The *nifLA* promoter of *K. pneumoniae* is a special promoter which needs for transcription the sigma-54 and the phosphorylated form of NtrC for its transcriptional activation. The *nifLA* operon is a regulatory operon for the rest of the *nif* genes and hence its expression needs to be tightly regulated in response to the environmental status and stimulii.

It has been shown that the phosphorylated NtrC binds to two sites located more than 100 nucleotides upstream of the -24, -12 region of the *nifLA* promoter and that the transcriptional activation by NtrC-P is face of the helix dependent, both *in vivo* and *in vitro* (Minchin *et al.*, 1989).

The mechanism of interaction of NtrC with E-σ54 bound downstream is not well understood. A deletion of DNA sequence between -135 and -53 which leaves both the NtrC binding sites intact, reduces the level of activation by 5 fold *in vivo* (Drummond *et al.*, 1983). It was therefore suggested that DNA loop formation is involved in NtrC mediated activation of the *nifLA* promoter.

DNA loop formation has been proposed as a general model for transcriptional activation at a distance (Ptashne, 1986). In an analogous situation, DNA looping is brought
about by the IHF protein which binds to a site intermediate between the upstream activator (NifA) binding site and the promoter downstream (nifH) (Santero et al., 1989). IHF does not bind to the intervening region of the nifLA promoter of K. pneumoniae (Hoover et al., 1990). We were therefore interested in finding out if sequence induced bending in this region [which could result when particular structural motifs like 'A' or 'T' tracts are repeated in phase with the DNA helical repeat (Crothers et al., 1990)] could be involved in facilitating protein-protein interactions between the NtrC and the RNA polymerase.

Work of somewhat similar kind has been reported, where the effect of curved DNA upstream of the -35 region (centered around -43), on the function of a promoter isolated from Bacillus subtilis bacteriophage SP82 was examined (McAllister and Achberger, 1985). By analysing a series of mutants in which the polyadenine containing curved DNA was either deleted or altered, they were able to show a decreased promoter activity in vivo and a decreased RNA polymerase binding in vitro. Short DNA insertions of 6-29 base pairs which simultaneously changed the linear placement and the rotational orientation, altered the activity of the promoter in vivo (McAllister and Achberger, 1989). It was therefore proposed that the function of the curved, adjacently upstream DNA presumably, was to increase RNA-
polymerase promoter interactions and facilitate open complex formation.

This however is not an identical situation like the sigma-54 dependent *nifLA* promoter of *K. pneumoniae*. Firstly, the promoter studied was a σ-70 dependent promoter which does not require the looping of the intervening region to promote interactions between the upstream activator and the promoter. Instead, the curved DNA [which is present very close to the upstream segment (-35) of the promoter] is thought to deflect the helix back towards the promoter bound RNA polymerase to assist the formation of an open complex.

The anomalous electrophoretic mobility of DNA fragment containing the *nifLA* upstream element is depicted in Fig. 3.2. It is evident that the 583 bp *BamHI-HindIII* fragment from pDJ22 (for details see Table 3.4a and b) moves like a 690bp fragment on PAGE, which points towards the presence of an intrinsic 'bend' in this DNA fragment. In addition, when an 83 bp region (which lies between the NtrC binding site and the *nifLA* promoter of *K. pneumoniae*), is deleted (between bases -135 and -53 with respect to the transcription start site of the *nifLA* promoter), the fragment moves virtually like linear, non-curved DNA which suggests that the bending motif lies within the 83 bp region (marked within boxes in Fig. 3.1). It can also be seen from Fig. 3.2 that disruption of an 'A' or 'T' tract in
isolation (as in pAN 3.1, pAN 5.1 or pAN 242.1) decreases the anomalous mobility marginally (relative to the wild type pDJ22 fragment). However when the two 'A' tracts are disrupted in conjunction (as in pAN 4.1) or an 'A' and the 'T' tract are disrupted together (as in pAN 241.1D), then the decrease in anomaly and the relative curvature is substantial. This suggests that the cumulative curvature seen in the pDJ22 fragment could be attributed to a synergistic effect of the 'A' and 'T' tracts present in the upstream region of the nifLA promoter of K. pneumoniae. The minicircle DNA fragment of Trypanosoma is highly bent (by around 300-400°) (Wu and Crothers, 1984) because of the presence of 18 phased A₄ tracts. On the other hand the relative curvature of the 583bp BamHI-HindIII fragment from pDJ22 is less (0.075) possibly due to just three A/T tracts.

For the upstream activator (NtrC) to interact effectively with the RNA polymerase sigma-54 complex, an optimal degree of DNA 'bending' or 'looping' would be required. It is therefore possible that the intrinsic curvature present in the upstream region of the nifLA promoter, though less, is adequate to allow protein-protein interaction between the NtrC and RNA polymerase. However the possibility of involvement of an external protein which would assist the preexisting sequence induced bend, to achieve optimal looping cannot be ruled out.
Electron microscopy of DNA fragments containing (wild type and mutagenised) \textit{nifLA} upstream element revealed a higher percentage of 'looped' or 'bent' molecules for pDJ22 and pAN241.1 fragments (see Fig. 3.5 and Table 3.2) as compared to deletion derivative (pDJ225) or the mutagenesied DNA fragment from pAN4.1. Electron microscopy (EM) offers a means of evaluating DNA bending directly. EM might also detect bends in DNA fragments which are too short or too long to be evaluated by other methods. EM of relaxed minicircles from the trypanosomatid \textit{Crithidia fasciculata} revealed 200-300 bp loops within the larger circles (Griffith et al., 1986). The EM studies in our case too, showed the presence of predominantly 'looped' DNA molecules in the 583 bp fragment from pDJ22 (See Fig.3.5) which were conspicuously absent in the corresponding fragments from pDJ225 and pAN4.1. This substantiated the finding from the earlier experiment and reenforced the idea of existence of an intrinsic curvature in the upstream region of the \textit{nifLA} promoter of \textit{K.pneumoniae}.

Currently, the most accurate way for mapping a 'bend' within a DNA fragment is to examine the electrophoretic behaviour of a set of circularly permuted bent DNA fragments (Wu and Crothers, 1984). This can be achieved by cloning the fragment of interest in the vector pBend2 (Kim et al., 1985). Fig.3.7b shows the electrophoretic mobility of
circularly permuted fragments containing the upstream element of \textit{nifLA}. A plot of the relative size (obtained from Table 3.3) against the mid point position, helped us to 'map' the 'bent' locus (Fig. 3.9) to a region very close to the 'A' tracts present in the upstream region of the \textit{nifLA} promoter.

A summary of the biological activity (in terms of $\beta$-galactosidase activity) of the \textit{lac} fusions of wild type and mutagenised \textit{nifLA} promoter and upstream sequence is given in Table 3.4a. It is significant that the decrease in relative curvature of pAN3.1, pAN5.1 and pAN242.1 (taken from Table 3.1) results in a concomitant decrease in the $\beta$-galactosidase activities of their corresponding \textit{lac} fusion constructs. Furthermore, mutagenising the 'A' and 'T' tracts in conjunction (as in pAN242.2D and pAN4.2) results in a further drop in activity of the \textit{nifLA} promoter. The effect of arbitrarily introduced upstream A-tracts has been shown to be context dependent (Ellinger \textit{et al.}, 1994). The authors showed that for the sigma-70 promoters in which binding of RNA polymerase is rate limiting, the introduction of upstream A-tracts stimulated promoter activity by enhancing the ability to compete for limiting RNA polymerase \textit{in vivo}. Kinetic studies have shown that the introduced 'A' tracts in the upstream region of sigma-70 promoters can increase the rate of complex formation between promoter and RNA polymerase and that they can also stabilise the complex
(Gartenberg and Crothers, 1991). We were also able to achieve an increase in the biological activity by the incorporation of two \( \text{`A}_{4} \)' tracts, in phase with the naturally preexisting ones.

In order to define the role of the intervening region and to decipher the role of negative supercoiling in the transcription of the \( \text{nifLA} \) promoter, we decided to replace the native upstream sequences of \( \text{nifLA} \) with \( \text{`foreign'} \) sequences and subsequently assess the effect on the activity of the \( \text{nifLA} \) promoter (Table 3.5 and 3.6). Replacement of the \( \text{`intervening region'} \) with a highly curved sequence from pHN1036 (Goodman and Nash, 1989), resulted in a substantial drop in promoter activity. In the past, Jose et al. (1994), replaced the IHF induced bend in the \( \text{nifH} \) promoter of \( \text{K.pneumoniae} \) with the curved fragment from pHN1036. Their best constricts showed only 60% of the wild type promoter activity.

In our case, it is therefore possible that pBJS.2 and pBJS.2R (Table 3.5), show a reduced promoter activity because a high degree of bending is not optimal for effective interactions between the NtrC and the RNA polymerase-sigma 54 complex.

The integration host factor (IHF) has been shown to stimulate NifA mediated activation of transcription from the \( \text{nifH} \) promoter by 20 fold in an \textit{in vitro} transcription system.
(Santero et al., 1992). The same group showed that when the NifA binding site is replaced by the NtrC binding site, IHF could still stimulate transcription, although to a reduced extent. When we exchanged the NtrC binding site with the NifA binding site, upstream of the nifLA promoter, we observed that the resultant promoter retained 40% of the wild type β-galactosidase activity while replacement of the intervening region (in isolation or along with the NifA binding site) resulted in a further loss in the promoter activity. In addition, sensitivity to coumermycin A1 was retained in all these clones. The lac fusion of the deletion derivative of nifLA viz. pSG1A1 (for details see Table 3.4a), also shows a further decrease in β-galactosidase activity (50 Miller units as compared to 151 Miller units in the absence of coumermycin A1) in the presence of DNA Gyrase inhibitor, coumermycin A1. Taken together, these results indicate the fact that the sensitivity to the level of negative supercoiling within a cell is not because of a localised region within the nifLA promoter or the upstream region, but is a cumulative effect.

Also, the overall promoter activity may be a net result of a precise geometry wherein the upstream activator can interact with the RNA polymerase and this in turn would be influenced by a correct degree of DNA bending, proper alignment, orientation and phasing of the DNA-protein complex. Excessive DNA bending may in fact be determinental
for the geometry of pre-initiation complex, thus resulting in a drop of biological activity of the *nifLA* promoter.

It has been suggested that if two divergently transcribing units which are close to each other, the moving RNA polymerase would generate two oppositely supercoiled gradients (Fig. 1.5). The local changes in DNA topology due to the transcription of one gene, therefore could potentially influence the transcription of the divergently transcribed gene (Wu et al., 1988). *nifLA* and *nifF* are two closely placed divergently transcribed promoters, such that NifA specific UAS at +59 with respect to the transcription start site of the *nifLA* promoter is required for the maximum activation of the *nifF* promoter (Buck et al., 1986; Minchin et al., 1988).

Since the *nifLA* promoter requires negative supercoiling for maximum activation, we were interested in determining whether the negative supercoils generated in the upstream region of *nifLA* promoter due to the transcription of the *nifF* promoter would affect the *nifLA* transcription. We blocked the *nifF* transcription by disrupting the consensus DNA sequence of the -24 and -12 region (by oligonucleotide directed, site specific substitutions) of the *nifF* promoter. The β-galactosidase activities of the resultant *lacZ* fusions (depicted in Table 3.7), indicate that blocking of *nifF* transcription did not affect the transcription from the
nifLA promoter and hence the mechanism by which negative supercoiling influences the transcription of the nifLA promoter and the elements involved in this response need to be further examined.

The NifA mediated activation of transcription of the nifH promoter of K. pneumoniae is known to be stimulated by IHF. In the absence of the IHF protein, the transcription from the nifH promoter falls to less than 10% of that in presence of IHF. In pSG1A1 (see Table 3.4a), where the 83bp intervening region is deleted, only around 35% of the wild type activity is retained. We were interested, therefore in determining if the intrinsic curvature present in the upstream region of the nifLA promoter was aided by any external protein(s) to bring about optimal activation. We, therefore, determined the β-galactosidase activity of the lacZ fusion of nifLA promoter and upstream sequence in E.coli strains mutated at the hupA, hupB and hns loci. The results are presented in Table 3.8. The nifLA promoter showed an increased activity in HU defective background while the expression dropped substantially in an hns⁻ background. The in vivo data thus suggested the involvement of these proteins in the transcription of the nifLA promoter. We were then interested in looking at binding of any protein in the upstream region of the nifLA promoter.
The low-molecular weight, heat stable histone like proteins are known to bind DNA in a relatively non-specific fashion and influence gene expression (Falconi et al., 1988; Hughes et al., 1992). Manna and Gowrishankar (1994) showed the stimulatory effect of HU and counteracting effect of H-NS on the expression of proU gene from *S. typhimurium* by *in vivo* studies. Martin and Lorenzo (1995) showed that the HU protein stimulated transcription initiation of the sigma-54 dependent *Ps* promoter from *Pseudomonas putida*. Based on our *in vivo* results under *hupA*-, *hupB*- and *hns*- background (Table 3.8), we argued that if the two proteins in question, influence the expression of the nifLA promoter by binding to the intervening region, then we should expect two distinct DNA binding activities from the partially purified protein fractions from *E. coli* GJ134 (hupA+, hupB+, hns+), while the two mutant strains viz. *E. coli* GJ1083 (hns-) and *E. coli* GJ1086 (hupA-, hupB-), being deficient in one of the two proteins should show only one type of DNA binding activity.

When each of the ten fractions from all the 3 *E. coli* strains were assayed for their DNA binding activity (discussed in Section 3.12.1), we observed the presence of two distinct protein peaks in *E. coli* GJ134 one of which was correspondingly absent in *E. coli* GJ1083 and *E. coli* GJ1086 (Fig. 3.12 A, B and C). From this data, we presume that the DNA binding activity obtained in fraction 4 from *E. coli*
GJ134 is due to a H-NS like protein while that found in number 7 protein fraction from E.coliGJ134 is a HU like protein (since these peaks are absent in their equivalent fractions in the respective mutant strains). For further discussion, therefore we would refer to these fractions as H-NS like (number four from E.coliGJ134) and HU like (for number seven fraction from E.coliGJ134).

Fig.3.15 depicts the relative affinities of these proteins when a mixture of different types of DNA molecules ('curved' and 'non-curved') are present in the solution. The H-NS like protein was found to interact preferentially with curved DNA (Fig.3.15 A and C). This finding is in accordance with that of Hughes et al (1992) where they observed that the purified H-NS protein interacted with curved DNA to influence DNA topology and gene expression. The HU like protein did not show a clear trend. Both proteins however bound all types of DNA molecules present in the solution. This emphasises the relative non-specific DNA binding property of these proteins while a preference for 'curved' DNA suggests that though they might interact with different types of DNA fragments they would regulate only a subset of promoters.

The HU and H-NS like protein fractions, when subjected to SDS-PAGE, revealed major bands which corresponded to a molecular weight, less than 14.5kD. Yaniv and Gros (1975)
reported a subunit molecular weight of 9kD for the HU protein while H-NS is a homodimer with a molecular weight of around 14kD (Spassky et al., 1984). The two proteins were also found to be heat stable (Fig. 3.14). Heat stability for HU and H-NS has been reported by Spassky et al., (1984).

We also carried out footprinting studies, to further assess the pattern of protein binding. The results depicted in Fig. 3.18 show the presence of hypersensitive bands at low protein concentration, while the DNA is uniformly protected at higher concentrations of the protein. The latter finding is in accordance with the footprint pattern obtained with purified H-NS protein with proU regulatory region of E.coli (Lucht et al., 1994). The presence of hypersensitive bands at low protein concentration is known to be an indicator of partial footprints where sites are partially occupied by DNA binding proteins.

Our in vitro results, thus are in accordance with the studies carried out with purified HU and H-NS proteins. The circumstantial evidence thus suggests that the DNA binding activities that we have obtained with fractions 4 and 7 from E.coliGJ134 could in fact be H-NS and the HU protein respectively. It thus appears that the looping of the DNA between NtrC binding site and the promoter of the nifLA operon of
*K. pneumoniae* is primarily facilitated by intrinsic bending of the DNA mediated by stretches of 'A's/'T's. H-NS appears to bind to this 'bent' DNA and either affects the extent of bending or the topology of DNA. HU apparently has a counteracting effect. It is possible that the relative efficiency of binding of HU and H-NS, which might be affected by environmental factors is involved in the fine tuning of the final optimum bending of DNA. Excessive bending of DNA is detrimental, as evident from the replacement of the natural intervening sequence with an artificial curved DNA fragment.