Review

Transcriptional takeover by σ appropriation: remodelling of the σ^{70} subunit of *Escherichia coli* RNA polymerase by the bacteriophage T4 activator MotA and co-activator AsiA

Deborah M. Hinton, Suchira Pande,† Neelowfar Wais,‡ Xanthia B. Johnson,§ Madhavi Vuthoori,‖ Anna Makela‡‖ and India Hook-Barnard

Laboratory of Molecular and Cellular Biology, National Institute of Diabetes Digestive and Kidney Diseases, National Institutes of Health, Bethesda, MD, USA

Activation of bacteriophage T4 middle promoters, which occurs about 1 min after infection, uses two phage-encoded factors that change the promoter specificity of the host RNA polymerase. These phage factors, the MotA activator and the AsiA co-activator, interact with the σ^{70} specificity subunit of *Escherichia coli* RNA polymerase, which normally contacts the −10 and −35 regions of host promoter DNA. Like host promoters, T4 middle promoters have a good match to the canonical σ^{70} DNA element located in the −10 region. However, instead of the σ^{70} DNA recognition element in the promoter’s −35 region, they have a 9 bp sequence (a MotA box) centred at −30, which is bound by MotA. Recent work has begun to provide information about the MotA/AsiA system at a detailed molecular level. Accumulated evidence suggests that the presence of MotA and AsiA reconfigures protein–DNA contacts in the upstream promoter sequences, without significantly affecting the contacts of σ^{70} with the −10 region. This type of activation, which is called ‘σ appropriation’, is fundamentally different from other well-characterized models of prokaryotic activation in which an activator frequently serves to force σ^{70} to contact a less than ideal −35 DNA element. This review summarizes the interactions of AsiA and MotA with σ^{70}, and discusses how these interactions accomplish the switch to T4 middle promoters by inhibiting the typical contacts of the C-terminal region of σ^{70}, region 4, with the host −35 DNA element and with other subunits of polymerase.

Overview

Upon infection of *Escherichia coli*, bacteriophage T4 establishes its own developmental cycle. Within 20 min, the phage programmes the generation of approximately 200 copies of its genome, the packaging of that DNA, and finally its escape from the host by lysis (reviewed by Miller *et al.*, 2003). Regulation of this cycle is achieved largely by phage promoters, which sequentially express early, middle and late phage genes. Because T4 does not encode its own RNA polymerase, it must direct the host transcriptional machinery to these phage promoters at the correct time during infection. T4 encodes factors that accomplish this takeover by altering the specificity of the host *E. coli* RNA polymerase as infection proceeds (reviewed by Miller *et al.*, 2003; Stitt & Hinton, 1994).

*E. coli* RNA polymerase consists of a core of five subunits (α₂, β, β', and ω), which contains the RNA-synthesizing activity, and a σ factor that binds to a specific promoter sequence and sets the start site for transcription (reviewed by Gruber & Gross, 2003; Paget & Helmann, 2003). The primary σ, σ^{70}, is the predominant recognition factor for the transcription of housekeeping genes. Other σ factors are used under certain growth conditions or at times of stress. RNA polymerase containing σ^{70} is the major polymerase species present during exponential growth, the condition under which T4 infects. When present in polymerase, σ^{70} usually recognizes two regions in promoter DNA: a −10 element, having a consensus sequence of 5'-TATAAT-3',...
and a −35 element, having a consensus sequence of 5′-TTGACA-3′ (Campbell et al., 2002; Gardella et al., 1989; Keener & Nomura, 1993; Murakami et al., 2002b; Siegela et al., 1989; Vassylyev et al., 2002; Waldburger et al., 1990). (All sequences are given as the top, i.e. the non-template, strand of DNA.) To a first approximation, the strength of a host promoter reflects the match between its −10 and −35 sequences and the canonical sequences for these regions. A subset of σ70-dependent promoters lack a good match to the canonical −35 consensus sequence, but nevertheless are recognized well by polymerase. These promoters, designated extended −10 promoters, contain the sequence 5′-TG-3′ at positions −15/−14 and thus have an extended −10 sequence of 5′-TGnTATAAT-3′ (Bown et al., 1997). In addition, contact between the x subunits of polymerase and the sequence between positions −40 and −60 (UP elements) can contribute to promoter recognition and strength (Ross et al., 2001; Ross & Gourse, 2005).

At the start of infection, early T4 promoters must compete with host promoters for the available RNA polymerase. T4 wins this competition with two main strategies. First, early promoters contain excellent matches to the canonical −10 binding element and although their −35 sequences differ somewhat from the host canonical −35 element, these promoters are still recognized well by host polymerase. In fact, when present on a plasmid, these T4 early promoters are stronger than excellent host promoters (Wilkins & Ruge, 1996). In addition, T4 injection of the phage Alt protein (reviewed by Miller et al., 2003; Stitt & Hinton, 1994), which ADP-ribosylates one of the σ subunits of the core, immediately increases recognition of the phage early promoters over host promoter sequences (Sommer et al., 2000; Wilkins et al., 1997). Thus, the strength of the T4 early promoters together with the modification of the x subunit ensures that the transcriptional machinery of the host switches very quickly from host DNA to the T4 genome.

Phage middle promoters become active about 1 min after infection at 37 °C (reviewed by Brody et al., 1995; Stitt & Hinton, 1994). These promoters contain the σ70−10 DNA element and are dependent on RNA polymerase containing σ70 for transcription, but they lack the σ70−35 DNA element. Instead they have a different consensus sequence (a MotA box, 5′-atGCTTTA-3′) centred at −30 (Marshall et al., 1999; Stitt & Hinton, 1994; Truncaite et al., 2003). Activation of middle promoters requires a T4 transcription activator, MotA (Hinton, 1991; Mattson et al., 1974), and a T4 transcription co-activator, AsIΔ (Hinton et al., 1996b; Ouhammouch et al., 1994, 1995; Stevens, 1973). By itself, AsIΔ also functions as an inhibitor of σ70-dependent transcription (Stevens, 1972, 1973). In addition, during middle gene expression, a second ADP-ribosylating enzyme, the T4 Mod protein (Tiemann et al., 2004; reviewed by Miller et al., 2003; Stitt & Hinton, 1994), ADP-ribosylates the other x subunit of RNA polymerase. Thus, by about 4 min after infection, both x subunits are fully modified. ADP-ribosylation may serve to decrease the strength of host promoters (Miller et al., 2003) by eliminating the ability of the x subunit of polymerase to bind to promoter UP elements present in the −40 to −60 region of some host promoter sequences (reviewed by Ross et al., 2001). In addition, by this time of infection, multiple phage-encoded nucleases are digesting the host DNA, eliminating the supply of any competing host promoter sequences and further ensuring the commitment to phage transcription (reviewed by Carlson et al., 1994; Miller et al., 2003). Phage DNA, which contains glucosylated, hydroxymethylated cytosines, is immune to this nuclease action.

T4 late transcription (reviewed by Miller et al., 2003) still requires host RNA polymerase core, but the σ70 subunit is replaced by a σ factor composed of the T4 gene products 55 and 33 (Kolesky et al., 2002; Nechaev et al., 2004). Polymerase containing this alternative σ, in the presence of the late transcription activator T4 gene product 45, recognizes and initiates transcription from phage late promoter sequences. 45 protein is also the DNA polymerase clamp (Moarefi et al., 2000; Nossal, 1992), and the activation of late promoters requires active DNA replication. This connection of late transcription to the replication of phage DNA serves to link the expression of late genes, whose functions are primarily DNA packaging and capsid assembly, with the level of phage DNA.

In this review, we focus on the activation of transcription from T4 middle promoters. As detailed below, the switch by polymerase from recognizing host and T4 early promoters to T4 middle promoters involves retaining some of the σ70−DNA contacts found in host promoter recognition while rearranging others. This type of activation is called ‘σ appropriation’.

The σ70 subunit of E. coli RNA polymerase and recognition of host promoters

σ70 belongs to a large group of bacterial σ factors (Gruber & Gross, 2003; Lonetto et al., 1992). Like all primary σ proteins, it can be divided into four main domains (regions 1–4; Fig. 1, top), which are further subdivided into sub-regions (1.1, 1.2, etc.). Two of these subregions, region 2.4 located in the middle of σ70 and region 4.2 located in the C-terminal portion of the protein, recognize host promoter DNA (Fig. 1). Crystal structures obtained using promoter DNA in a complex with Thermus aquaticus RNA polymerase (Murakami et al., 2002a) or a −35 DNA element in a complex with region 4 of the primary σ of T. aquaticus (Campbell et al., 2002) have demonstrated that residues in an x-helix in region 2.4 are responsible for contacts with the −10 DNA element while region 4.2 contacts the −35 promoter element through a classic helix–turn–helix motif (H3-T-H4 in Fig. 2). Residues within region 4 that contact the −35 element DNA in the crystal structure of region 4 with this DNA are indicated in magenta in Fig. 2. Amino acid substitutions at σ70 residues R584 (Gregory et al., 2005; Siegela et al., 1989), E585 (Keener & Nomura, 1993) or R588 (Gardella et al., 1989) render σ70 better able to use
promoters with specific base-pair changes at positions $-31$, $-33$ or $-33$, respectively, suggesting that these residues directly interact with base moieties at these positions. The crystal structure of \textit{T. aquaticus} $\sigma$ region 4 with a $-35$ sequence element confirms the direct interaction of R584 and E585 with bases at $-31$ and $-33$, respectively. However, in the structure, DNA contact by R588 is mediated indirectly, and it is unclear how R588 provides specific base discrimination. Besides R588, many other residues within $\sigma$ region 4 also make indirect contact with the $-35$ region of the DNA from positions $-39$ to $-30$, primarily by interacting with the phosphate backbone, by van der Waals' contacts, or by water-mediated contacts (Campbell \textit{et al.}, 2002). Thus, the surface of $\sigma$ region 4 in contact with DNA is extensive, involving two residues in region 4.1 and nine residues in region 4.2 and extending for 9 bp along the DNA.

In RNA polymerase holoenzyme, interactions between residues in $\sigma$ region 4 and components of the core are needed to correctly position regions 2.4 and 4.2 so that the $-10$ and $-35$ elements are simultaneously contacted (Mekler \textit{et al.}, 2002; Murakami \textit{et al.}, 2002b; Vassylyev \textit{et al.}, 2002). As shown in blue in Fig. 2, several residues in $\sigma$ region 4 are involved in this contact with the core. In particular, some of these residues interact with a structure in

---

**Fig. 1.** Interaction of MotA and AsiA with $\sigma^{70}$ region 4 changes protein–protein and protein–DNA interactions in the upstream region of a T4 middle promoter. Top: relative locations of regions 1–4 within the 613 residues of $\sigma^{70}$ are indicated. Middle: cartoons depict RNA polymerase alone at a host or a T4 early promoter (left) or RNA polymerase together with MotA and AsiA at a T4 middle promoter (right). RNA polymerase consists of a core of proteins ($\beta$, $\beta'$, $\alpha$ and $\omega$) plus the $\sigma^{70}$ specificity factor. At a $\sigma^{70}$–dependent promoter, $\sigma^{70}$ region 2.4 contacts the $-10$ sequence element and $\sigma^{70}$ region 4.2 contacts the $-35$ sequence element. At a T4 middle promoter, the contact between $\sigma^{70}$ region 2.4 and the $-10$ element is retained, but AsiA and the N-terminal domain (NTD) of MotA interact with $\sigma^{70}$ region 4 and the C-terminal domain (CTD) of MotA interacts with a MotA box element. Canonical sequences for the DNA elements are shown. Bottom: speculative model for activation by MotA and AsiA based on the structures of \textit{Thermus aquaticus} (Murakami \textit{et al.}, 2002b) and \textit{Thermus thermophilus} (Vassylyev \textit{et al.}, 2002) holoenzyme and on FRET analysis of \textit{E. coli} holoenzyme in an open complex with DNA (Mekler \textit{et al.}, 2002). Core subunits $\beta$ and $\beta'$ (in blue) are shown as well as $\sigma^{70}$ regions 2–4 (in yellow). (For simplicity the core subunits $\alpha$ and $\omega$ and $\sigma^{70}$ region 1 are not shown.) Left, at a host promoter, $\sigma^{70}$ region 4 is positioned to contact the $-35$ region of promoter DNA in part because of its interaction with the $\beta$-flap. Right, at a T4 middle promoter, $\sigma^{70}$ region 4 interacts with AsiA and MotA, and thus is now unable to interact with the $\beta$-flap or with the $-35$ region of the DNA. Instead the MotA CTD now interacts with the MotA box centred at position $-30$. 

---

promoters with specific base-pair changes at positions $-31$, $-33$ or $-33$, respectively, suggesting that these residues directly interact with base moieties at these positions. The crystal structure of \textit{T. aquaticus} $\sigma$ region 4 with a $-35$ sequence element confirms the direct interaction of R584 and E585 with bases at $-31$ and $-33$, respectively. However, in the structure, DNA contact by R588 is mediated indirectly, and it is unclear how R588 provides specific base discrimination. Besides R588, many other residues within $\sigma$ region 4 also make indirect contact with the $-35$ region of the DNA from positions $-39$ to $-30$, primarily by interacting with the phosphate backbone, by van der Waals' contacts, or by water-mediated contacts (Campbell \textit{et al.}, 2002). Thus, the surface of $\sigma$ region 4 in contact with DNA is extensive, involving two residues in region 4.1 and nine residues in region 4.2 and extending for 9 bp along the DNA.

In RNA polymerase holoenzyme, interactions between residues in $\sigma$ region 4 and components of the core are needed to correctly position regions 2.4 and 4.2 so that the $-10$ and $-35$ elements are simultaneously contacted (Mekler \textit{et al.}, 2002; Murakami \textit{et al.}, 2002b; Vassylyev \textit{et al.}, 2002). As shown in blue in Fig. 2, several residues in $\sigma$ region 4 are involved in this contact with the core. In particular, some of these residues interact with a structure in
the core called the \( \beta \)-flap, residues 884–917 of the \( E. \ coli \) \( \beta \) subunit of RNA polymerase (diagrammed in Fig. 1, bottom) (Murakami et al., 2002b; Vassylyev et al., 2002). The interaction of \( \sigma^{70} \) region 4 with the \( \beta \)-flap appears to be crucial for positioning the DNA-binding residues of region 4 relative to the DNA-binding helix of region 2.4 since polymerase lacking the \( \beta \)-flap residues is incompetent to transcribe from promoters that require recognition of the \( \sigma^{70} \)-DNA element (Kuznedelov et al., 2002; Nickels et al., 2005).

Recognition of the TG sequence at \( -15/-14 \) arises through an \( \alpha \)-helix in region 3 (Barne et al., 1997; Campbell et al., 2002; Sanderson et al., 2003). [This region was originally called 2.5 (Campbell et al., 2002).] As expected, the interaction between \( \sigma^{70} \) region 4 and the \( \beta \)-flap is not absolutely required for transcription from \( \sigma^{70} \)-dependent extended \( -10 \) promoters (Kuznedelov et al., 2002; Nickels et al., 2005).

The T4 transcription co-activator AsiA and transcription activator MotA

AsiA is a 90 amino acid protein of T4 that is expressed early (Ouhammouch et al., 1994) and binds tightly to \( \sigma^{70} \) (Stevens et al., 2001) to promote transcription from multiple promoters.
& Rhoton, 1975) with a stoichiometry of 1:1 (Adelman et al., 1997). Wild-type AsiA exists as a homodimer in solution (Lambert et al., 2001; Urbauer et al., 2001, 2002), and the homodimer interface involves mostly hydrophobic interactions between residues in the first half of each AsiA monomer (Urbauer et al., 2002). Although two differing AsiA structures were initially reported (Lambert et al., 2001; Urbauer et al., 2002), the structure of Urbauer et al. (2002) is correct (Lambert et al., 2004a; Urbauer et al., 2002). AsiA was first identified as a 10 kDa factor that significantly inhibits transcription initiation from σ70-dependent promoters (Stevens, 1972, 1973). Subsequent work assigned this protein as the product of the T4 asiA gene (Ouhammouch et al., 1994) and demonstrated that AsiA specifically inhibits σ70 recognition of the −35 DNA element, since extended −10 promoters are not inhibited by AsiA (Colland et al., 1998; Pahari & Chatterji, 1997; Severinova et al., 1998). In addition, AsiA is required as a co-activator for transcription from T4 middle promoters (Hinton et al., 1996b; Ouhammouch et al., 1995). Thus, the binding of AsiA to σ70 operates as a molecular switch that simultaneously represses transcription from host promoters, which are recognized by σ70 RNA polymerase, and co-activates T4 middle promoters, which are recognized by the AsiA-bound polymerase together with the T4 transcription activator MotA. However, it appears that AsiA is not required for repression of T4 early promoters since T4 early transcription is still inhibited in a T4 asiA− infection (Pene & Uzan, 2000). In addition, in vitro work has demonstrated that transcription from several T4 early promoters is only mildly affected by the presence of AsiA (Orsini et al., 2004).

Although AsiA is found associated with RNA polymerase holoenzyme, direct binding of AsiA to holoenzyme occurs slowly, if at all (Hinton & Vuthoori, 2000). Instead, AsiA binds very readily to free σ70, and it is the resulting AsiA−σ70 complex which then binds to core to form the AsiA-associated polymerase (Hinton & Vuthoori, 2000). When bound to polymerase, AsiA inhibits recognition of promoters requiring a σ70 contact with the −35 element by severely retarding the formation of the stable polymerase–promoter complex (Orsini et al., 2001). However, after an extended incubation, AsiA-associated polymerase can stably bind to these promoters without the dissociation of AsiA (Orsini et al., 2001; Pal et al., 2003). This promoter binding in the presence of AsiA requires the DNA-binding region of the σ subunits of RNA polymerase, suggesting that with a long enough incubation, σ–DNA contacts can eventually compensate for the lack of contacts between σ70 region 4 and the −35 DNA element (Orsini et al., 2001).

The T4 MotA protein is a transcription activator that binds as a monomer (Cicero et al., 1998; Li et al., 2002) to the MotA box element (Brody et al., 1983; Guild et al., 1988; Hinton, 1991; Schmidt & Kreuzer, 1992) with an apparent dissociation constant of 100–200 nM (Cicero et al., 1998; Sharma et al., 1999a). MotA also interacts with σ70 (Gerber & Hinton, 1996; Pande et al., 2002). MotA was first identified through the isolation of T4 motA− phages, which were shown to be defective in the expression of a set of T4 middle gene products (Mattson et al., 1974, 1978). Structural and biochemical studies have indicated that MotA contains two distinct domains: an N-terminal domain (NTD) that is formed by five α-helices and a short β-ribbon (Li et al., 2002) and houses a transcription activation function (Finnin et al., 1997; Gerber & Hinton, 1996; Pande et al., 2002) and a C-terminal domain (CTD) that by itself is capable of binding DNA (Pande et al., 2002) and is composed of a novel ‘double wing’ motif of three α-helices interspersed with six β-strands (Li et al., 2001).

### T4 middle promoters and a model for middle promoter activation

The major sequence determinants for a T4 middle promoter are a canonical σ70−10 DNA element and a MotA box sequence, 5′-tGCTTtA-3′, with the C centred at position −28, −29 or −30. An analysis of more than 30 middle promoters has indicated that for most of these promoters, there is an excellent match to this consensus sequence, particularly in the core GCTT motif (Marshall et al., 1999; Stitt & Hinton, 1994; Truncaite et al., 2002, 2003). However, mutational analyses of a typical middle promoter sequence indicate that single base-pair changes within the MotA box are usually acceptable, and functional middle promoters with deviant sequences have been identified (Marshall et al., 1999). An examination of how the loss or modification of base determinants within the MotA box sequence affects MotA binding and activation suggests that MotA uses minor groove contacts upstream and major groove contacts downstream of the centre GC, but surprisingly, it does not require any particular base feature at the highly conserved centre C-G base pair (located at position −28, −29 or −30) (Sharma et al., 1999a). Thus, a strong match to the core GCTT motif is not an absolute requirement for MotA function. In contrast, an excellent match to the canonical σ70−10 sequence is an invariant feature of middle promoters (Marshall et al., 1999; Stitt & Hinton, 1994; Truncaite et al., 2003). These results have suggested that a good interaction between the σ70 region 2.4 and the −10 DNA element is crucial for middle promoter usage.

Despite the requirement for MotA and AsiA in vivo, some T4 middle promoters can be recognized by polymerase alone in vitro. This is probably because middle promoters have an excellent σ70−10 element and in addition, some also have the extended −10 sequence and/or a poor, but usable, σ70−35 sequence element. The in vivo modification of cytosines in wild-type T4 DNA, which places a bulky glucosyl moiety in the major groove, most likely explains why RNA polymerase does not use these promoters during infection in the absence of MotA and AsiA. Such a modification should severely limit the ability of RNA polymerase to make needed major groove contacts (Sharma et al., 1999b). Thus, RNA polymerase recognition of middle promoters in the absence of MotA and AsiA is not biologically relevant.
However, this recognition has proven useful since it allows a comparison of protein–DNA contacts made by polymerase alone with those made by MotA–AsiA–polymerase. Such a comparison demonstrates that it is the protein–DNA contacts in the upstream promoter region that are significantly altered by the presence of MotA and AsiA (Hinton et al., 1996a). These results have led to the idea that MotA and AsiA affect $\sigma^{70}$ contacts with the upstream promoter sequences without disturbing the typical $\sigma^{70}$ contacts with the $-10$ element (Hinton et al., 1996a; Pande et al., 2002). In this type of activation, called $\sigma$ appropriation, the DNA-binding CTD of MotA, rather than $\sigma^{70}$ region 4.2, contacts the upstream sequences, the MotA box, of a middle promoter (Fig. 1, middle and bottom, right). This switch from $\sigma^{70}$ region 4.2 contact with the DNA to MotA contact with the DNA is possible because of the interaction of $\sigma^{70}$ with AsiA and with the NTD of MotA. As detailed below, the known interactions of AsiA and MotA with residues within $\sigma^{70}$ region 4 support this model and help to explain how these interactions replace the typically extensive contacts of region 4 with DNA and with residues of the core.

$\sigma$ appropriation is fundamentally different from the well-characterized class I or II models of prokaryotic activation, in which the tight binding of an activator with its site together with the interaction of the activator with polymerase forces $\sigma^{70}$ to contact DNA-binding elements with non-canonical $\sigma^{70}$ sequences (reviewed by Barnard et al., 2004). To date, MotA/AsiA activation is the only known example of this type of system in prokaryotes. However, in some aspects it resembles how some eukaryotic TBP (TATA-binding protein) associated factors (TAFs) may function to change the specificity of the RNA polymerase II for different core promoter sequences (reviewed by Albright & Tjian, 2000; Chen & Hampsey, 2002).

Interaction of $\sigma^{70}$ region 4 with AsiA

The ability of MotA and AsiA to alter the protein–upstream DNA contacts at a T4 middle promoter while retaining the interactions of polymerase with the $-10$ element has suggested that one or both of the phage proteins specifically target $\sigma^{70}$ region 4. In fact, AsiA interacts with $\sigma^{70}$ residues in both regions 4.1 and 4.2 (Collard et al., 1998; Lambert et al., 2004b; Pahari & Chatterji, 1997; Severinov & Muir, 1998; Severinova et al., 1996; Sharma et al., 1999b; Simeonov et al., 2003; Urbauer et al., 2001). Two groups, Simeonov et al. (2003) and Lambert et al. (2004b), have recently provided structural analyses of the AsiA–$\sigma^{70}$ region 4 interaction. Both analyses indicate that the AsiA interface present in the AsiA–$\sigma^{70}$ heterodimer is similar to the face that is buried in the homodimer of two AsiA proteins (Urbauer et al., 2002). Thus, the formation of the AsiA–$\sigma^{70}$ heterodimer is thought to arise through an exchange of an AsiA partner for $\sigma^{70}$ (Lambert et al., 2001; Minakhin et al., 2001; Urbauer et al., 2002). However, this homodimer to heterodimer exchange may not be obligatory since AsiA bearing an N-terminal hexahistidine tag or a K20A substitution has been reported to be monomeric in solution and functional for $\sigma^{70}$ binding (Gregory et al., 2004; Lambert et al., 2004b).

In the NMR structure of AsiA in a complex with $\sigma^{70}$ region 4 (residues 533–613) (Lambert et al., 2004b), 18 AsiA residues, all within the N-terminal half of the protein (indicated as the red ribbon in Fig. 2, bottom right), interact with $\sigma^{70}$ residues in regions 4.1 and 4.2 (indicated by the black lines in Fig. 2, top). In this structure, many of the residues that $\sigma^{70}$ would normally use to contact the $-35$ region of DNA or to contact the core (Campbell et al., 2002; Kuznedelov et al., 2002; Murakami et al., 2002b; Vassylyev et al., 2002) interact with AsiA. In addition, the structure shows that the binding of AsiA to regions 4.1 and 4.2 dramatically reconfigures region 4 such that $\sigma^{70}$ H3-T-H4 becomes one continuous pseudohelix (Fig. 2, bottom). As a consequence, some of the $\sigma^{70}$ residues that normally interact with DNA are positioned away from the surface, further limiting DNA contact, and portions of region 4 that would normally interact with core are also repositioned. Thus, the structure argues that AsiA inhibits in two ways: (1) it directly interacts with some of the $\sigma^{70}$ residues that normally contact the $-35$ element and the $\beta$-flap; and (2) it remodels region 4, creating a fundamentally different structure that lacks the architecture needed for DNA binding and the correct positioning of region 4 by core.

A previous NMR analysis (Simeonov et al., 2003) was obtained using AsiA and peptides that contain $\sigma^{70}$ region 4.1 (residues 540–565) and $\sigma^{70}$ region 4.2 (residues 570–599). This analysis also demonstrates that the N-terminal half of AsiA constitutes the AsiA interface in the AsiA–$\sigma^{70}$ heterodimer and that the interaction of AsiA with $\sigma^{70}$ should interfere with the interaction of $\sigma^{70}$ region 4 with the $\beta$-flap. However, some findings in this NMR analysis differ from those of the AsiA–$\sigma^{70}$ region 4 structure (Lambert et al., 2004b). In the work by Simeonov et al. (2003), the interaction of AsiA with region 4.2 starts at residue 587, rather than residue 580 as reported by Lambert et al. (2004b). In addition, some of the specific $\sigma^{70}$ residues that AsiA contacts in regions 4.1 and 4.2 are not the same as those found in the structure of Lambert et al. (2004b). In particular, AsiA does not interact directly with $\sigma^{70}$ residues that normally contact DNA. Simeonov et al. (2003) suggest that the ability of AsiA to inhibit the interaction of $\sigma^{70}$ with the $-35$ element is manifested primarily through its disruption of the normal interactions between region 4 and the $\beta$-flap, which causes a reorientation of $\sigma^{70}$ region 4 relative to core. Using luminescence resonance energy transfer, they found that the distance between $\sigma^{70}$ regions 2.4 and 4.2 is shorter in AsiA-associated polymerase than polymerase without AsiA. This finding supports the idea that when AsiA is present, $\sigma^{70}$ region 4 is not positioned correctly for its needed interaction with the $\beta$-flap. The authors also argue that the ability of AsiA to bind to the region 4.1 or 4.2 peptides individually suggests that under
certain conditions AsiA may have the flexibility to limit its interaction to just region 4.1 or 4.2.

Structural analyses are invaluable for understanding protein–protein interactions at a molecular level. However, it is important to combine these structures with biochemical and genetic data to test the relevance of the structures to the biological system. Substantial biochemical data support many features of the AsiA–σ^70 structural analyses. The importance of some of the AsiA residues that directly contact σ^70 in these structures has been examined. In two investigations (Pal et al., 2003; Sharma et al., 2002), AsiA mutant proteins were generated both randomly and by site-specific mutagenesis. AsiA substitutions V14D, L18S, L18F and I40N, which are located within the N-terminal half of AsiA, decrease the interaction of AsiA with σ^70. Similarly, substitutions V14D, L18S, L18F and I40N, which are located within the N-terminal residues of AsiA are not deleterious (Pal et al., 2003; Sharma et al., 2002). Furthermore, even a deletion of AsiA residues 47–90 results in a protein that can still interact with σ^70 region 4 and has some ability to inhibit transcription in the absence of MotA and activate transcription in the presence of MotA (Pal et al., 2003). In the AsiA–σ^70 region 4 structure, the N-terminal half of AsiA contains all the residues that interact with σ^70, and V14, L18 and I40 are residues that make direct contacts (Lambert et al., 2004b). In another investigation, a pull-down assay has demonstrated that an alanine substitution at D6, E10, K20, F36 or E39 disrupts the interaction between AsiA and σ^70 region 4 (Lambert et al., 2001). E10, K20 and F36 also directly contact σ^70 in the structure. Together, these studies provide strong support for the conclusion that the AsiA interface is composed of the N-terminal half of the protein and that some of the AsiA residues that contact σ^70 in the structure are indeed important for this interaction.

The importance of specific σ^70 residues in the interaction of σ^70 with AsiA has also been screened in various biochemical and two-hybrid assays. Two-hybrid assays using AsiA and σ^70 region 4 with single substitutions within regions 4.1 or 4.2 have yielded only very modest effects, even when the σ^70 substitution occurs within the known binding site for AsiA (S. Pande, N. Wais, M. Vuthoori, X. B. Johnson & D. M. Hinton, unpublished). However, a modification of this assay has confirmed the importance of σ^70 residue F563, which contacts AsiA residues in the solution structure (Lambert et al., 2004b) and in the NMR analysis of Simeonov et al. (2003). The significance of F563 was revealed using a variant of AsiA that has a K20A substitution and a variant of σ^70 region 4 that has a D581G substitution. The D581G change makes the helix–turn–helix motif of region 4.2 (H3–T–H4 in Fig. 2) more similar to that found in typical H–T–H DNA-binding proteins, whereas the K20 mutation is reported to result in AsiA being monomeric rather than dimeric (Gregory et al., 2004).

When used together, the AsiA K20A and σ^70 D581G variants interact very strongly in the two-hybrid assay, resulting in a sixfold increase in the level of the reporter gene signal over that observed with wild-type AsiA and wild-type σ^70 region 4 (Gregory et al., 2004). How the AsiA K20A substitution results in a higher signal in this assay is not clear, given that this substitution disrupts the AsiA–σ^70 interaction in a pull-down assay (Lambert et al., 2001). Nevertheless, with such a high signal, Gregory et al. (2004) were able to screen a set of randomly introduced mutations in σ^70 region 4 for ones that decrease the AsiA–σ^70 region 4 interaction. This screen produced F563Y. In vitro transcription assays using wild-type AsiA and σ^70 containing only the F563Y change demonstrated that the F563Y substitution renders σ^70 much less susceptible to AsiA inhibition, providing independent biochemical evidence that this σ^70 residue is important for the σ^70–AsiA interaction (Gregory et al., 2004).

Structural work with polymerase holoenzyme (Murakami et al., 2002b; Vassylyev et al., 2002) has assigned F563 as a residue that is normally involved in the σ^70–core interaction, directly interacting with the β-flap. The interaction between the β-flap and σ^70 region 4 can also be observed in the two-hybrid assay provided that the σ^70–core interaction (Gregory et al., 2004). The targeting of F563 by AsiA is consistent with the idea that AsiA inhibits σ^70-dependent transcription at least in part by disrupting the contact of σ^70 with the β-flap. Interestingly, in the two-hybrid assays, the effects of the F563 substitutions are highly specific. The F563Y change has little effect on the σ^70 interaction with the β-flap while the F563L substitution does not affect σ^70–AsiA. These results suggest that although F563 contacts AsiA, this interaction differs from its interaction with the core. Finally, combining the F563Y mutation with other mutations in region 4 that disrupt the interaction of region 4 with the β-flap renders the polymerase once again susceptible to AsiA inhibition (Gregory et al., 2004). This result suggests that AsiA can compete with the β-flap for binding to this region of σ^70. Given that the binding of wild-type AsiA to wild-type σ^70 occurs when σ^70 is not bound to the core (Hinton & Vuthoori, 2000), it may be that these σ^70 mutations that disrupt the σ^70 interaction with the β-flap now allow AsiA to access σ^70 when it is associated with the core. Alternatively, they may shift the dynamic equilibrium between holoenzyme and free σ^70 plus free core toward the free components, thus, making more free σ^70 available to bind to AsiA at any given time.

Recently, Gregory et al. (2005) used a different assay to identify another mutation that renders σ^70 less susceptible to AsiA inhibition. This assay uses a plasmid containing the σ^70 substitution R584A, which changes the preferred σ^70 −35 DNA-binding element from 5′-TTGACA-3′ to 5′-TTGAAA-3′. With this plasmid, one can screen for other σ^70 mutations in vivo that interfere with the ability of AsiA
to inhibit, including mutations that would be deleterious if present in the chromosomal wild-type $\sigma^{70}$ that E. coli must use to express its genes. This assay revealed two substitutions that affect AsiA inhibition: F563Y, which was found in the earlier screen, and T552A. Transcription assays using a $\sigma^{70}$ with only the T552A substitution confirmed that this change lessens AsiA inhibition in vitro. T552 is not identified as a contact residue for AsiA in the AsiA–$\sigma^{70}$ region 4 structure (Lambert et al., 2004b) or in the earlier NMR analysis of AsiA interaction with $\sigma^{70}$ region 4 peptides (Simeonov et al., 2003), but it is adjacent to the contact residue L551.

The effect of specific substitutions within $\sigma^{70}$ region 4.2 on its binding to AsiA have also been examined by using a series of $\sigma^{70}$ 4.2 peptides, each with a single alanine substitution, in a competition assay with the wild-type 4.2 peptide (Minakhin et al., 2001). Again, no single amino acid substitution had a dramatic effect, although substitutions at T569, V576, I590, K593, L595 and R596 were the most deleterious. In addition, polymerase with a $\sigma^{70}$ bearing either a K593E or R596E substitution required more AsiA to inhibit transcription in vitro. The AsiA–$\sigma^{70}$ region 4 structural work (Lambert et al., 2004b; Simeonov et al., 2003) reveals a direct interaction between AsiA residues and the $\sigma^{70}$ residues I590 and L595. Although no direct interaction between T569, V576, K593 or R596 and AsiA residues is observed in these analyses, K593 and R596 lie next to contacted residues.

**Interaction of $\sigma^{70}$ region 4 with MotA**

The interaction of $\sigma^{70}$ with MotA is not as strong as its interaction with AsiA. Unlike AsiA, MotA is not found associated with RNA polymerase after T4 infection (Hinton et al., 1996b), and in the E. coli two-hybrid assay, the interaction of MotA with $\sigma^{70}$ region 4 is weak, but reproducible (Pande et al., 2002). Using this assay, Pande et al. (2002) demonstrated that the NTD of MotA is sufficient for an interaction with $\sigma^{70}$, consistent with the idea that this portion of MotA contains an activation domain. A screen of various $\sigma^{70}$ region 4 single substitutions has yielded three, T552A, E585D and R608C, that significantly disrupt this interaction and one, E555A, that has about a twofold effect, while having little effect on the interaction of $\sigma^{70}$ region 4 with wild-type AsiA in this assay (P. S. Pande, N. Wais, M. Vuthoori, X. B. Johnson & D. M. Hinton, unpublished).

The Far C-terminal region of $\sigma^{70}$ contains R608 and is an $\alpha$-helix in the crystal structure of $\sigma$ region 4 of Thermotoga maritima (Lambert et al., 2004b) (H5 in Fig. 2). Evidence suggests that this region is crucial for the MotA–$\sigma^{70}$ interaction. A substitution of the last 17 amino acid residues of $\sigma^{70}$ with those of the stationary-phase $\sigma$, $\sigma^{3}$, makes multiple substitutions in this region. This $\sigma^{3}$$/\sigma^{70}$ exchange eliminates the interaction of region 4 with MotA in the two-hybrid assay, but only decreases its interaction with AsiA by about twofold (Pande et al., 2002). In addition, the deletion of $\sigma^{70}$ residues 608–613, which removes much of H5 (Fig. 2), nearly eliminates the ability of MotA and AsiA to activate transcription from the T4 middle promoter $P_{457}$, but does not significantly affect the ability of AsiA to inhibit transcription from this promoter in the absence of MotA (Pande et al., 2002).

The importance of $\sigma^{70}$ residues T552, E555 and E585 for the interaction between MotA and $\sigma^{70}$ is not yet clear. In the two-hybrid assay, replacement of either T552 or E555 with an alanine is deleterious, which is consistent with the involvement of the side chains at these residues (S. Pande, N. Wais, M. Vuthoori, X. B. Johnson & D. M. Hinton, unpublished results). Thus, there may be $\sigma^{70}$–MotA contacts within regions 4.1 and 4.2, and if so, these contacts would be physically very close to those of AsiA–$\sigma^{70}$. However, as yet there is no other evidence to support an involvement of these 4.1 and 4.2 residues in the interaction of MotA with $\sigma^{70}$. In addition, there is no evidence as yet to indicate that MotA and AsiA physically interact.

A genetic screen has also been employed to investigate interactions between $\sigma^{70}$ and MotA (Cicero et al., 2001). The $\sigma^{70}$ substitutions D570N, Y571C, Y571H, L595P or S604P suppress the growth defect of a particular T4 motA mutant phage in vivo (Cicero et al., 2001). This MotA mutant (D30A/F31A) is a positive control mutant. It is partially defective for activation of transcription but not for binding to the MotA box DNA (Finnin et al., 1997). The identification of the suppressor S604P is consistent with an assignment of a MotA interaction site to the far C-terminal region of $\sigma^{70}$, and the identification of other suppressing residues in region 4 again suggests the possibility of other contact points for MotA in $\sigma^{70}$. However, purified $\sigma^{70}$ proteins containing each of these substitutions do not suppress the mutant MotA protein in vitro, suggesting that the in vivo results may arise from an indirect effect (Cicero et al., 2001).

In summary, the biochemical and genetic data support an interaction between MotA and the very C-terminal region of $\sigma^{70}$ and suggest that there may be other contact points within regions 4.1 and 4.2. Two models for how both MotA and AsiA interact with $\sigma^{70}$ region 4 have been offered. Pande et al. (2002) have proposed that the interaction between the DNA-bound MotA and the C-terminal region of $\sigma^{70}$ serves as a molecular bridge between $\sigma^{70}$ and the DNA, and thus substitutes functionally for the interaction of $\sigma^{70}$ with its −35 DNA element. MotA alone binds to a MotA box with a $K_{d}(\text{app})$ of 0.1–0.2 μM (Cicero et al., 1998; Sharma et al., 1999a), and the MotA box overlaps the −35 element for $\sigma^{70}$. Thus, in this model, the interaction of AsiA with $\sigma^{70}$ region 4 is required to promote the formation of the MotA bridge because MotA alone cannot compete with $\sigma^{70}$ effectively for the −30 to −35 region of a promoter. The AsiA–$\sigma^{70}$ region 4 structure (Lambert et al., 2004b) has features that are consistent with this idea. In this structure, the far C-terminal region of $\sigma^{70}$ is disordered, suggesting that the binding of
AsiA to $\sigma^{70}$, and the ensuing conformational change, may cause a change in the C-terminal helix H5. Such a change could facilitate the connection between MotA and $\sigma^{70}$, while the interaction of AsiA with $\sigma^{70}$ regions 4.1 and 4.2 could allow MotA better access to its DNA binding site. Thus, AsiA would represent a co-activator that works by remodelling one portion of $\sigma^{70}$ so that another portion is now available for the transcriptional activator. In the other model for MotA and AsiA interaction with $\sigma^{70}$, Minakhin et al. (2003) have proposed that the interaction of MotA with the far C-terminal region of $\sigma^{70}$ lessens AsiA binding to its contact residues in region 4.2, limiting AsiA to its 4.1 contacts. Thus, in this model, it is the interaction of AsiA with region 4.1 that causes the activation of transcription, although MotA may also make a contribution. This model is based on the finding that in the absence of MotA, AsiA activates transcription from the T4 middle promoter P_{RHB} provided that $\sigma^{70}$ lacks region 4.2 (Minakhin et al., 2003). The finding that AsiA can bind to region 4.1 and 4.2 peptides separately supports the idea that AsiA might be able to limit its binding to just the 4.1 region (Simeonov et al., 2003; Urbauer et al., 2001). Despite their differences, both models share the idea that the interaction of $\sigma^{70}$ region 4 with AsiA and MotA precludes its normal interactions with the $-35$ element and with core.

Several class II prokaryotic activators also bind to $\sigma^{70}$ region 4. However, in many of these cases, the activator facilitates the interaction of $\sigma^{70}$ region 4 with a non-ideal $-35$ sequence. Investigation of the specific residues in $\sigma^{70}$ region 4.2 that are involved in the class II interaction has identified residues 588–603, which are just N-terminal to H5 (Landini & Busby, 1999; Lonetto et al., 2002; Rhodius & Busby, 2000). There is no indication that the far C-terminal region of $\sigma^{70}$ is needed for class II activation. Thus, the T4 system reveals another patch of $\sigma^{70}$ region 4 that can be manipulated for regulation.

**Final thoughts**

The recent availability of polymerase and activator structures has greatly increased our knowledge of RNA polymerase and the mechanisms of transcription initiation. As depicted in the structure-based cartoon at the bottom left of Fig. 1, it is now known that $\sigma^{70}$ interacts extensively with both the $\beta$ and $\beta'$ subunits of the core, and through these interactions positions its DNA-binding regions 2.4 and 4.2 so that they are in the correct spatial orientations for promoter binding. The interaction of $\sigma^{70}$ with the $\beta$-flap appears to be particularly important for the correct placement of region 4.2. In the case of activation by MotA and AsiA, structural analyses are supporting the model of $\sigma$ appropriation that had been developed from previous biochemical analyses. This model postulates that interactions of MotA and AsiA with $\sigma^{70}$ region 4 overcome the extensive interactions between $\sigma^{70}$ region 4 and DNA and $\sigma^{70}$ region 4 and core. Consequently, a different upstream promoter sequence can now be recognized. Although structural analyses of the MotA–AsiA–holoenzyme complex are not yet available, we can build a simple model based on the structure of holoenzyme and what is now known about the interactions of AsiA and MotA with $\sigma^{70}$ region 4 (bottom right of Fig. 1). In this model the co-activator AsiA and the NTD of the activator MotA function by loosening the grip of the $\beta$-flap on region 4, thus, freeing region 4 from its normal role of contacting the DNA. The interaction of MotA NTD with $\sigma^{70}$ then positions the CTD of MotA so that it is poised for contact with the $-30$ MotA box. However, this remodelling of region 4 is accomplished without disturbing the needed interactions between $\sigma^{70}$ region 2.4 and the $-10$ region of the T4 middle promoter. Thus, this T4 system suggests that region 4 of $\sigma^{70}$ is quite flexible and has the capacity to be changed without significantly affecting the required protein–protein and protein–DNA interactions of other $\sigma^{70}$ regions.

The MotA/AsiA example of $\sigma^{70}$ appropriation is as yet unique. However, it would be surprising if T4 middle transcription were the only case where $\sigma^{70}$ region 4 is manipulated in this way. Recent work has indicated that there is a family of proteins with similarity to AsiA in T4-type phages and bacteria (Pineda et al., 2004). Phage orthologues have been found in the genomes of the T4-type phages RB69, 44RR, KVP40 and Aeh1, which infect a variety of Gram-negative bacteria. These phage proteins share conserved amino acids at many of the T4 AsiA residues that contact region 4 in the structure, and the KVP40 AsiA behaves like T4 AsiA in $\sigma^{70}$ binding and in transcription assays. The bacterial proteins Rsd (Jishage & Ishihama, 1998), an anti-$\sigma^{70}$ protein of E. coli, and AlgQ (Deretic & Konyecsni, 1989), a regulator of alginate production in Pseudomonas aeruginosa, appear to be members of this family also (Pineda et al., 2004). Rsd and AlgQ have conserved residues at positions corresponding to T4 AsiA E10, L18, A35, F36 and V42, residues that directly contact $\sigma^{70}$ in the T4 AsiA–$\sigma^{70}$ structure. Both of these proteins also interact with residues in $\sigma^{70}$ regions 4.1 and 4.2. As was observed with AsiA, the $\sigma^{70}$ substitution F563Y diminishes the Rsd–region 4 or AlgQ–region 4 interaction (Pineda et al., 2004). In addition, an R596H substitution lessens the interaction of Rsd with $\sigma^{70}$ region 4 (Dove & Hochschild, 2001), and contact sites for Rsd in region 4.2 have been mapped to residues 1590, L595 and L598 (Jishage et al., 2001; Westblade et al., 2004), residues that interact with AsiA in the AsiA–$\sigma^{70}$ region 4 structure (Lambert et al., 2004). Thus, activation systems similar to that of MotA/AsiA may exist in other organisms. However, a MotA orthologue has not yet been identified in the KVP40, Aeh1, E. coli or P. aeruginosa genomes. Further work should determine the generality of the T4 system.

**Acknowledgements**

The authors thank N. Nossal, A. Hochschild, and B. Gregory for helpful discussions. We are especially grateful to L. Westblade for his suggestion of the phrase ‘$\sigma$ appropriation’.
References


Interactions of T4 MotA and AsiA with σ70


