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The prokaryotic enhancer binding protein NTRC has an ATPase activity which is phosphorylation and DNA dependent

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The prokaryotic activator protein NTRC binds to enhancer-like elements and activates transcription in response to nitrogen limitation by catalysing open complex formation by σ^{54} RNA polymerase holoenzyme. Formation of open complexes requires the phosphorylated form of NTRC and the reaction is ATP dependent. We find that NTRC has an ATPase activity which is activated by phosphorylation and is strongly stimulated by the presence of DNA containing specific NTRC binding sites.

Key words: ATP hydrolysis/DNA binding/protein phosphorylation/transcriptional activator

Introduction

The ability of some prokaryotic activators to stimulate transcription at a distance has led to the identification of enhancer-like elements in prokaryotes (Buck *et al.*, 1986; Reitzer and Magasanik, 1986; Herendeen *et al.*, 1989). In the most well-studied cases the enhancer binding proteins activate transcription at promoters recognized by a minor form of RNA polymerase containing an alternative sigma factor. One family of such activators stimulates transcription by the sigma 54 holoenzyme form of RNA polymerase ($E\sigma^{54}$) which has a unique promoter specificity (Kustu *et al.*, 1989). Activation of transcription by members of this family is face-of-the-helix dependent suggesting that the enhancer-bound activator may contact $E\sigma^{54}$ via the formation of a DNA loop (Buck *et al.*, 1987; Birkmann and Bock, 1989; Minchin *et al.*, 1989; Reitzer *et al.*, 1989). Activation of transcription *in vitro* can occur *in trans* provided that the enhancer is tethered near the promoter on a singly linked catenane (Wedel *et al.*, 1990). Moreover DNA loops formed between upstream-bound activator and promoter-bound $E\sigma^{54}$ have been visualized directly by electron microscopy (Su *et al.*, 1990).

Members of the family of σ^{54} dependent activator proteins each contain a highly conserved central domain (see Figure 1) which is essential for their positive control function (Morett *et al.*, 1988; Huala and Ausubel, 1989; Drummond *et al.*, 1990). This domain is predicted to interact with $E\sigma^{54}$ during transcriptional activation and contains a putative nucleotide binding site (Ronson *et al.*, 1987). A mutation in this nucleotide binding motif in the NTRC activator protein prevents isomerization of closed promoter complexes with $E\sigma^{54}$ to open promoter complexes (Drummond *et al.*, 1990; Austin *et al.*, 1991). Furthermore, ATP is necessary for NTRC to catalyse formation of open promoter complexes by $E\sigma^{54}$ *in vitro*. Since ATP cannot be substituted by non-hydrolysable analogues, ATP hydrolysis is most likely required for transcriptional activation (Popham *et al.*, 1989). The presence of the nucleotide binding motif in all known σ^{54} dependent activators suggests a common mechanism in which ATP hydrolysis functions to catalyse formation of open complexes by the σ^{54} modified form of RNA polymerase (Kustu *et al.*, 1989).

The activity of the NTRC is controlled by phosphorylation and dephosphorylation of an aspartate residue in its N-terminal domain (Figure 1) by the histidine protein kinase NTRB. A homologous domain is found in a family of bacterial response regulator proteins whose activity is controlled by phosphorylation (Stock *et al.*, 1989). In common with other members of this family, the aspartyl-phosphate linkage of NTRC-phosphate is very unstable and the protein exhibits autophosphatase activity under non-denaturing conditions (Keener and Kustu, 1988; Weiss and Magasanik, 1989). Only the phosphorylated form of NTRC is competent to activate transcription (Ninfa *et al.*, 1987). Phosphorylation also influences the DNA binding activity of NTRC, increasing its affinity for 'weak' binding sites which lack 2-fold rotational symmetry (Minchin *et al.*, 1988). Mutant forms of NTRC which have phosphorylation independent activity have been obtained in several laboratories (Popham *et al.*, 1989; Weglenski *et al.*, 1989; Dixon *et al.*, 1991). Some of these mutant proteins have single amino acid substitutions located close to the putative ATP binding site in the primary sequence, raising the possibility that phosphorylation activates ATP hydrolysis by NTRC.

We find that only the phosphorylated form of NTRC has significant ATPase activity and that this activity is strongly stimulated in the presence of DNA containing cognate binding sites. We infer that the association of phosphorylation dependent ATPase activity with site-specific DNA binding ensures that ATP hydrolysis by NTRC is primarily coupled to the formation of open complexes during transcription initiation.

Results

Unphosphorylated S160F NTRC has an intrinsic ATPase activity which is stimulated by DNA

We have shown previously that substitutions at serine residue 160 increase the activity of *Klebsiella pneumoniae* NTRC in the absence of NTRB (Dixon *et al.*, 1991). The Ser160→Phe mutant NTRC (S160F) can activate transcription in the absence of phosphorylation and this mutant protein has been used to demonstrate the ATP requirement for open complex formation in the absence of the kinase function of NTRB (Popham *et al.*, 1989). To characterize the potential ATPase activity of NTRC we chose initially to use the S160F mutant as this protein could be studied without the need to phosphorylate it first with NTRB.

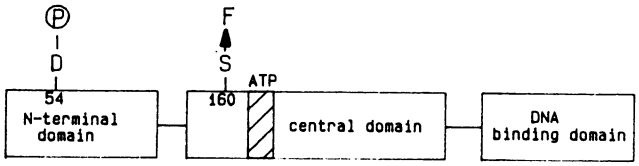

Fig. 1. Diagramatic representation of the domain structure of NTRC. D54 and the circled 'P' indicate the phosphorylation site on the N-terminal domain. The position of the S160F mutation in the central domain is also indicated.

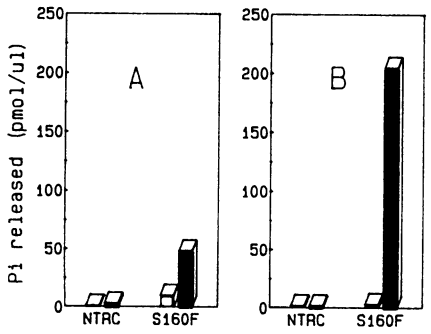

Fig. 2. Comparison of ATP hydrolysis by non-phosphorylated wild-type and S160F mutant proteins. (**A**) Reactions containing 1 μM wild-type or S160F protein were incubated in chloride buffer (see Materials and methods) at 25°C in the absence (open bars) or presence (filled bars) of pRD581 supercoiled plasmid DNA (final concentration 20 nM). Hydrolysis was initiated by addition of 3 mM [γ-^{32}P]ATP (73 c.p.m./pmol ATP) and incubation was continued for 1 h. 1 μl aliquots were removed for analysis of phosphate release by TLC as described in Materials and methods. (**B**) As for (A) but incubations were carried out in acetate buffer.

The amount of inorganic phosphate released from incubations containing [γ-^{32}P]ATP was measured under various conditions using highly purified protein from *K.pneumoniae*. Figure 2 compares the ATPase activity of unphosphorylated wild-type and the S160F mutant form of NTRC in two different buffers, one chloride based, the other acetate based. We examined the effect on ATPase activity of adding DNA by using supercoiled plasmid pRD581 which contains tandem NTRC binding sites located upstream of the *nifL* promoter (Minchin *et al.*, 1988). In both buffers there was a low level of ATP hydrolysis by wild-type NTRC and addition of DNA did not increase this level at all in acetate buffer and only slightly in chloride buffer. The S160F protein also had a low level of ATPase activity in the absence of DNA in both buffers but addition of DNA caused a 200-fold stimulation in acetate and a smaller stimulation (5-fold) in chloride buffer. The stimulation of activity by acetate-based buffer is of interest since we have previously shown that transcriptional activation by non-phosphorylated S160F protein is strongly stimulated in this buffer compared with chloride buffer (Dixon *et al.*, 1991). Therefore acetate buffer was used in all further experiments.

Figure 3A shows a time course of ATP hydrolysis by S160F NTRC which was linear for up to 1 h at 25°C in the presence and absence of pRD581 DNA. DNA dependent ATP hydrolysis was linear with time for up to 1 h with 1 μM protein at ATP concentrations above 0.5 mM. The apparent K_m for ATP was ~600 μM in the presence of 20 nM pRD581 DNA and apparent V_{max} was 5.2 pmol/min/pmol S160F dimer (at 1 μM protein). We could not measure an apparent K_m in the absence of DNA since

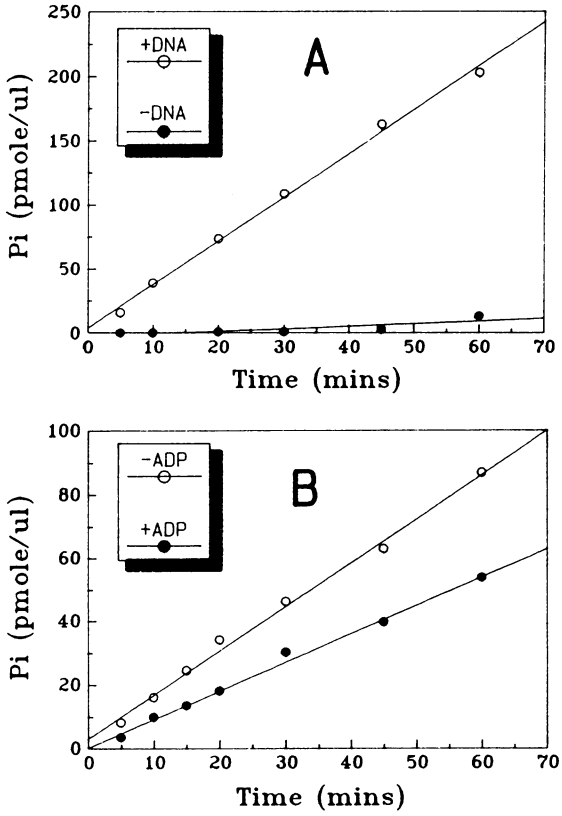

Fig. 3. Time course of ATP hydrolysis by S160F. (**A**) 1 μM S160F was preincubated in acetate buffer for 5 min at 25°C either in the absence (closed circles) or presence (open circles) of plasmid pRD581 DNA (final concentration 20 nM). Reactions (final vol 10 μl) were initiated by addition of 3 mM [γ-^{32}P]ATP (95 c.p.m./pmol ATP). 1 μl samples from each incubation were removed at 5, 10, 20, 30, 45 and 60 min and diluted with 9 μl 0.5% SDS, 2 mM EDTA. 2 μl aliquots were analysed for phosphate release as described in Materials and methods. (**B**) 1 μM S160F NTRC was incubated as above with 20 nM pRD581 DNA. ADP was added to one reaction to a final concentration of 100 μM (closed circles) whereas a second reaction contained no ADP addition (open circles). Reactions were initiated with 0.6 mM [γ-^{32}]ATP (476 c.p.m./pmol ATP). 1 μl samples were removed from each incubation at 5, 10, 15, 20, 30, 45 and 60 min and treated as in A.

under these conditions the ATPase activity of S160F showed a sigmoidal response to ATP concentration (data not shown).

Radioactively labelled ADP was released from [α-^{32}P]ATP as well as [^{32}P]phosphate from [γ-^{32}P]ATP. The released ADP was not hydrolysed further indicating that the S160F form of NTRC is not a phosphatase. We investigated whether ADP produced by the hydrolysis of ATP was itself inhibitory to the reaction. Using a substrate concentration of 0.6 mM ATP, addition of 0.1 mM ADP at the start of the reaction caused a reduction in the rate of ATP hydrolysis by 40% (Figure 3B). Addition of 0.1 mM ADP after 15 min of incubation also caused a decline in the rate of hydrolysis (data not shown).

Specificity of DNA dependent stimulation of ATP hydrolysis by S160F protein

We found that other supercoiled plasmid DNAs including those without NTRC binding sites could stimulate ATPase activity. The apparent K_m for pRD581 DNA was ~10 nM compared with ~30 nM for pTE595 DNA, an analogous plasmid lacking both NTRC binding sites. However, the

apparent V_{max} values were identical with both DNA substrates. Synthetic double-stranded DNA templates could also stimulate the ATPase activity. Addition of poly(dA)-(dT), poly d(A-T) and poly d(I-C) stimulated activity to 21, 46 and 80% respectively of the level shown with pRD581 DNA when equimolar concentrations of these cofactors were compared in terms of nucleotide equivalents (data not shown).

We hypothesized that the lack of specificity of DNA dependent ATPase activity was due to non-specific DNA binding by NTRC which occurs at high protein concentrations (Hawkes *et al.*, 1985). In order to overcome this and correlate ATPase activity with site-specific DNA binding we investigated the ability of short double-stranded oligonucleotides containing NTRC binding sites to stimulate hydrolysis (Figure 6A). We have shown previously that NTRC binds cooperatively to tandem binding sites (sites 1 and 2) upstream of the *nifL* promoter and that phosphorylation increases the occupancy of NTRC at these sites (Minchin *et al.*, 1988). Oligonucleotide A was a 51mer containing both sites 1 and 2. Oligonucleotide B was identical to A with the exception of a C→T substitution in site 1 corresponding to position −169 in the *nifL* promoter (Figure 6A). The analogous mutation reduces transcriptional activation from this promoter *in vivo* by 55% compared with the wild-type sequence and also decreases the affinity of NTRC for both sites 1 and 2 by 2-fold *in vitro* as judged by DNase I footprinting using a 360 bp DNA fragment. Oligonucleotide C was a 30mer containing site 2 only. By analogy, deletion of site 1 leaving site 2 intact reduces transciptional activation by 10-fold *in vivo* (Minchin *et al.*, 1988). Oligonucleotide A stimulated ATP hydrolysis most as would be predicted from our previous DNA binding studies (Figure 6D). The ATPase activity of the protein alone was only 0.2% of that seen in the presence of oligonucleotide A which corresponds to over 500-fold stimulation of activity (Figure 6D). This stimulation was reduced by 60% using oligonucleotide B with the C→T substitution in site 1 and by 98% with oligonucleotide C which contains site 2 only. The single-stranded forms of these oligonucleotides stimulated ATPase activity by <2-fold suggesting that single-stranded DNA is not an effective co-factor (data not shown). These results are therefore in accord with our previous DNA binding data and suggest that the ATPase activity of S160F is stimulated by site-specific DNA binding. Equilibrium experiments to establish relative binding constants for these three oligonucleotides are discussed later.

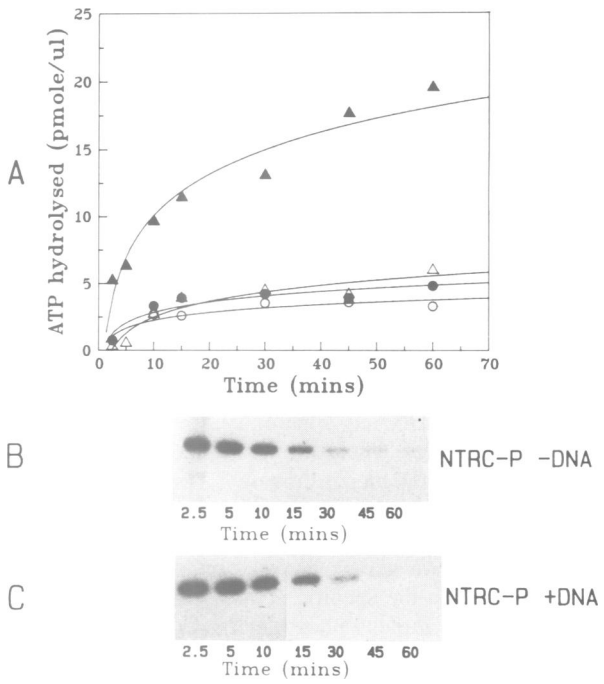

Fig. 4. Time course of ATP hydrolysis by wild-type NTRC. (**A**) Wild-type NTRC was phosphorylated as described in Materials and methods. Reactions containing 1 μM NTRC (0.07 mol phosphate/mol NTRC dimers) were incubated in acetate buffer at 25°C in the absence (open triangles) or presence (closed triangles) of pRD581 DNA (final concentration, 20 nM). Parallel reactions containing 1 μM non-phosphorylated NTRC were incubated under the same conditions either in the absence (open circles) or presence (closed circles) of DNA. Reactions were initiated by addition of 0.3 mM [α-^{32}P]ATP (163 c.p.m./pmol ATP). 2 μl aliquots were withdrawn at 2.5, 5, 10, 15, 30, 45 and 60 min and diluted with 5 μl 0.5% SDS, 2 mM EDTA. 2 μl aliquots of the diluted samples were analysed for ADP release as described in Materials and methods. (**B**) and (**C**) The remaining 5 μl samples from each time point were subjected to SDS−PAGE and autoradiography of the dried gel. The amount of NTRC-phosphate remaining at each time point in the absence of DNA (B) or presence of DNA (C) was estimated by densitometric scanning of the autoradiograph.

ATPase activity of wild-type NTRC is phosphorylation dependent and stimulated by site-specific binding to DNA

Experiments to examine the effect of phosphorylation on ATP hydrolysis are complicated by the necessity to remove NTRB and ATP after the phosphorylation reaction and by the instability of the aspartyl-phosphate linkage on NTRC, which has a half life of ~3 min at 37°C (Keener and Kustu, 1989). Although it is possible to generate NTRC-phosphate in the reaction mixture by providing NTRB, this approach makes it difficult to distinguish phosphate released by the autophosphatase activity of NTRC from that produced as a consequence of ATP hydrolysis. We developed a procedure involving phosphorylation of NTRC by NTRB using [γ-^{32}P]ATP followed by removal of NTRB and unhydrolysed ATP by chromatography on heparin agarose (see Materials and methods). The phosphorylated protein was then concentrated and desalted by dialysis into acetate buffer containing 50% glycerol and stored at 4°C. Since the autophosphatase activity of NTRC-phosphate (Weiss and Magasanik, 1989) and the related response regulator protein, phospho-CheY (Lukat *et al.*, 1990) is dependent on divalent cations such as Mg^{2+}, EDTA was present at all stages subsequent to the phosphorylation reaction. Under these conditions the protein dephosphorylated slowly with an estimated $t_{½}$ of ~5 days. The extent of phosphorylation varied from 0.05−0.1 mol phosphate per mol of NTRC dimers or 2.5−5% dimers with both sites phosphorylated. (It is not yet known whether a single site or both sites are phosphorylated in the dimer.) In order to estimate ATPase activity, the phosphorylated protein was rapidly diluted into reaction buffer (which by necessity contains Mg^{2+}) and immediately incubated with [α-^{32}P]ATP at 25°C. ATP hydrolysis was quantitated by determining the level of ^{32}P-labelled ADP released. This strategy therefore distinguishes ATPase activity from the release of ^{32}P-labelled phosphate by the autophosphatase activity of the phosphorylated protein. Exchange of released phosphate into either ADP

or ATP was not detectable under the conditions of the assay (data not shown). Figure 4A shows a time course of ATP hydrolysis by NTRC-phosphate and non-phosphorylated NTRC at 25°C. The addition of plasmid DNA containing the *nifL* NTRC sites caused a stimulation of ATP hydrolysis by NTRC-phosphate. In the absence of DNA there was only a small increase in ATP hydrolysis by NTRC-phosphate compared with the non-phosphorylated protein. The rate of hydrolysis gradually declined during the time course as the protein dephosphorylated. Quantitation of the amount of phosphorylated protein remaining at each time point gave a first-order rate constant for dephosphorylation of 0.067 min^{-1} ($t_{½}$ = 10.3 min) at 25°C in the presence or absence of DNA. The rate of ATP hydrolysis by NTRC-phosphate with or without DNA is apparently linear for the first 30 min of the reaction when the first-order decay constant is taken into account (data not shown). To provide a very crude estimate of the level of stimulation of ATP hydrolysis by phosphorylation we compared the specific activity of the non-phosphorylated protein in terms of moles NTRC dimer in comparison with the specific activity of NTRC-phosphate calculated as moles phosphate incorporated. These calculations indicate a ~25-fold stimulation by phosphorylation and ~250-fold stimulation when the phosphorylated protein was incubated in the presence of pRD581 DNA (Table I).

To show that the ATPase activity of NTRC-phosphate was a consequence of phosphorylation we preincubated the protein for varying lengths of time at 37°C in the presence of Mg^{2+} to decrease its phosphorylation state. At each time point an aliquot of the incubation was removed to measure the amount of phosphate remaining in the protein and to assay its ability to hydrolyse ATP at 25°C. Figure 5A shows the decline of ATP hydrolysis with preincubation time. During the course of the preincubation there was a 40-fold decrease in ATP hydrolysis when DNA was present. The first-order rate constant for dephosphorylation at 37°C was 0.178 min^{-1} ($t_{½}$ = 3.9 min) and the presence of DNA did not significantly influence the autophosphatase activity of NTRC-phosphate (Figure 5B). The relationship between concentration of NTRC-phosphate and ATP hydrolysis was not linear with or without DNA (Figure 5C). The presence of DNA gave rise to a non-linear increase in ATP hydrolysis with increasing NTRC-phosphate concentration suggesting that DNA stimulates hydrolysis by promoting cooperative interactions between NTRC molecules.

We examined the ability of the NTRC binding site oligonucleotides to stimulate ATP hydrolysis by wild-type NTRC (Figure 6). The non-phosphorylated protein showed little activity with any of the oligonucleotides (Figure 6B). Oligonucleotide A containing both sites 1 and 2 stimulated ATPase activity of the phosphorylated protein nearly 9-fold compared with the level with the protein alone (this corresponds to a stimulation of ~200-fold when the concentration of NTRC-phosphate is taken into account (Table I). This was reduced by 71% with oligonucleotide B containing the C→T mutation in site 1 and by 85% with oligonucleotide C with only site 2 present (Figure 6C). These results are similar to those obtained with the S160F form of NTRC and provide good evidence for the stimulation of ATPase activity by site-specific DNA binding.

Phosphorylation of S160F stimulates ATPase activity in the presence and absence of DNA

We prepared purified S160F-phosphate using the procedure for the wild-type protein. The protein showed the same stability characteristics as wild-type NTRC in the absence of Mg^{2+} in acetate buffer containing 50% glycerol. In these experiments the protein contained 0.15–0.2 mol phosphate per mol S160F dimers or 7.5–10% of possible sites phosphorylated.

Figure 7 shows a time course of ATP hydrolysis by S160F-phosphate in the presence and absence of DNA. With the plasmid pRD581 and oligonucleotide A and B as substrates, an initial rapid increase in hydrolysis was observed which declined to a slower rate as the protein dephosphorylated. Surprisingly, in contrast to results obtained with non-phosphorylated S160F (Figure 6D), the rate of ATP hydrolysis was almost identical in the presence of either oligonucleotide A or oligonucleotide B. An identical rate was also observed in the presence of pRD581 whereas a lower rate was observed with oligonucleotide C. In the absence of DNA the activity reached a plateau value reflecting a decline in the concentration of S160F-phosphate below the level required to stimulate ATP hydrolysis by the protein alone. The ATPase activity of the non-phosphorylated and phosphorylated S160F proteins in response to the three oligonucleotides is compared in

Table I. Comparison of the ATPase activities of S160F and wild-type NTRC

	Protein			
DNA co-factor[a]	NTRC[b]	NTRC-phosphate[c]	S160F[b]	S160F-phosphate[c]
None	0.1	2.5	<0.1	23.2
pRD581	0.1	25.7	3.4	121.4
Oligonucleotide A	0.2	32.8	6.4	117.8
Oligonucleotide B	0.1	9.4	2.6	110.7
Oligonucleotide C	0.1	4.9	<0.1	47.0

Since the ATPase activity is not linear with protein concentration, particularly with the phosphorylated proteins, these values represent a considerable underestimate. The first order rate constant for dephosphorylation has also not been taken into account.

[a] DNA was added to a final concentration of 40 nM for pRD581 and 500 nM for oligonucleotides.

[b] For non-phosphorylated proteins, data are expressed as pmol ATP hydrolysed/min/pmol NTRC dimers. (The final protein concentration in all reactions was 1 μM.)

[c] For phosphorylated proteins data are expressed as pmol ATP hydrolysed/min/pmol NTRC-phosphate or S160F-phosphate. (The total protein concentration in these reactions was either 1 μM NTRC or 0.5 μM S160F.)

Figure 6D and E. Clearly phosphorylation increases ATP hydrolysis by S160F in the presence and absence of DNA and in particular, unlike wild-type NTRC, activity on oligonucleotide B is similar to the level observed with oligonucleotide A. The influence of these oligonucleotides on the ATPase activity of S160F-phosphate correlates with its DNA binding activity (see below).

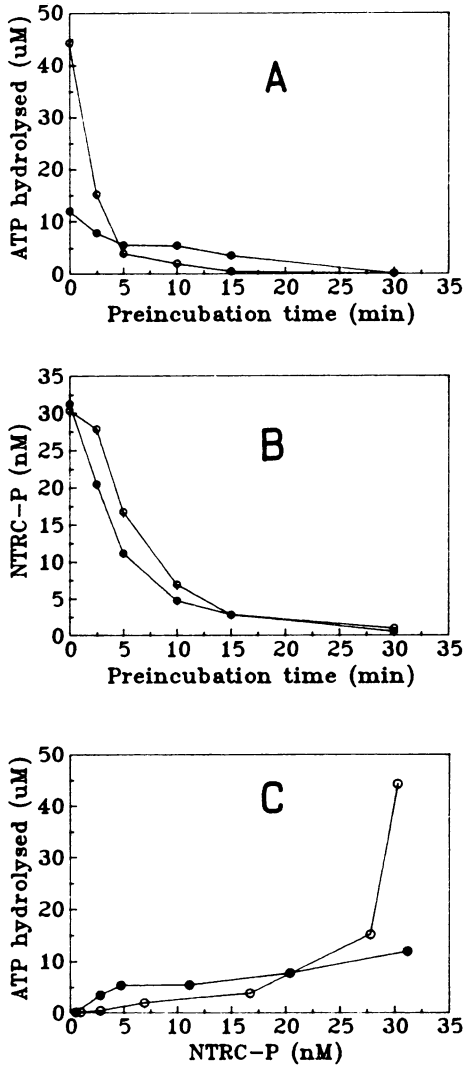

Fig. 5. Dependence of ATP hydrolysis on phosphorylation of wild-type NTRC. (**A**) Wild-type NTRC was phosphorylated as described in Materials and methods. 20 μl reactions containing 1 μM NTRC dimers (0.03 mol phosphate/mol NTRC dimers) were preincubated in acetate buffer in the absence (closed circles) or presence (open circles) of pRD581 DNA (40 nM) at 37°C for 0, 2.5, 5, 10, 15 and 30 min. After preincubation the reactions were chilled on ice for 30 s and then ATP hydrolysis was initiated by addition of 1 mM [α-^{32}P]ATP (220 c.p.m./pmol ATP). Incubations were at 25°C for 1 h. 1 μl aliquots were analysed for ADP release as described in Materials and methods. (**B**) At each preincubation time point, 10 μl samples were removed from the reactions before addition of ATP to measure the amount of phosphorylated NTRC remaining after preincubation. The samples were analysed on SDS–polyacrylamide gels and the radioactivity in NTRC determined by counting the stained band corresponding to NTRC in the excised gel slice as described in Materials and methods. Closed circles indicate reactions lacking DNA and open circles indicate those containing DNA. (**C**) The data in A and B are re-plotted to show the dependence of ATP hydrolysis on concentration of NTRC-phosphate. Symbols are the same as in A and B.

Figure 8A shows the effect of dephosphorylation of S160F-phosphate on its ATPase activity following preincubation at 37°C in the presence of Mg^{2+}. Without preincubation ATP hydrolysis was stimulated by phosphorylation both in the absence and presence of pRD581 DNA (see also Table I). Little activity was detectable after 30 min preincubation in the absence of DNA since the ATPase activity of non-phosphorylated S160F is DNA dependent (Figure 2). In a control experiment preincubation of unphosphorylated S160F at 37°C in these conditions had no effect on the DNA dependent stimulation of ATPase activity when subsequently measured at 25°C (data not shown). Thus preincubation at 37°C does not itself inactivate the ATPase and the decline in activity in the preincubated phosphorylated protein must therefore be due to dephosphorylation. As with the wild-type NTRC, the presence of DNA had little influence on the autophosphatase activity of S160F-phosphate at 37°C (Figure 8B), the $t_{½}$ was 3 min in the absence of DNA and 3.7 min when DNA was present.

The relationship between ATP hydrolysis and S160F-phosphate concentration is shown in Figure 8C. In the absence of DNA, activity increased slowly with increasing S160F-phosphate concentrations whereas in the presence of plasmid pRD581 a biphasic curve was observed with an initial sharp increase, followed by a response similar to that observed without DNA at S160F-phosphate concentrations above 50 nM. It is possible that the DNA binding sites are saturated above this level of phosphorylated protein. The activity of S160F-phosphate was significantly greater than that of NTRC-phosphate (compare Figures 5 and 8). However, insufficient phosphate was incorporated into wild-type NTRC to compare activities above 30 nM phosphorylated protein. The unusual and complex relationship between ATPase activity and concentration of phosphorylated protein is discussed later.

DNA binding studies

The pattern of stimulation of ATP hydrolysis by specific oligonucleotides (Figure 6) suggests that DNA increases the ATPase activity of NTRC as a consequence of site-specific binding. In order to determine relative binding constants for the three oligonucleotides we set up equilibrium reactions in which NTRC, S160F and their phosphorylated derivatives were incubated with oligonucleotide mixtures using the approach of Liu-Johnson *et al.* (1986). Bound complexes were separated from free oligonucleotides by gel retardation and the resolved bands were excised from the gel, purified and analysed on denaturing sequencing gels. As shown in Figure 9A, non-phosphorylated NTRC bound relatively poorly to oligonucleotide C (30mer) and there were relatively more counts in the bound fractions containing oligonucleotide A than those containing oligonucleotide B, indicating a higher affinity for A compared with B (compare lanes 1 and 2 with lanes 3 and 4 in Figure 9A). Quantitation of the bound and unbound species by densitometry allowed us to determine relative binding constants for these oligonucleotides. Both phosphorylated and non-phosphorylated NTRC showed ~50-fold higher affinity for oligonucleotide A than oligonucleotide C and ~13-fold greater affinity for oligonucleotide B compared with oligonucleotide C (K_{51}/K_{30} values, Table II). Although these ratios were

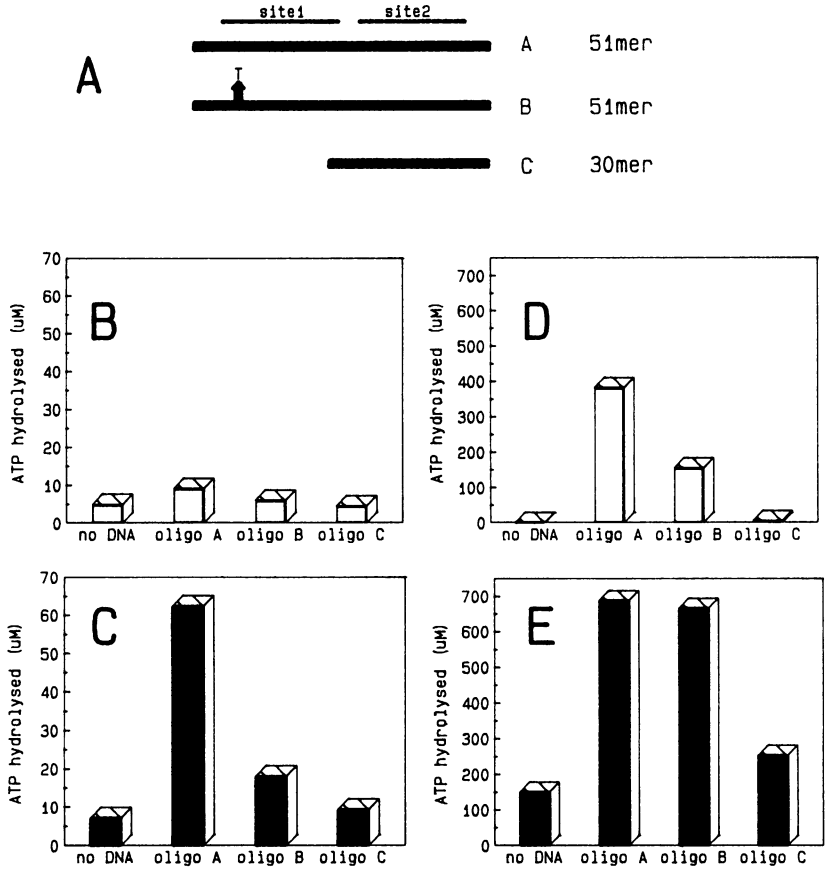

Fig. 6. Stimulation of ATP hydrolysis by NTRC binding site oligonucleotides. (**A**) Diagram of double-stranded oligonucleotides containing NTRC binding sites 1 and 2 from the *K.pneumoniae nifL* promoter. Oligonucleotide A is a 51mer with both sites. Oligonucleotide B is similar to A but has a C→T substitution equivalent to position −169 in site 2 (marked with an arrow). Oligonucleotide C is a 30mer with site 2 only. (**B**) Reactions containing 1 μM non-phosphorylated wild-type NTRC were incubated in acetate buffer at 25°C for 1 h. Oligonucleotides were added to give a final concentration of 500 nM where indicated. Hydrolysis was initiated by addition of 1 mM [α-^{32}P]ATP (220 c.p.m./pmol ATP). 1 μl aliquots were analysed for ADP release as described in Materials and methods. (**C**) Wild-type NTRC was phosphorylated as described in Materials and methods. Reactions containing 1 μM NTRC (0.03 pmol phosphate/pmol NTRC dimers) were incubated and analysed for ADP release as in (B). (**D**) 1 μM non-phosphorylated S160F protein was incubated under the same conditions as in (B) except that 3 mM [γ-^{32}P]ATP (73 c.p.m./pmol) was added to initiate ATP hydrolysis. (**E**) S160F protein was phosphorylated as in Materials and methods. Reactions containing 0.4 μM S160F (0.175 mol phosphate/mol S160F dimers) were incubated as in (D).

similar for both NTRC and NTRC-phosphate, phosphorylation of the protein apparently increased the individual binding constants for all three oligonucleotides (Table II). This observation is in agreement with previous results using DNase I footprinting which showed that phosphorylation increases the affinity of NTRC for sites 1 and 2 (Minchin *et al.*, 1988). The relatively lower affinity for oligonucleotide B, which contains the C to T mutation in site 1, compared with oligonucleotide A (both sites wild-type) is also in accord with our previous observations.

With non-phosphorylated S160F protein, relative binding constants were similar to those observed with NTRC-phosphate; a 50-fold greater affinity was found for oligonucleotide A compared with C and a 16-fold greater affinity for B compared with C (Figure 9B and Table II). However, with S160F-phosphate the relative binding constant for oligonucleotide B (39-fold higher than oligonucleotide C, Table II) approached that of oligonucleotide A (47-fold). This result directly parallels the observed stimulation of ATPase activity of S160F-phosphate by oligonucleotides A and B (Figure 6D) and suggests that phosphorylation of S160F protein increases its affinity for the mutant NTRC binding site.

The results of the equilibrium competition experiments suggest a close relationship between relative DNA binding affinity and ATP hydrolysis. It seems possible that phosphorylation could influence ATP hydrolysis as a consequence of its affect on DNA binding, although it is obvious, particularly with S160F protein, that phosphorylation also influences activity in the absence of DNA.

Discussion

We have shown that wild-type NTRC possesses an ATPase activity which is phosphorylation dependent and strongly stimulated by site-specific binding to DNA. Since ATP hydrolysis is required for isomerization of closed complexes between Eσ^{54} and promoters to open complexes (Popham *et al.*, 1989) it seems likely that the ATPase activity of NTRC performs an important catalytic function during open complex formation. Only phosphorylated NTRC is able to activate transcription (Ninfa and Magasanik, 1986) and only this form of the protein has significant ATPase activity. Phosphorylation therefore influences either ATP binding or the rate of ATP hydrolysis by NTRC. In accordance with

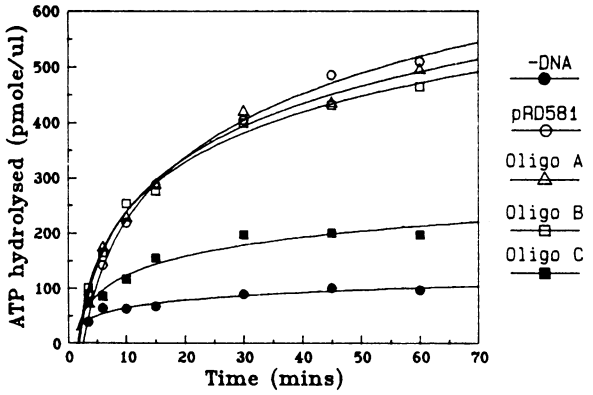

Fig. 7. Time course of ATP hydrolysis by phosphorylated S160F. S160F NTRC was phosphorylated as described in Materials and methods. Reactions containing 0.4 μM S160F (0.175 pmol phosphate/pmol S160F dimers) were incubated in acetate buffer at 25°C. pRD581 DNA was added to a final concentration of 40 nM (open circles) and oligonucleotides, A (open triangles), B (open squares) and C (filled squares) were added to a final concentration of 500 nM. Reactions without DNA addition are shown as filled circles. ATP hydrolysis was initiated by addition of 3 mM [α-^{32}P]ATP (50 c.p.m./pmol ATP). 1 μl samples were withdrawn at 2.5, 5, 10, 15, 30, 45 and 60 min and diluted with 9 μl 0.5% SDS, 2 mM EDTA. 1 μl aliquots of the diluted samples were analysed for ADP release as described in Materials and methods and the remaining 9 μl samples from each time point were subjected to SDS–polyacrylamide electrophoresis and autoradiography of the dried gel as described in Figure 4B and C.

this conclusion, the S160F mutant form of NTRC, which is capable of activating transcription in the absence of phosphorylation (Popham *et al.*, 1989; Dixon *et al.*, 1991) gives significant ATP hydrolysis in its non-phosphorylated form. Like wild-type NTRC the ATPase activity of S160F is stimulated by the presence of DNA.

While this work was in progress Weiss *et al.* (1991) reported the initial observation that the ATPase activity of NTRC is dependent upon phosphorylation. Although their experiments are complicated by the presence of the phosphotransferase NTRB in the reaction mixture, our results confirm their conclusion that phosphorylation is required to activate ATP hydrolysis. However, we have also shown that the ATPase activity is stimulated by site-specific binding to DNA, giving rise to significantly increased rates of ATP hydrolysis. In the presence of DNA containing NTRC binding sites, the activity curve for ATP hydrolysis showed an unusual relationship to the NTRC-phosphate concentration (Figure 5C), exhibiting a non-linear increase with increasing levels of phosphorylated protein. We interpret this response as a cooperative influence of DNA on ATP hydrolysis, arising from the binding of NTRC-phosphate molecules to multiple binding sites. The ultrasensitive response to the increase in NTRC-phosphate concentration suggests that oligomerization of NTRC enhances ATPase activity. Recall that the DNA template contains tandem NTRC binding sites and that the interaction between phosphorylated dimers at these sites is apparently cooperative (Minchin *et al.*, 1988; Contreras and Drummond, 1988). The titration data with the wild-type protein therefore suggest a model in which multiple binding sites on DNA stimulate ATP hydrolysis as a consequence of cooperative interactions between bound dimers of NTRC-phosphate. The titration curve obtained with the phosphorylated S160F mutant (Figure 8C) does not show the same response as wild-type NTRC-phosphate but

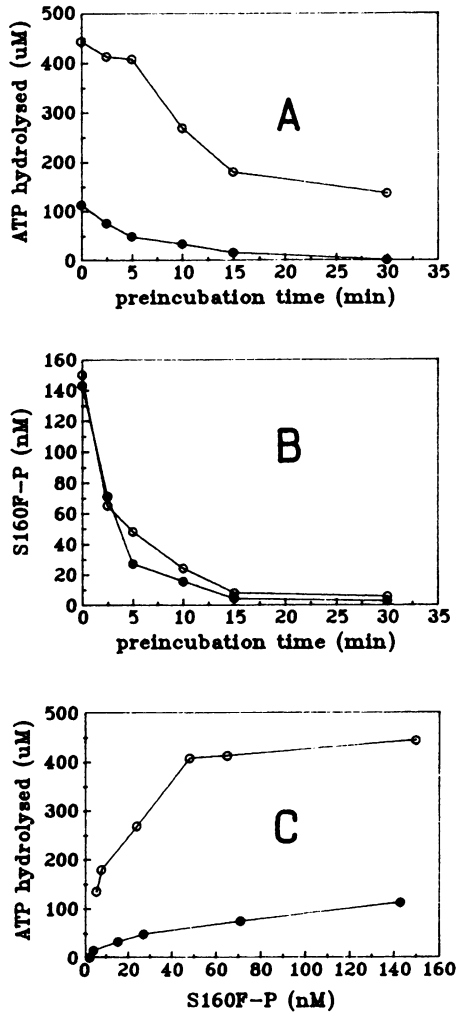

Fig. 8. Influence of phosphorylation on the ATPase activity of S160F. (**A**) S160F was phosphorylated as described in Materials and methods. 20 μl reactions containing 0.7 μM S160F (0.2 mol phosphate/mol S160F dimers) were preincubated in acetate buffer at 37°C for 0, 2.5, 5, 10, 15 and 30 min in the absence (filled circles) or presence (open circles) of pRD581 DNA at a final concentration of 40 nM. After preincubation the reactions were chilled on ice for 30 s and then ATP hydrolysis was initiated by addition of 3 mM [α-^{32}P]ATP (73 c.p.m./pmol ATP). Incubations were at 25°C for 1 h. 1 μl aliquots were analysed for ADP release as described in Materials and methods. (**B**) Decay of phosphate from S160F-P during preincubation is analysed as in Figure 5B. Symbols are the same as in (A) above. (**C**) Dependence of ATP hydrolysis on concentrations of S160F-phosphate as determined from the data in (A) and (B).

in this case the data are more difficult to interpret: first, the non-phosphorylated form of this protein has ATPase activity in the presence of DNA, which may relate to its higher affinity for binding sites compared with non-phosphorylated wild-type protein (Dixon *et al.*, 1991). Secondly, phosphorylated S160F gives significant levels of ATP hydrolysis in the absence of DNA which could reflect an increased propensity to oligomerize. Indeed, Weiss *et al.* (1991) have suggested that S160F has a greater tendency to aggregate than NTRC and that phosphorylation increases the aggregation state of both proteins, consequently influencing ATP hydrolysis. According to our model, multiple DNA binding sites permit the ordered assembly of NTRC-phosphate oligomers on DNA, thus facilitating cooperative stimulation of ATP hydrolysis. The presence of

Table II. Relative binding constants for oligonucleotides

Protein	Oligonucleotide mixture	K_{51}[a]	K_{30}[b]	K_{51}/KZ_{30}[c]
NTRC	A + C	1.69	0.03	56
	B + C	0.41	0.03	14
NTRC-phosphate	A + C	7.60	0.13	58
	B + C	1.18	0.10	12
S160F	A + C	4.80	0.09	53
	B + C	2.22	0.14	16
S160F-phosphate	A + C	8.90	0.19	47
	B + C	3.90	0.10	39

Data were obtained by scanning several different exposures of the gels represented in Figure 9.
[a] Binding constant for individual 51mers (A or B) defined as the band intensity of DNA in the complex divided by the band intensity of DNA in the free fraction.
[b] Binding constant for the 30mer (oligonucleotide C) defined as in footnote a.
[c] Relative binding constants calculated using the equation $K_{51}/K_{30} = (C_{51}/D_{51})/(C_{30}/D_{30})$ where C represents the amount of DNA in the complex and D the amount of DNA in the free DNA fractions. The subscript 51 designates either oligonucleotide A or B whereas the subscript 30 designates oligonucleotide C.

multiple activator binding sites is a common feature of NTRC-activatable promoters and indeed σ^{54} dependent promoters in general (Collado-Vides *et al.*, 1991). For example, the *glnAp2* promoter contains five NTRC binding sites in the upstream regulatory region, three of which are occupied *in vivo* (Sasse-Dwight and Gralla, 1988) whereas at the *glnHp2* promoter, NTRC binds primarily to two overlapping sites (Clavarie-Martin and Magasanik, 1990). Other examples of multiple activation sites in σ^{54} dependent promoters occur in *K.pneumoniae nifU* and *nifE* (Cannon *et al.*, 1990, 1991), *Azospirillum brasilense nifH* (de Zamonoczy *et al.*, 1989; Fani *et al.*, 1989), *Bradyrhizobium japonicum nifH* and *nifD* (Alvares-Morales *et al.*, 1986) and *Rhizobium meliloti* and *Rhizobium leguminosarum dctA* (Ledebur *et al.*, 1991).

Activation of transcription at σ^{54} dependent promoters can occur in the absence of specific DNA binding sites, provided that the activator is present at high concentration. In this case the activator could either contact Eσ^{54} from solution or bind non-specifically to DNA. Mutagenesis of either the C-terminal helix-turn-helix motif of NTRC required for DNA recognition (Contreras and Drummond, 1988) or removal of the entire C-terminal DNA binding domain inactivates NTRC (Drummond *et al.*, 1990), suggesting that DNA binding is essential for the activation process. A further interpretation of this result is that in the absence of the DNA binding domain, NTRC has insufficient ATPase activity to activate transcription. This may not be the case with other σ^{54} dependent activators such as NIFA, which can still function as an activator when the DNA binding domain is removed (Morett *et al.*, 1988; Huala and Ausubel, 1989). The potential ATPase activity of NIFA may therefore not be so tightly coupled to DNA binding as in the case of NTRC. It also seems likely that the ATPase activities of only those members of the family which share a homologous N-terminal domain in common with NTRC are regulated by phosphorylation.

The dependence of ATP hydrolysis on both phosphorylation and DNA binding presumably has important physiological consequences, ensuring that the catalytic activity of NTRC only functions when the activator is correctly positioned to make contact with σ^{54} RNA polymerase. Activation of ATP hydrolysis is potentiated with small increases in phosphorylation state and provides efficient coupling to the signal transduction pathway. If ATP hydrolysis by NTRC is primarily confined to the process of open complex formation one might also expect that Eσ^{54} would act as an effector of ATP hydrolysis in addition to DNA. However, preliminary experiments with the S160F protein indicate that ATPase activity in the presence of promoter DNA is stimulated only 2- to 3-fold by addition of σ^{54} holoenzyme (unpublished results). We cannot rule

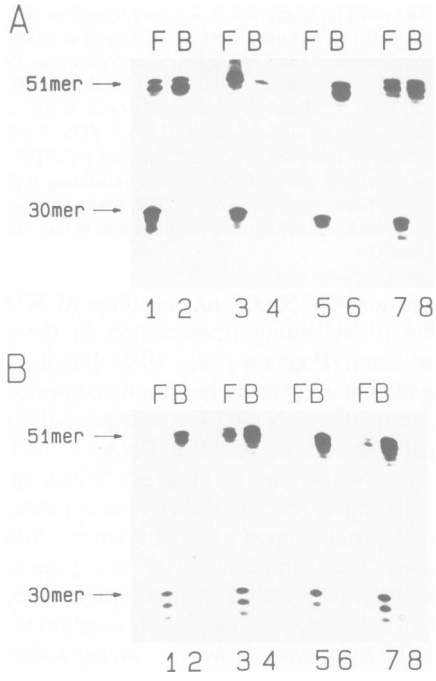

Fig. 9. Equilibrium binding of NTRC and S160F to double-stranded oligonucleotides. The oligonucleotides shown schematically in Figure 6A were mixed together in a single binding reaction: reactions analysed in lanes 1, 2, 5 and 6 contained an equimolar mixture of oligonucleotides A and C whereas reactions in lanes 3, 4, 7 and 8 contained a mixture of oligonucleotides B and C. Samples were run on native polyacrylamide gels and free DNA (lanes marked 'F') and bound DNA (lanes marked 'B') fractions were excised, purified and analysed on a denaturing polyacrylamide gel. The figure shows an autoradiogram of the denaturing gel obtained by direct autoradiography. Longer exposure of the gels revealed bands corresponding to the 30mer (oligonucleotide C) in the bound fraction. (**A**) Reactions contained either NTRC (lanes 1–4) or NTRC-phosphate (lanes 5–8). (**B**) Reactions contained either S160F (lanes 1–4) or S160F-phosphate (lanes 5–8).

out the possibility that our experimental conditions are inappropriate to detect a significant level of stimulation by σ^{54} RNA polymerase. Alternatively, σ^{54} holoenzyme may not be an effector of ATPase activity and for example ATP hydrolysis may be required to promote a conformational change in the activator necessary for its interaction with σ^{54} or RNA polymerase.

Finally, our results show that NTRC is an example of a large number of proteins whose ATPase activity is DNA dependent. We note apparent similarities with the three phage T4-encoded DNA polymerase accessory proteins which comprise an enhancer binding complex and exhibit a DNA dependent ATPase activity required for the stimulation of open complex formation at T4 late promoters (Herendeen *et al.*, 1989). However, in this case the moving replication fork acts as a mobile enhancer rather than a specific binding site on DNA. A requirement for ATP at steps prior to transcription initiation is a more common phenomenon in eukaryotes. For example a factor required for transcription of vaccinia virus early genes has DNA dependent ATPase activity (Broyles and Moss, 1988). ATP is also required as a cofactor for transcription initiation by RNA polymerase II. Interestingly one of the proteins associated with the general transcription factor TFIIF (also known as RAP 30/74 or β) has a DNA helicase function which is predicted to melt DNA at the transcription start-site (Sopta *et al.*, 1989). Another accessory transcription factor has an ATPase activity which is strongly stimulated by short DNA fragments containing the TATA region of promoters (Conaway and Conaway, 1989). As in the case of the bacterial enhancer-like systems, ATP hydrolysis is not required for formation of the closed RNA polymerase II transcription complex but is specifically utilized for formation of open complexes, thus revealing mechanistic similarities in transcription initiation which cross the prokaryotic–eukaryotic boundary (Wang *et al.*, 1992).

Materials and methods

Purification of proteins

Klebsiella pneumoniae wild-type NTRC and the mutant S160F derivatives of NTRC were purified from overproducing strains as described previously (Austin *et al.*, 1987; Dixon *et al.*, 1991) with minor modifications. Crude cell extracts containing NTRC were subjected to ammonium sulfate precipitation (0–50%) followed by chromatography on heparin sepharose (Pharmacia) or heparin agarose (BRL). NTRC was eluted with a linear salt gradient of 0.05–0.75 M NaCl in either case. Further purification was achieved by fast protein liquid chromatography on MONO Q (Pharmacia) in TGED buffer (Burgess and Jendrisak, 1975). The column was developed with a gradient of 0–1 M NaCl and NTRC eluted at 0.2 M NaCl. It was necessary to remove a small number of contaminating proteins remaining after this step which might include those with ATPase activity. To achieve this, NTRC was further purified either by re-chromatography on MONO Q or by chromatography on Superose 12 (Pharmacia) where NTRC eluted as a dimer with a molecular weight of 112 000. To eliminate the possibility of trace contamination by DNA dependent ATPases, a small amount of the Superose 12 purified NTRC was further purified on single-stranded DNA agarose. This material showed the same specificity of ATP hydrolysis on plasmid DNA and oligonucleotides as NTRC which had not been chromatographed on DNA agarose. The most highly purified NTRC and S160F preparations were estimated to be at least 99% pure and showed no other contaminating bands as judged by inspection of stained bands on an overloaded SDS–polyacrylamide gel.

NTRB was purified as described previously (Austin *et al.*, 1987). No DNA dependent ATPase activity was detectable in this preparation.

Protein concentrations were determined both by measurement of absorbance at 280 nM, using $A_{1\%}$ = 9.1 for NTRC (Weiss and Magasanik, 1988) and by using a commercial protein assay employing Coomassie Brilliant Blue G-250 as reagent (Pierce). For comparative purposes, protein concentrations were also estimated by densitometric scanning of stained bands on SDS–polyacrylamide gels.

Phosphorylation of NTRC and purification of NTRC-phosphate

Wild-type NTRC was phosphorylated in a buffer containing 65 mM Tris–HCl pH 7.9, 70 mM sodium chloride, 5 mM magnesium chloride and 0.5 mM EDTA. NTRC and NTRB were added to a final concentration of 9 μM and 1.25 μM respectively. Phosphorylation was performed at 37°C in a final vol of 250 μl. The reaction was initiated by addition of 0.4 mM [γ-^{32}P]ATP at 1 Ci/mmol. S160F protein was phosphorylated under the same conditions except that S160F and NTRB were added to give final concentrations of 4.5 μM and 0.625 μM respectively in a reaction vol of 500 μl.

After 20 min incubation the reactions were chilled on ice and immediately diluted with 3 vol of a buffer containing 10 mM Tris–HCl pH 7.9, 5% glycerol, 50 mM sodium chloride, 1 mM EDTA and 0.1 mM DTT. The diluted reaction mix was then applied to a heparin agarose column (1 ml bed volume) equilibrated in the same buffer. The column was washed with 20 ml of the same buffer to remove NTRB and unincorporated ATP. The phosphorylated NTRC was eluted with three 1 ml aliquots of 10 mM Tris–Cl pH 7.9, 1 mM EDTA and 1 M sodium chloride. The eluted protein was dialysed against 1 l of 35 mM Tris-acetate pH 7.9, 70 mM potassium acetate, 0.1 mM EDTA, 0.1 mM DTT and 50% glycerol for 5 h at 4°C and the phosphorylated protein was stored at this temperature. The concentration of NTRC-phosphate was determined by SDS–PAGE of 10 μl samples of the phosphorylated protein and excision of the stained band corresponding to NTRC from the gel. The radioactivity in the gel slices was then determined by counting in Cocktail T scintillation fluid (BDH).

ATPase assay

ATPase assays were performed in an acetate buffer containing 35 mM Tris-acetate pH 7.9, 70 mM potassium acetate, 5 mM magnesium acetate, 19 mM ammonium acetate, 0.7 mM DTT with the exception of Figure 1 where a chloride-based buffer was used. This contained 25 mM Tris–HCl pH 7.8, 5 mM magnesium chloride, 50 mM potassium chloride, 0.05 mM EDTA, 0.05 mM DTT and 25 μg/ml BSA. Assays were performed at 25°C in a final reaction vol of 10 μl. Reactions were initiated by addition of unlabelled ATP mixed with either [γ-^{32}P]ATP (3000 Ci mmol, Amersham) or [α-^{32}P]ATP (600 Ci/mmol, ICN). The final ATP concentration in the assay was 3 mM and the protein concentration was 1 μM unless stated otherwise in the figure legends. The reactions were terminated by adding one tenth vol of 5% SDS, 20 mM EDTA followed by freezing in liquid nitrogen. 1 μl samples were spotted onto polyethyleneimine cellulose plates (Macherey-Nagel) and dried. Nucleoside mono, di and triphosphates and free phosphate were separated by ascending chromatography in 0.75 M potassium dihydrogen phosphate buffer pH 3.5. The positions of the reaction products were confirmed by UV shadowing of unlabelled standards. The dried plates were subjected to autoradiography to identify the position of the radioactive spots. These were cut out of the plate and the radioactivity counted in Cocktail T scintillation fluid (BDH). The values for phosphate or ADP release were calculated as a percentage of the total amount of radioactivity in each 1 μl sample. A background value obtained from a control reaction with buffer alone was then subtracted to give the percentage hydrolysis due to NTRC. The values obtained were expressed as pmoles ATP hydrolysed per μl of reaction over the period of the assay.

Plasmid DNA cofactors and oligonucleotides

Plasmid pRD581 contains a 360 bp *Eco*RI–*Bam*HI fragment carrying the *nifL* promoter in pTE103 (Dixon *et al.*, 1988). pTE595 is a derivative of pRD581 in which the *nifL* sequence upstream of −138 is deleted, effectively removing NTRC binding sites 1 and 2 (Minchin *et al.*, 1989). Oligonucleotides were synthesized on an Applied Biosystems 381A synthesizer and purified by FPLC using a MONO Q column. Three oligonucleotides were used to construct synthetic NTRC binding sites:

A 5′-TTGCTTTGCACTACCGCGGCCCATCCCTGCCCCAAAACG-ATCGCTTCAGCC-3′

B 5′-TTGCTTTGTACTACCGCGGCCCATCCCTGCCCCAAAACG-ATCGCTTCAGCC-3′

C 5′-CATCCCTGCCCCAAAACGATCGCTTCAGCC-3′

These were made double-stranded by annealing to a primer:

3′-GGGTTTTGCTAGCGAAGTCGG-5′ and filling in the 5′-extension with the Klenow fragment of DNA polymerase I in the presence of all four deoxynucleotide triphosphates. For experiments requiring radioactively labelled oligonucleotides, the primer was first end-labelled with [γ-^{32}P]ATP using polynucleotide kinase.

Determination of relative binding constants

10 μl binding reactions contained 250 nM of each oligonucleotide 1 μM NTRC or S160F protein and 5 mM ATP in acetate buffer (as for ATPase assays) and were incubated at 25°C for 30 min. When necessary proteins were pre-phosphorylated by incubation in the same buffer with NTRB (final concentration, 300 nM) for 10 min at 37°C before addition to the binding reaction. Protein–DNA complexes were resolved from free DNA on 5% native polyacrylamide gels in a buffer containing 50 mM Tris, 10 mM sodium acetate, 5 mM magnesium acetate, 1 mM EDTA, pH 7.8. Both the gel and running buffer contained 400 μM ATP. Following autoradiography, the resolved complex and free DNA bands were excised from the gel and eluted into a buffer containing 0.5 M ammonium acetate, 10 mM magnesium acetate, 1 mM EDTA and 0.1% SDS at 37°C overnight. Samples were precipitated with ethanol, resuspended in sequencing loading dye and then analysed on 6% polyacrylamide sequencing gels. Following autoradiography for different exposure times, band intensities were quantitated on a Molecular Dynamics computing densitometer. Oligonucleotides were slightly contaminated with coupling failures of length $n-1$, $n-2$ and $n-3$ (where n = full length oligomer) but only full length products were analysed quantitatively. Relative binding constants were calculated by using the equation $K_{51}/K_{30} = (C_{51}/D_{51})/(C_{30}/D_{30})$ where C_{51} and C_{30} represent the band intensities of the labelled 51mer and 30mer competing fragments respectively in the complex and D is the corresponding intensity of labelled DNA in the unbound fraction (Liu-Johnson *et al.*, 1986).

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