Specific binding of the transcription factor sigma-54 to promoter DNA

Martin Buck & Wendy Cannon

AFRC Nitrogen Fixation Laboratory, University of Sussex, Brighton BN1 9RQ, UK

A CENTRAL event in transcription is the assembly on DNA of specific complexes near the initiation sites for RNA synthesis. Activation of transcription by one class of enhancer-binding proteins requires an RNA polymerase holoenzyme[1] containing the specialized transcription factor, sigma-54 (σ^{54}). We report here that σ^{54} alone specifically binds to promoter DNA and is responsible for many of the close contacts between RNA polymerase holoenzyme and promoter DNA, a property proposed for the major σ^{70} protein family. Binding of σ^{54} to promoter DNA is not equivalent to that of holoenzyme suggesting that there is a constraint on σ^{54} conformation when bound with core RNA polymerase. Footprints indicate σ^{54} is at the leading edge of DNA-bound holoenzyme. Like the holoenzyme[1-4], σ^{54}-binding to promoter DNA does not result in DNA strand separation. Instead the specific DNA-binding activity of σ^{54} assists assembly of a closed promoter complex. This complex can be isomerized to the open (DNA melted) complex by activator protein[5,6], but promoter-bound σ^{54} alone cannot be induced to melt DNA. The pathway leading to productive transcription is similar to that proposed for eukaryotic RNA polymerase II systems.

The promoter sequence recognized by the holoenzyme ($E\sigma^{54}$) is generally characterized by the presence of GG and GC doublets 24 and 12 base pairs, respectively, upstream of the transcription initiation point (Table 1; refs 1, 3), and activation requires binding of the appropriate activator protein upstream of $E\sigma^{54}$ at enhancer sequences[7-9]. Using conditions for detecting closed promoter complexes at the σ^{54}-dependent *Klebsiella pneumoniae* nitrogen fixation (*nif*) promoters[10] we footprinted the *nifH* promoter and a mutant variant, *nifH049* (Table 1) which forms a stronger closed complex[3,11]. We were able to detect $E\sigma^{54}$ complexes bound at the *nifH* and *nifH049* promoters, and σ^{54} bound at the *nifH049* promoter (Figs 1*a* and 3). The *nifH* promoter did not detectably bind σ^{54} (Fig. 3, lanes 16, 17). The complexes with $E\sigma^{54}$ and the *nifH* and *nifH049* promoters were not equivalent. The *Exo*III digestion block was 3–4 base pairs (bp) closer to the transcription initiation site in the *nifH049* promoter. Thus $E\sigma^{54}$ seemed to establish more contacts at the *nifH049* promoter. In the absence of core RNA polymerase subunits, σ^{54} bound to the *nifH049* promoter to produce a clear *Exo*III block (Fig. 1*a*), but only partially protected conserved guanine residues at −24, −25 and −13 from dimethylsulphate attack (Fig. 1*b*). The σ^{54}-dependent *Exo*III block at the *nifH049* promoter was 3–4 bp closer to the transcription initiation site than when $E\sigma^{54}$ bound (Fig. 1*a*), indicating σ^{54} is conformationally constrained by core polymerase. Unlike $E\sigma^{54}$ (ref. 11), binding of σ^{54} did not increase reactivity of the T at −9 towards $KMnO_4$ (data not shown). Thus σ^{54} did not seem to distort the DNA. DNase 1 footprints revealed that the DNA was covered to a similar extent by σ^{54} and $E\sigma^{54}$, but that the major $E\sigma^{54}$ footprint extended about 7 bases further upstream than did the σ^{54} footprint (Fig. 1*c*). Gel mobility shift assays also demonstrated that the complex forming with σ^{54} was different to that forming in the presence of $E\sigma^{54}$, the latter complex having a much reduced mobility (data not shown).

To extend our analysis of σ^{54} binding to include a naturally occurring promoter sequence (Table 1) we examined the binding of $E\sigma^{54}$ (ref. 3) and σ^{54} to the *Rhizobium meliloti nifH* promoter (Fig. 2). Both σ^{54} and $E\sigma^{54}$ produced clear blocks to *Exo*III digestion, with the σ^{54} block being about 4 bp closer to the transcription initiation site than the $E\sigma^{54}$ block. Dimethylsulphate and DNase 1 footprints again revealed that σ^{54} was responsible for most of the close contacts detected between holoenzyme and the *R. meliloti nifH* promoter (Gs at −14, −25, −26; Fig. 2). Holoenzyme covered ~32 bp and σ^{54} covered 27 bp of promoter DNA as judged by DNase 1 footprinting (Fig. 2). Footprints on the bottom strand showed a single *Exo*III block for σ^{54} and $E\sigma^{54}$ at around −34, protection of Gs −13, −22 and −24 by σ^{54} and $E\sigma^{54}$ and a DNase 1 footprint from −34 to −5 for σ^{54} and $E\sigma^{54}$.

The *nifH049* and *R. meliloti nifH* promoters have a 'T'-tract from −14 to −17 that distinguishes them from the *nifH* promoter[11] and is necessary for the binding of σ^{54} (Table 1). *R. meliloti* and *nifH049* templates in which dT was replaced by dU, effectively removing the 5-methyl group of T, bound $E\sigma^{54}$ but not σ^{54} (Fig. 3; Table 1). In complementary experiments we constructed wild-type sequence *K. pneumoniae nifH* templates in which dC was replaced by dm^5C and found that σ^{54} then bound (Fig. 3). The sequence from −14 to −17 is therefore critical for σ^{54} binding. Specifically the methyl groups in the DNA major groove are important (Table 1).

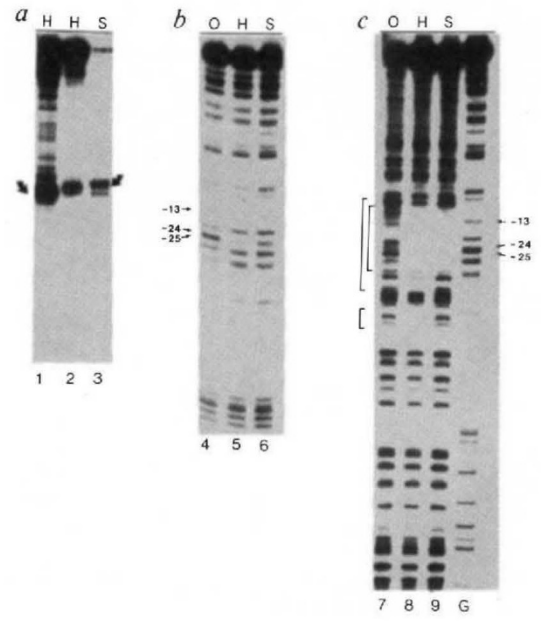

FIG. 1 Footprints demonstrating the binding of $E\sigma^{54}$ and σ^{54} to the *K. pneumoniae nifH* and *nifH049* promoters. *a*, Exonuclease III footprints; *b*, dimethylsulphate footprints; *c*, DNase 1 footprints, H, Holoenzyme; S, σ^{54}; O, no addition. Arrows mark *Exo*III blocks due to $E\sigma^{54}$ or σ^{54} binding; brackets, the extent of the DNase 1 footprint. Lane 1, wild-type *nifH* promoter; lanes 2–9, *nifH049* promoter. Lane G, Chemical cleavage of DNA at G residues. $E\sigma^{54}$ bound the *nifH* and *nifH049* promoters, σ^{54} bound the *nifH049* promoter. Footprints were conducted as described previously[10] in 25 mM Tris-acetate, 8 mM magnesium acetate, 10 mM KCl, 1 mM dithiothrietol, 3.5% (w/v) polyethylene glycol, pH 8.0. Template DNA (2.4 nM) was incubated with 125 nM core RNA polymerase (E) and 280 nM σ^{54} ($E\sigma^{54}$) (E and σ^{54} were previously combined and warmed at 30 °C for 5 min before addition) at 30 °C in a 50-μl assay. When σ^{54} (560 nM) alone was used, this was also preincubated before adding to template DNA. Binding was for 20 min before addition of footprinting reagent. In DNase 1 footprints, salmon sperm DNA (270 μg ml^{-1}) was included in the incubations. *K. pneumoniae* σ^{54} was free of β and β' RNA polymerase subunits as judged by stained, overloaded gels. Equivalent exonuclease III and DMS footprints were obtained when the binding buffer was supplemented with 100 mM KCl or footprints were conducted in 50 mM Tris-acetate, 100 mM potassium acetate, 8 mM magnesium acetate, 27 mM ammonium acetate, 1 mM dithiothrietol, 3.5% (w/v) polyethylene glycol[2]. Thus binding of σ^{54} persists at physiologically relevant salt concentrations. Positions of digestion blocks were estimated from G and C+T chemical cleavage sequencing ladders.

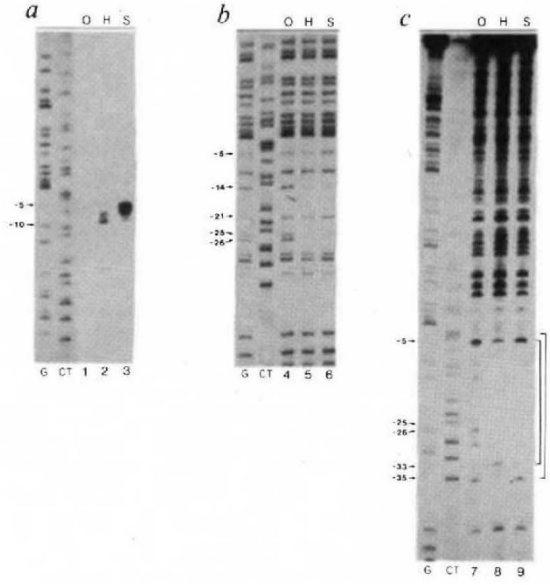

FIG. 2 Footprints demonstrating the binding of Eσ^{54} (H) and σ^{54} (S) to the *R. meliloti nifH* promoter. *a*, Exonuclease III footprints; *b*, dimethylsulphate footprints; *c*, DNase 1 footprints. Conserved Gs at −14, −25, −26 were protected from dimethylsulphate attack by σ^{54} and Eσ^{54}. Assays were as for Fig. 1, and footprints persisted in buffer supplemented with 100 mM KCl or when the 100 mM potassium acetate-containing buffer was used. The σ^{54} *Exo*III block persisted down to 15 nM σ^{54}, and shifting the σ^{54} block to that characteristic of Eσ^{54} needed a minimum of 2 nM E at 280 nM σ^{54}. Serial dilution of Eσ^{54} resulted in a gradual loss of the Eσ^{54} *Exo*III block without the appearance of the block characteristic of σ^{54} alone.

TABLE 1 Promoter sequences and template requirements for binding σ^{54}

Promoters		σ^{54} Binding
Wild-type *nifH*	CTGGTATGTTCCCTGCA	−
nifH049	CTGGTATGTTTTTTGCA	+
R. meliloti nifH	CTGGCACGACTTTTGCA	+

	Template sequences	Footprints Eσ^{54}	Footprints σ^{54}
nifH	CCCTGCA	+++	−
nifH049	TTTTGCA	+++	+++
RmH	TTTTGCA	+++	+++
nifH049-dU	UUUUGCA	+++	−
RmH-dU	UUUUGCA	+++	−
nifH-dm^{5}C	m^{5}Cm^{5}Cm^{5}CTGm^{5}CA	+++	+

Binding sites for Eσ^{54} are characterized by GG and GC doublets separated by 10 bp and centred roughly 18 bp upstream of the transcription initiation site. A T-tract upstream of the GC doublet is important for the binding of Eσ^{54} and σ^{54} (refs 3, 22). The *nifH049* and *R. meliloti nifH* promoters bound σ^{54} but binding to the wild-type *nifH* promoter was not detected (Figs 1–3). When templates were prepared in which the 5-methyl group of T was absent (dU), σ^{54} did not detectably bind (Fig. 3). Introduction of 5-methyl (dm^{5}C) into templates increased binding of σ^{54} (Fig. 3).

The closed complex that forms at the *nifH049* promoter can be isomerized to the open complex by the NtrC S160F enhancer-binding activator protein[11–13]. But when prebound σ^{54} at *nifH049* was incubated with NtrC S160F protein no evidence for DNA melting (using DNA reactivity towards $KMnO_4$ as an index of denaturation) was obtained (data not shown). Thus σ^{54} seems unable to function in the process of open complex formation unless bound as holoenzyme.

In summary, σ^{54} functions through assisting assembly of a preinitiation complex consisting of σ^{54}-holoenzyme and promoter DNA at a site centred about 18 bp upstream of the transcription initiation point. A predominant event in binding holoenzyme to promoter DNA is recognition of DNA by σ^{54}. The nonequivalence in the σ^{54} recognition sequence when the −25 and −13 elements are compared presumably enables the orientation of DNA-bound σ^{54} to define the direction of transcription.

Detection of Eσ^{54} binding when σ^{54} binding is not evident indicates core RNA polymerase reduces the promoter dissociation rate of σ^{54}. It is possible that the binding specificities of σ^{54} and Eσ^{54} are different, suggesting a source of favourable binding energy for the holoenzyme. Notably σ^{54} functions with the same core RNA polymerase subunits as does the major eubacterial sigma factor, σ^{70}, but seems to associate (at least in the absence of DNA template) relatively weakly with core RNA polymerase[14]. It is possible that σ^{54} can bind the template and then associate with core RNA polymerase, possibly in contrast to σ^{70} which seems to bind only as holoenzyme[15]. It seems that σ^{54} is at the leading edge of the holoenzyme, positioned to extend over DNA to be melted during open complex formation, implying σ^{54} might directly participate in DNA melting. Open promoter complex formation occurs efficiently in response to

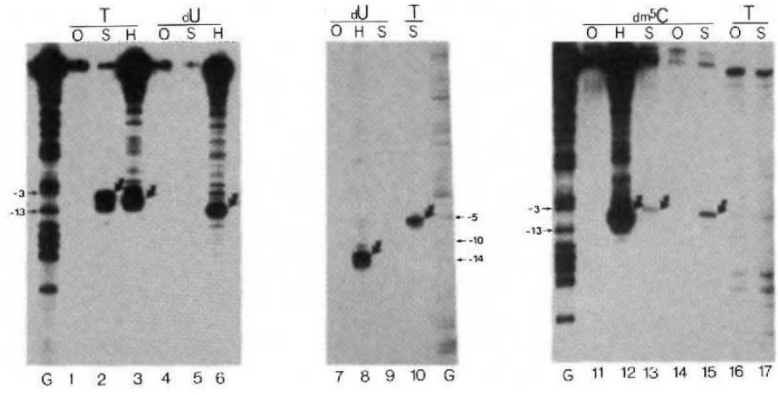

FIG. 3 Binding of Eσ^{54} (H) and σ^{54} (S) to templates altered by the replacement of dT by dU or of dC by dm^{5}C (see also Table 1). Binding was assayed by exonuclease III footprinting and blocks are marked with arrows. Lanes 1–3, *nifH049* dT template footprints; lanes 4–6, *nifH049* dU template footprints; lanes 7–9, *R. meliloti nifH* dU template footprints; lane 10, *R. meliloti nifH* dT template footprint. Wild-type *K. pneumoniae nifH* dm^{5}C template footprints, lanes 11–15. For comparison, footprints of the wild-type *K. pneumoniae* dT template are shown in lanes 16 and 17. Assays for lanes 15 and 17 contained 1.2 μM σ^{54}. Lanes 16 and 17 were twofold overloaded. Lane G, chemical cleavage of DNA at G residues. Templates were generated by polymerization using nucleotide triphosphate mixes containing either dUTP or dm^{5}CTP rather than dTTP or dCTP, and thus the top nontemplate strand only is modified (the strand visualized in the footprints). Substituting dT by dU effectively removes the C^5-methyl group of dT found in the major groove of DNA, and replacement of dC by dm^{5}C effectively introduces C^5-methyl. No binding of σ^{54} to the wild-type *nifH* dT template was detected (lane 17 and unpublished data). Replacement of dT by dU greatly reduced σ^{54} binding (lanes 5, 9). Replacement of dC by dm^{5}C increased σ^{54} binding (lanes 13, 15). Differences in DNA geometries may also contribute to the preferential binding of σ^{54} to dT rather than dm^{5}C containing templates. As anticipated from the conservation of G·C base pairs at −24, −25 and −13 and their protection from methylation by Eσ^{54} and σ^{54} (Fig. 2; refs 2, 10) replacement of dG by C^7-deaza guanine resulted in templates that failed to bind Eσ^{54} or σ^{54} as judged by DNase 1 footprinting (data not shown). The sequence TTCGCT rather than TTTGCA from −16 to −11 in the enteric *glnA*p2 promoters may explain why the binding of σ^{54} is not readily detected at this sequence[19,20]. Proposed domain structures for σ^{54} indicate that DNA recognition may involve a helix–turn–helix structure[18,21].

the activator only when σ^{54} is bound as holoenzyme at the promoter. The conformation of σ^{54} in the holenzyme, or the RNA polymerase subunits themselves, may also be important for DNA melting. That σ^{54} binds to promoter DNA but does not melt it is consistent with all known σ^{54}-dependent transcription systems that have a strict requirement for an activator protein[1]. Separation of promoter recognition and DNA melting functions permits $E\sigma^{54}$ to respond to many stimuli of gene expression through their influences on the activity of the activator protein. Activator dependence and assembly of the preinitiation complex in its absence strongly suggests a broad similarity to eukaryotic RNA polymerase II transcription[16,17]. □

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Different β-subunits determine G-protein interaction with transmembrane receptors

C. Kleuss*, H. Scherübl†, J. Hescheler†, G. Schultz† & B. Wittig*

* Institut für Molekularbiologie und Biochemie, and
† Institut für Pharmakologie, Freie Universität Berlin, Arnimallee 22, D-1000 Berlin 33, Germany

REGULATORY GTP-binding proteins (G proteins) are membrane-attached heterotrimers (α, β, γ) that mediate cellular responses to a wide variety of extracellular stimuli[1,2]. They undergo a cycle of guanine-nucleotide exchange and GTP hydrolysis, during which they dissociate into α-subunit and $\beta\gamma$ complex[1]. The roles of G-protein α-subunits in these processes and for the specificity of signal transduction are largely established; the β- and γ-subunits are essential for receptor-induced G-protein activation and seem to be less diverse and less specific. Although the complementary DNAs for several β-subunits have been cloned[2,5–8], isolated subunits have only been studied as $\beta\gamma$ complexes[3,9–12]. Functional differences have been ascribed to the γ-subunit on the basis of extensive sequence similarity among β-subunits and apparent heterogeneity in γ-subunit sequences[13,14]. $\beta\gamma$ complexes can interact directly or indirectly with different effectors[10,11,15–20]. They seem to be interchangeable in their interaction with pertussis toxin-sensitive α-subunits[3], so we tested this by microinjecting antisense oligonucleotides into nuclei of a rat pituitary cell line to suppress the synthesis of individual β-subunits selectively. Here we show that two out of four subtypes of β-subunits tested (β_1 and β_3) are selectively involved in the signal transduction cascades from muscarinic M_4 (ref. 4) and somatostatin receptors, respectively, to voltage-dependent Ca^{2+} channels.

We have established nuclear microinjection of short selective antisense oligonucleotides as a general method to study G protein function in intact cells[21]. The effects of 'knocking out' the expression of individual G-protein α-subunits are measured in a single cell either electrophysiologically or by immunofluorescent labelling of the targeted proteins. Here we apply this approach to elucidate the role of β-subunit subtypes in the differential coupling of G proteins to the same receptor/G protein/effector systems in GH_3 cells, a rat pituitary cell line.

Cells that have been injected with the antisense oligonucleotide β-com (see Fig. 1 legend), which targets messenger RNA of all known four β-polypeptides, contain reduced amounts of immunostainable β-subunits (data not shown). The disappearance of β-subunits parallels the functional loss of inhibitory hormonal effects on Ca^{2+} currents. Ca^{2+} currents in GH_3 cells are probably due to the activation of L-type channels and are inhibited to 70–80% of control currents by carbachol and somatostatin[21]. One day after injection of the oligonucleotide β-com, neither hormone reduced the Ca^{2+} current (Fig. 1); this effect lasted for one more day. The original hormone sensitivity of the Ca^{2+} channel was restored by ~60 h after injection.

To study the question of preferential interaction between the activated receptor and G_O proteins containing a given β-subtype, GH_3 cells were injected with antisense oligonucleotides that can specifically hybridize with the mRNA of one particular β-subtype (Figs 2 and 3). Neither β_2- nor β_4-specific antisense

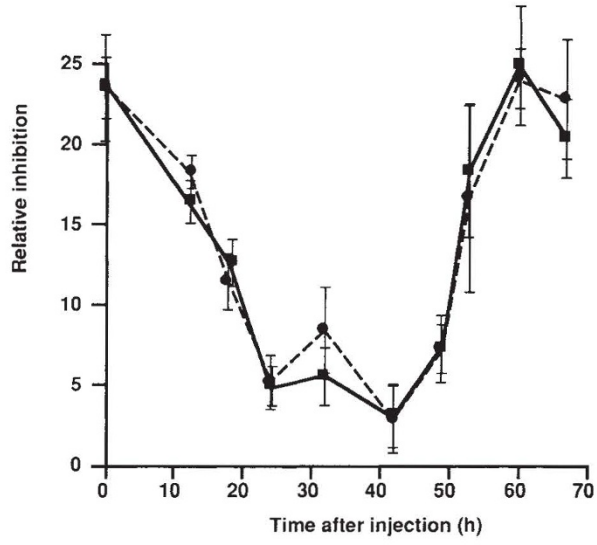

FIG. 1 Time course of Ca^{2+} current inhibition by somatostatin or carbachol in GH_3 cells. At time zero, cells were injected with antisense oligonucleotide β-com (see Methods). At the indicated time points, the Ca^{2+} current was measured in the presence of 1 μM somatostatin (dotted line) or 10 μM carbachol (solid line). Mean values with s.e.m. are shown ($n \geqq 5$).
METHODS. Microinjection (about 10,000 full-length oligonucleotides per nucleus) and electrophysiological measurements were done as described[21], but during current recording cells were perfused with a solution containing 120 mM choline-Cl, 10.8 mM $BaCl_2$, 1 mM $MgCl_2$, 5.4 mM CsCl, 10 mM glucose and 10 mM tetraethylammonium-HEPES (pH 7.4 at 37 °C). Sequence of injected β-com oligonucleotide: 5′-TTGCAGTTGAAGTCGTCRTA-3′, corresponding to nucleotides 825–844 of the identical strand of the β_1 gene sequence[5]. It can hybridize with the mRNAs of β_1, β_2, β_3 and β_4. Abbreviations for wobbled positions: R (G or A), M (A or C), Y (T or C), S (G or C). The oligonucleotide β-com is different from the previously used oligonucleotide anti-β[21]. Oligonucleotide β-com can hybridize perfectly with β_1, β_2, and β_3 mRNAs, and the longest continuously matching stretch in β_4 mRNA is 16 bases. The much-improved hybridization characteristics compared with oligonucleotide anti-β may explain why less β-com (<10,000 molecules, as compared with 50,000 for anti-β) is needed for effective repression of hormone responses.