggested that binding of CovR to the downstream region was cooperative.

The amount of unbound and bound DNA at different concentrations of CovR was determined and the binding of CovR was analyzed using a Hill plot. The data from one experiment is shown in Fig. 3. The combined results of four experiments gave a value of 4.4 ± 0.4 for the Hill coefficient, indicating a high degree of cooperativity in the binding of CovR to the downstream region. When only the data points around 50% binding are included in the calculation the value of the Hill coefficient is higher, 6.4 ± 0.4. These results imply that 4–6 molecules of CovR bind cooperatively to this region of DNA, consistent with the narrow range of concentrations required for inhibition of transcription (Fig. 2). We were unable to carry out a similar analysis of binding of CovR-P to this segment of DNA, because although a single slower migrating species appeared after incubation of the DNA probe with CovR-P, the majority of the bound DNA did not migrate into the gel. Thus, individual bound species of DNA could not be identified and accurately quantitated. However, binding of CovR-P to the downstream region occurred over the same narrow concentration range as for CovR, implying a similar degree of cooperativity of binding to that shown by CovR.

Identification of Thymine Residues Important for CovR Binding to sag DNA—We used uracil interference assays (16) to identify thymine residues important for CovR binding using as a template the same 210-bp radiolabeled fragment containing the downstream region (Fig. 1, labeled B) that we used in the gel mobility shift assays described above. DNA fragments were prepared by PCR using different ratios of dUTP to TTP and then incubated with CovR. Bound and free DNA were separated by electrophoresis, and the resulting DNA preparations were cleaved at incorporated deoxyuridine residues. Enhancement of cleavage at a particular position in unbound DNA or diminution of cleavage at the same position in bound DNA indicates that incorporation of uracil at that position interferes with protein binding. The results shown in Fig. 4 demonstrate that incorporation of deoxyuridine in place of thymidine at four positions in the “top” strand (Fig. 4, panel 1) and two positions in the “bottom” strand (Fig. 4, panel 2) resulted in enhancement of cleavage in unbound DNA and/or diminution of cleavage at the same position in bound DNA. No enhancement of cleavage in unbound DNA or diminution of cleavage in bound DNA, indicative of a role for the base in CovR binding, was observed for any thymine residue within the ATTARA sequence. Control experiments (data not shown) showed that we could reproduce the reported results for Phas (13) revealing essential thymine residues at each ATTARA sequence within the has promoter region.

In contrast to the results obtained with the downstream region, when we repeated the uracil interference experiments using a DNA fragment containing the upstream region (Fig. 1, labeled A), the only indication of altered cleavage patterns in bound and unbound DNA occurred within the single ATTARA binding site (Fig. 4, panels 3 and 4). Thus, we found no evidence for an array of essential thymine residues in either region of Psag as has been found in the has and cov promoters Those residues identified as being important for CovR binding in the upstream and downstream regions of Psag may participate in nucleation sites for CovR binding in each region.

CovR-P Has a Higher Affinity than CovR for sag DNA—To compare directly the binding of CovR and CovR-P to sag DNA we carried out DNase I protection assays using as template the same 210-bp radiolabeled fragment containing the downstream region (Fig. 1, labeled B) as we used in the gel mobility shift and uracil interference assays. As shown in Fig. 5, both CovR and CovR-P bound and protected a similar region of the fragment, except at the highest concentrations of CovR-P. Binding by either species of CovR produced the same site of enhanced cleavage, unlike binding at Pcov, where only CovR-P produces enhancements of cleavage (12). There was no evidence of discrete binding sites separated by unprotected DNA within the protected region or of binding sites with different affinities for the protein, both of which have been seen in other regions of DNA protected by CovR from nuclease cleavage. However, the most striking difference between the two species of CovR, compared with binding at other promoters, was that binding of CovR-P occurred at a 32-fold lower concentration of protein than did CovR. Partial protection of the DNA by CovR was observed at 12.5 μM, whereas CovR-P produced partial protection at 0.4 μM. This stimulation of binding of CovR by phosphorylation was much greater than the 2–3-fold stimulation observed previously with other DNA templates (12, 13). At high concentrations of CovR-P (<12.5 μM), the region of protection spread along almost the entire fragment. Such extension of the CovR footprint because of phosphorylation has been previously observed at Pcov (12). Extension of the protected region at these higher concentrations was not seen with unphosphorylated CovR at either

**FIGURE 5.** Protection from DNase I cleavage by CovR of a 210-base pair fragment containing the downstream region from the sag promoter. The arrow in the top part of the figure shows the extent of the DNA probe. The arrowhead denotes the 3’-end of the labeled strand. Lanes 1–6, 2-fold decreases in concentration of unphosphorylated CovR from 50 to 1.7 μM; lane 7, no CovR; lane 8, probe alone without CovR or DNase I; lanes 9–12, DNA sequencing reactions; lanes 13–22, 2-fold decreases in concentration of phosphorylated CovR from 50 to 0.06 μM; lane 23, no CovR; lane 24, probe alone without CovR or DNase I. The extent of the protected region with respect to the start point of transcription is indicated on the left of the bottom part of the figure. The asterisks indicate a position of DNase I cleavage enhanced by CovR binding. Concentrations of CovR showing partial protection are indicated.
promoter, suggesting that interaction between CovR monomers is facilitated by phosphorylation.

To determine the effect of phosphorylation on binding of CovR to the entire sag promoter fragment, we carried out a similar footprinting analysis to that shown in Fig. 5. We used a 403-bp radiolabeled fragment as probe that contained both the upstream promoter region of the sag operon (Fig. 1, labeled A) and the downstream untranslated leader region of the sagA gene (Fig. 1, labeled B). CovR binding to the upstream promoter region of this fragment is shown in Fig. 6. The binding of CovR and CovR-P to the promoter and upstream region was similar to that at the downstream region alone. Again, the most significant difference between CovR and CovR-P was the stimulation (~16-fold) in binding caused by the phosphorylation of CovR. Also, at the highest concentration of CovR, the protected region extended from the promoter toward the downstream region.

The Upstream Promoter Region and the Downstream Untranslated Leader Region of the sagA Gene May Interact to Facilitate CovR Binding

The results in Fig. 6 show that 0.4 μM CovR-P caused partial protection of the upstream region. This is the same concentration that caused partial protection of the downstream region (Fig. 5). However, the concentration of CovR that caused partial protection of the upstream region was 6.8 μM, 2-fold lower than that causing partial protection of the downstream region. These results suggest that when both regions are present on the same DNA fragment, as in the experiment shown in Fig. 6, the two regions might interact to facilitate CovR binding. To investigate this possibility further, we repeated the footprinting analysis of the 403-bp fragment used in Fig. 6 with the other strand labeled. As shown in Fig. 7, the pattern of binding of both CovR and CovR-P to the downstream region of this fragment, containing both upstream and downstream regions, was essentially indistinguishable from binding to the same region of the 210-bp fragment containing only the downstream region (Fig. 5). However, although partial protection of the downstream region by CovR-P occurred at 0.4 μM, partial protection by CovR occurred at 3.4 μM, a 4-fold lower concentration than that
observed to cause partial protection of this region when it is the sole CovR binding region present on the DNA probe (Fig. 4). Thus, because the binding affinity of CovR (but not CovR-P) to Psag appears to be enhanced when both the upstream and downstream regions are present, the two regions may interact to facilitate CovR binding.

**DISCUSSION**

The results presented here extend our understanding of the regulation of the sag operon in five ways. First, we have shown that binding of CovR is sufficient to repress transcription from the sag promoter in vitro. Second, repression is more effective if the CovR is phosphorylated by treatment with acetyl phosphate. Third, we have identified a previously uncharacterized region of the sagA gene downstream of the promoter which is bound by CovR. Fourth, we have demonstrated that in contrast to other CovR-regulated promoters binding of CovR near the sag promoter is highly cooperative. Fifth, also in contrast to other genes where binding of CovR has been characterized, phosphorylation of CovR causes a substantial change in its apparent DNA binding affinity in vitro.

**Interaction of CovR with the sag Promoter**—The pattern of binding of CovR and CovR-P to the sag promoter region was significantly different from binding to the has and cov promoters (Fig. 1) (12, 13). At Psag, binding occurred to two segments of DNA. Within each region, binding was highly cooperative and protected a contiguous stretch of DNA from nuclease digestion. In contrast, at the has promoter, a number of discrete protected regions was observed, with intervening stretches of bases susceptible to nuclease cleavage (13). Mutation of nucleotides shown to be essential for CovR binding by uracil interference assays abolished binding to individual sites in the has promoter, but mutations within one binding site did not affect binding to other sites, indicating that binding in vitro of CovR at Psag is not cooperative (13). Rather, there are a series of individual binding sites with similar affinities. At the cov promoter, there are also discrete binding sites for CovR, but here, mutations of nucleotides predicted to be essential for binding to some
individual sites do affect binding to other sites, indicating that cooperative interactions occur between at least some of the CovR binding sites (12). These differences in binding of CovR to three different promoters suggest that there are differences at the DNA sequence level that underlie these different patterns of recognition by CovR.

**DNA Sequence Recognition by CovR**—It was originally suggested that TT/AATTTTTAAA/TAAAAC/A represents a binding site for CovR because all CovR-protected regions examined at that time, including the upstream region of the sag promoter, contain this sequence motif (14). A similar sequence is found in the CovR-protected region that we have identified located downstream of the sag promoter. However, uracil interference experiments using the has promoter subsequently identified the sequence ATTARA as being important for CovR binding (13). At Phas, this sequence occurs at irregularly spaced intervals, sometimes in inverted orientation, with these occurrences defining individual CovR binding sites revealed in nuclease protection assays (Fig. 1). At the cov promoter this sequence also occurs at irregularly spaced intervals, sometimes in inverted orientation, but related sequences ATTACA and ATTAGA are also found to bind CovR (Fig. 1) (12). Site-directed mutagenesis showed that thymine residues in these related sequences, as well as in the ATTARA sequences, are essential for Cov binding in vitro at the cov promoter (12). However, there are also regions of the cov promoter that bind CovR where ATTARA or the related sequences are not found.

Each region of the sag operon that is bound by CovR contains a single ATTARA sequence (Fig. 2). The sequences ATTACA and ATTAGA are not found at Psag. However, despite the presence of only a single ATTARA CovR binding sequence, the regions of the sag promoter that are protected from nuclease cleavage by CovR are as long as the assemblies of sites at the has and cov promoters. The upstream region of Psag is 150-bp long and the downstream region is 80-bp long, so each region binds more than one molecule of CovR.

Uracil interference and mutagenesis experiments carried out with the has and cov promoters have identified thymine nucleotides in the motif TTA, contained within ATTARA and related sequences, as essential for CovR binding (12). The uracil interference experiments reported here show that the only discernible effects on CovR binding in the upstream region are because of the replacement of thymine residues in the ATTARA sequence. Presumably this sequence acts as a nucleation site for binding to this region. In contrast however, in the downstream region, replacement of thymine residues in the ATTARA sequence has no discernible effect on binding. Rather, replacement of thymine residues in the TT/AATTTTTAAA/TAAAAC/A sequence affects binding (Fig. 8). Thus, in the downstream region this sequence, rather than the ATTARA sequence, appears to act as a nucleation site for binding.

Examination of the Psag sequences bound by CovR shows that in both protected regions nucleotides in the TTA motif occur with a periodicity of 10.9 base pairs along the DNA from the proposed nucleation sites (Fig. 8). Crystallographic analysis of PhoB, which is related to CovR, bound to its recognition sequence showed that monomers of PhoB make a limited number (two or three) of base-specific contacts with GT or TT dinucleotides occurring at a periodicity of 11 base pairs (19). Thus, we propose that at Psag, CovR makes base-specific contacts with thymine residues spaced with the correct periodicity along the DNA. According to this proposal, the upstream region of Psag that overlaps the promoter could bind as many as 13 monomers of CovR and the downstream region could bind 6–8 molecules of CovR. This latter number is consistent with an upper estimate of the Hill coefficient for binding to the downstream region of 6.4.

**Effect of Phosphorylation on Binding of CovR to Psag**—The second major difference in binding of CovR at the sag promoter, compared with binding to the has and cov promoters, is the effect of phosphorylation of CovR. At the sag promoter, phosphorylation causes an 8–32-fold increase in affinity of CovR for its binding sites. At the has and cov promoters, phosphorylation causes a 2–3-fold increase in affinity of CovR (12, 13). Because greater than 90% of the protein is phosphorylated under the conditions used in our DNA binding experiments, we suggest that the reason for this difference in the effect of phosphorylation on CovR binding affinity lies in the nucleotide sequence of the protected regions of the three promoters. If phosphorylation promotes cooperative interactions between monomers of CovR then phosphorylation would be expected to stabilize binding of several monomers to a segment of DNA that contains essential nucleotides distributed along the DNA with the correct periodicity. The has and cov promoters do not contain extended distributions of the TTA motif with an 11-base periodicity as do the protected regions of the sag operon.

Thus, we propose that CovR can interact with DNA in at least two different ways. The protein can bind to individual sites containing ATTARA and related sequences. The number of contacts with these sequences is sufficient to permit binding without a significant contribution from cooperative interactions between protein monomers, and thus binding to DNA containing aperiodic arrangements of these sequences such as those found at Phas may not be greatly stimulated by phosphorylation of CovR. The protein can also bind to stretches of DNA containing repeats of the TTA motif with regular periodicity. Here cooperative interactions between protein monomers contribute significantly to binding, and so binding to these regions of DNA is strongly stimulated by phosphorylation. At any given promoter, both

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5 J. Gao, unpublished results.
forms of binding may occur. This appears to be the case at Pcov, because there is evidence for cooperative interactions between some of the CovR binding sites (12). This proposal is supported by the observation that higher concentrations (6.8–12.5 μM) of non-phosphorylated CovR were required to bind to the sag promoter than to the has and cov promoters (2–4 μM), where cooperative interactions are presumed to be less important than at Psag.

Based on the notion that CovR can interact with promoters in different ways that respond differently to phosphorylation, we imagine that the CovR system operates to regulate expression of a significant fraction of the GAS genome in response to environmental and internal metabolic signals. The wide range of affinities of phosphorylated CovR for the different promoters examined to date suggests that variations in the fraction of CovR that is phosphorylated in response to environmental signals provide a means for differential regulation of many individual genes within the CovR regulon. An examination of additional promoters will help establish the repertoire of interactions of CovR with DNA.

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