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Enhanced activity of the bacteriophage λ P_L promoter at low temperature

(*Escherichia coli*/integration host factor/temperature response)

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ABSTRACT **The response of the early phage λ P_L promoter to temperature was investigated. Experiments with *lacZ* reporter gene fusions demonstrated that the activity of the phage λ P_L promoter is inversely dependent on temperature. The bacterial DNA-binding protein integration host factor (IHF) further enhances λ P_L promoter activity at low temperature, although no apparent changes in the cellular level of IHF protein were observed at the different temperatures. IHF protein binds DNA *in vitro* more avidly at low temperatures. *In vitro* transcription assays further revealed that the temperature response of P_L is the result of an intrinsic property of the promoter as well as its activation by IHF.**

Bacteriophage λ can follow a temporal program of gene expression along two pathways, lytic or lysogenic. Each pathway is regulated at the transcriptional and at the posttranscriptional levels. The P_L and P_R promoters are expressed early in bacteriophage λ infection, and the regulation of both promoters is critical for λ development (1). Both promoters are regulated by the λ *cI* and *cro* gene products. However, little is known about the effects of different physiological conditions of the infected cell on the activity of the early promoters.

We have previously shown that the *Escherichia coli* protein integration host factor (IHF) binds with high affinity to two tandem sites upstream of the phage λ P_L promoter and stimulates transcription by enhancing closed complex formation between RNA polymerase and the promoter (2).

IHF is an abundant protein that binds to specific DNA sequences and causes significant DNA bending (for review, see ref. 3). IHF participates in a number of cellular processes such as λ site-specific recombination, transposition and inversion, plasmid and phage DNA replication, and the positive and negative control of gene expression (3–5).

We have constructed P_L–*lacZ* reporter gene fusions and inserted them into the *E. coli* chromosome. We found that transcription from P_L increased as the temperature was lowered. IHF was found to increase P_L activity at low temperature both *in vivo* and *in vitro.*

MATERIALS AND METHODS

Bacterial Strains, Phages, and Plasmids. Strains A7655, A7506, A7521, A8112, and A7870 are all derivatives of A6826 [CSH50 (F^-, *ara*, Δ(*lac-pro*), *rpsL, thi*)] obtained by lysogenization with phages λAO1110, λAO1107, λAO1109, λAO1181, and λAO1136, respectively. Strains A7665 and A7507 are *hip*Δ*3*::*cat* derivatives of strains A7655 and A7506, and strain A7533 is a *himA*Δ*82*:Tn*10* derivative of A7507. The phages are the result of recombination between λB299 and the respective plasmids pHG87, pHG91K, pHG91, pIKEL, and pSK1. λB299 (obtained from R. Weisberg, National Institutes of Health, Bethesda) carries the *supF* gene flanked by truncated *lacZ* and *bla* genes. Recombination results in replacement of the *supF* gene by the promoter–*lacZ* fusion. All the above plasmids are derivatives of the *lacZ* operon fusion vector pHG86 (6). Plasmid pHG87 carries a 350-bp P_L promoter fragment (−240 to +110) (6); plasmid pHG91 carries a similar fragment containing an A → G mutation at −9 and a GGCC sequence substituting for AATT at −50 (see Fig. 1*c*) (6); plasmid pHG91K was derived from pHG91 by insertion of a kanamycin-resistance gene into the *Pst* I site of the *bla* gene. The *cspA* promoter in plasmid pIKEL is a 290-bp-length fragment, from −209 to +81, generated by PCR of phage 9F6 from Kohara's library (21). Plasmid pIKEL carries the kanamycin-resistance gene inserted within the *bla* gene. Plasmid pSK1 carries a 40-bp synthetic *lacUV5* promoter extending from −37 to +3 relative to the transcription start site.

Preparation of DNA Fragments. DNA fragments used for binding assays were obtained by PCR using *Taq* polymerase (Promega). To obtain the 275-bp P_L DNA fragment we used primers 1921: 5′-AAGAATTCGGGTTTTCTTT-3′ (pL positions −228 to −217) and 1526: 5′-AAGAGCGTCACCTTC-3′(P_L positions +40 to +26) and plasmid pHG244 DNA as a template (described in ref. 6). The 78-bp DNA fragment containing the H′ site of phage λ was generated as described (7). DNA fragments were end-labeled with [γ-^{32}P]ATP (Amersham) and purified on a 5% polyacrylamide gel.

Biochemical Assays. β-Galactosidase assays were done according to Miller (8). Binding of IHF to end-labeled DNA fragments was done as described (7).

In vitro transcription assays were done as described (6). Where indicated, IHF at a final concentration of 50 nM was added to the reaction, 15 min before the addition of *E. coli* RNA polymerase (Boehringer Mannheim). The transcription reactions were allowed to proceed for 20 min. The gels were analyzed and the bands were quantitated by the Fujix BAS100 PhosphoImager (Tokyo).

RESULTS

Temperature Response of the P_L Promoter *in Vivo*. The wild-type λ P_L promoter and the attenuated P_L-9G-50 promoter were fused to the *lacZ* reporter gene and inserted into the bacterial chromosome as a single copy within the *att* site (strains A7655 and A7506, respectively). P_L is constitutively expressed in these strains. Cultures of strains A7655 and A7506 and their respective IHF^- derivatives, A7665 and A7507, were grown at various temperatures to saturation and assayed for β-galactosidase levels. The data obtained with both wild-type and mutant P_L–*lacZ* fusions show that the specific activity of β-galactosidase declined precipitously as a function of temperature (Fig. 1). Between 15°C and 37°C, in the presence of IHF, the activity of wild-type P_L and of P_L-9G-50 decreased 5- and 14-fold, respectively. Moreover, in the absence of IHF, wild-type P_L activity decreased ≈10-fold as the temperature of growth was increased from 15°C to 37°C, suggesting the

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Abbreviation: IHF, integration host factor.

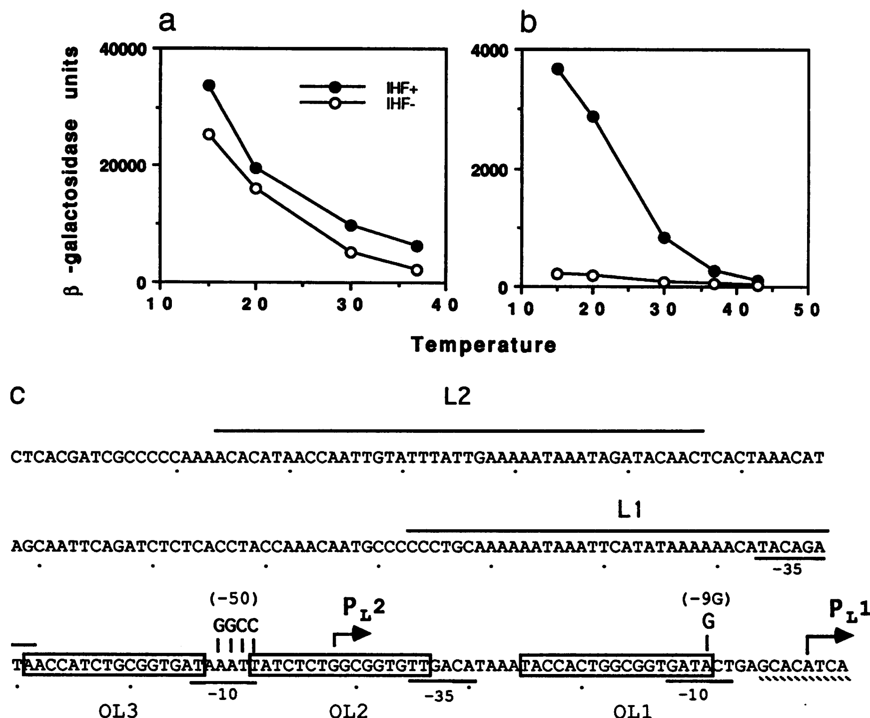

FIG. 1. Temperature response of the P_L promoter. Strains A7655 (IHF^+) and A7665 (IHF^-) carrying the wild-type P_L–*lacZ* fusion (*a*) and A7506 (IHF^+) and A7507 (IHF^-) carrying the mutant P_L-9G-50–*lacZ* fusion (*b*) were grown to stationary phase in LB medium at various temperatures and assayed for β-galactosidase activity. (*c*) DNA sequence of the P_L promoter region. The P_L1 and P_L2 promoter start sites are shown by arrows; the −10 and −35 regions are underlined; the −9G and −50 mutations are shown above the sequence; the L1 and L2 IHF binding sites are overlined; the boxes denote the O_L1, O_L2, and O_L3 λ repressor-binding sites; the hatched underline denotes the 8-bp sequence homology with the *cspA* promoter.

presence of an IHF-independent component. The temperature dependence of P_L promoter activity was also observed when the experiment was done with cells grown at different temperatures to the exponential stage of growth (Table 1 and Fig. 2*a*).

Temperature Shift Experiments. To study the rate of the response to temperature changes, we performed temperature-shift experiments using the attenuated P_L-9G-50 promoter. Transferring exponentially growing A7506 cells from 37°C to 15°C resulted in a dramatic increase in β-galactosidase levels (Fig. 2*a*), in spite of the long lag in cell growth induced by the cold shock (Fig. 2*b*). Analysis of the promoter activity by primer extension with reverse transcriptase revealed a 3-fold increase in the mRNA level at 1 hr after the transfer of an exponentially growing A7506 culture from 37°C to 15°C (data not shown). In the reciprocal experiment, cells were first

Table 1. Effect of temperature on promoter activity *in vivo*

		Fold induction†					
	Units*	20°C			15°C		
Promoter	(37°C)	1 hr	2 hr	4 hr	1 hr	2 hr	4 hr
P_L	1717	1.5	4.4	7.8	1.1	1.9	3.9
P_L-9G-50	64	2.9	5.1	11.5	1.3	4.4	12.2
P_{cspA}	3295	2.0	3.2	5.9	1.4	2.0	3.5
P_{lacUV5}‡	1862	1.5	1.6	2.1	0.9	1.2	1.8

Each promoter was fused to *lacZ*, transferred to phage λB299 by homologous recombination, and inserted into the bacterial chromosome at the *att* site by lysogeny. Cell cultures were grown in LB medium supplemented with antibiotic to OD_{600} of 0.2 unit and then transferred to 20°C or 15°C. β-Galactosidase activity was measured at 0, 1, 2, and 4 hr after transfer to the lower temperature.

*β-Galactosidase units.

†The β-galactosidase units obtained at each time point, divided by the amount obtained at 37°C.

‡A synthetic 40-bp promoter.

grown to exponential phase at 15°C and then transferred to 37°C. As seen in Fig. 2*c*, a rapid cessation in β-galactosidase expression was observed. The rate of the decrease in the specific activity of β-galactosidase correlated with the expected rate of dilution of the preexisting enzyme by cell division (Fig. 2*d*), suggesting that transcription from P_L was turned off rapidly after the shift to the high temperature.

Activation of P_L-9G-50 by Mutant IHF Proteins. We used the P_L-9G-50–*lacZ* fusion to test the activity of two IHF mutants, βR87G and βA90D, both carrying mutations at the C terminus of the hip subunit. These mutant IHF proteins were shown to be defective in their specific DNA-binding activity (7). Plasmids carrying the wild-type *hip* gene or one of the two *hip* point mutants βR87G or βA90D were introduced into the hip^- strain A7507 and tested for P_L-9G-50 induction at various temperatures. The presence of a plasmid carrying the wild-type *hip* gene fully restored the effect of IHF on P_L-9G-50. The βR87G *hip* gene complemented partially (≈50%) for IHF activity at low temperatures, leading to a shift in the P_L-9G-50 temperature–response curve without changing its general shape. The mutant βA90D was found to be inactive at all temperatures (data not shown). Thus, the activity of the P_L-9G-50–*lacZ* fusion depends on both temperature and IHF and can be used as a quantitative assay for IHF activity in the stimulation of transcription initiation.

Determination of Cellular Levels of IHF. The temperature-dependence curve of P_L-9G-50 promoter activity could be due to higher IHF levels in the cell at low temperatures. We therefore measured the level of IHF protein in A7506 cells grown to saturation at 37°C or 20°C, by immunoblot analysis (Fig. 3). The results show that the level of IHF at 37°C was ≈1.5-fold higher than at 20°C. Thus, the increased activity of P_L observed at 20°C is not due to increased IHF levels in the cell.

Temperature Response of Other Promoters. To test the specificity of the temperature response of P_L, we compared

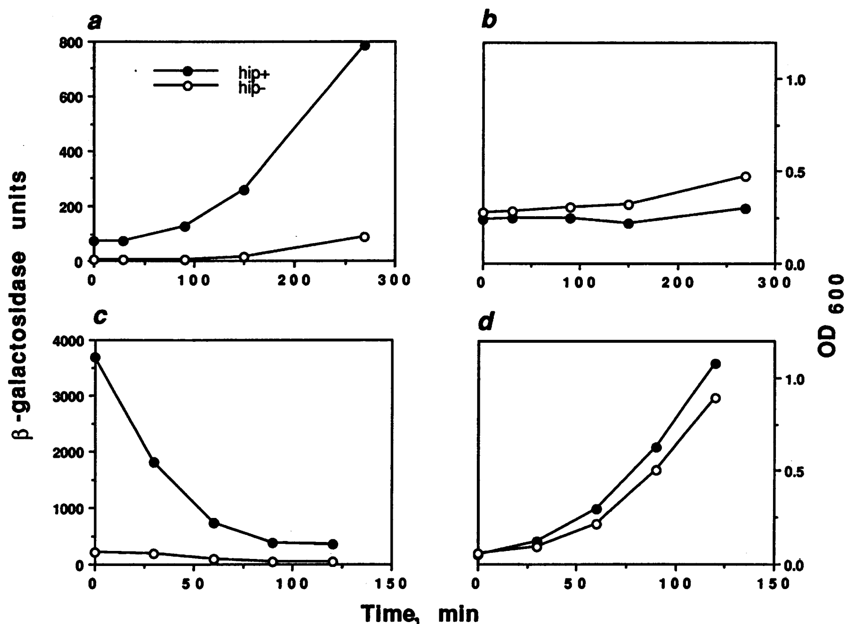

FIG. 2. Response of the P_L-9G-50 promoter to temperature shifts. Temperature down-shift (*a* and *b*) and up-shift (*c* and *d*) experiments. Cell cultures of strains A7506 (*hip*$^+$) and A7507 (*hip*$^-$) were grown to exponential phase at 37°C (*a* and *b*) or 15°C (*c* and *d*) and then transferred to the reciprocal temperature. β-Galactosidase levels (*a* and *c*) and cell density (*b* and *d*) were determined at different time points after the temperature shift.

β-galactosidase levels expressed at 15°C, 20°C, and at 37°C, from wild-type P_L and the mutant P_L-9G-50, with those expressed from two additional promoters: the promoter of the major cold-shock gene *cspA* (10) and a synthetic 40-bp *lacUV5* promoter (11). Each of the promoters was fused to the *lacZ* reporter gene, and the fusion was inserted into the *E. coli* chromosome as a single copy, via a λ prophage. Cells carrying the various *lacZ* operon fusions were grown at 37°C to an OD_{600} of 0.2 unit and then transferred to 20°C or 15°C. Samples for β-galactosidase assay were taken at different time intervals after the temperature shift. The mutant P_L-9G-50 promoter showed the highest degree of induction at low temperature (12-fold) (Table 1). Wild-type P_L showed 8- and 4-fold induction at 20°C and 15°C, respectively, similar to the levels of induction of the *cspA* promoter, which is known to be induced at low temperature (12). The synthetic *lacUV5* promoter, on the other hand, exhibited a smaller increase at both low temperatures (2-fold). These results suggest that the higher β-galactosidase levels observed for P_L and P_{cspA} at low temperatures are due to increased level of transcription of these promoters.

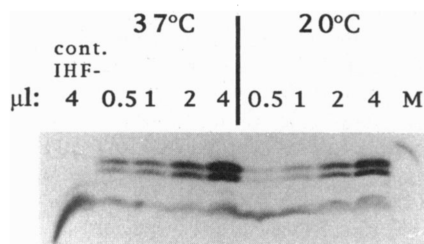

FIG. 3. Immunoblot analysis of IHF. Strain A7506 and its IHF$^-$ derivative A7533 (cont. IHF$^-$) were grown to stationary phase at 37°C and 20°C. Increased amounts of cell extracts were loaded on a 16.5% Tricine/SDS/polyacrylamide gel (9). Proteins were transferred to a nitrocellulose membrane and treated with rabbit anti-IHF antibodies (from H. Nash, National Institutes of Health) followed by protein A–horseradish peroxidase conjugate (Amersham). Luminescent visualization was done with an ECL immunoblotting kit (Amersham). The two closely migrating bands comprise the two IHF subunits, with the HimA subunit migrating the more slowly of the two. M, lysozyme 14.3-kDa marker.

Temperature Response of P_L *Transcription in Vitro*. To test whether the enhanced activity of P_L at low temperature requires the participation of cold-specific factors, we performed *in vitro* transcription assays using a purified system containing vegetative RNA polymerase Eσ70 and IHF. For DNA template a supercoiled plasmid carrying the wild-type P_L promoter, cloned upstream of the *rrnB* t1t2 transcription terminators, was used (6). The λ P_L promoter region contains two promoters, P_L1, the major P_L promoter that is stimulated by IHF, and a second weak promoter, P_L2, which is repressed by IHF. Thus, inhibition of P_L2 by IHF provides an independent assay for the binding of IHF at the P_L region. In addition to wild-type P_L, we analyzed the effect of temperature on the P_{tac} promoter and the ColE1 plasmid-born RNAI promoter (13, 14).

Fig. 4*a* shows an *in vitro* transcription assay performed with wild-type P_L (*Left*) and P_{tac} (*Right*). Quantitative analysis of the results (Fig. 4*b*) revealed that the activity of the P_L promoter was highest at 20°C and declined as the temperature was raised. The intrinsic transcription activity in the absence of IHF was also highest at 20°C. Repression of P_L2 by IHF was also strongest at 20°C (Fig. 4*a*, compare lanes 4, 6, and 8). In contrast to P_L, the activity of the P_{tac} promoter increased as a function of temperature, reaching maximal levels at 37°C and was not significantly influenced by the presence of IHF (Fig. 4 *a Right* and *b*).

An additional promoter, directing the synthesis of the short RNAI transcript (15), was expressed from the plasmid templates that were used in the transcription assays (Fig. 4*a*, lower band). We found that this promoter was not significantly affected by temperature or by IHF (Fig. 4*b*).

We have previously shown that DNA supercoiling stimulated P_L ≈16-fold, due to an increased rate of isomerization (k_f) of RNA polymerase from closed to open complex (6). To test whether the response of the P_L promoter to temperature is due to changes in DNA conformation that may occur in the structurally constrained supercoiled DNA, we carried out transcription assays with a linear DNA template. Our results (data not shown) demonstrated that transcription from P_L on

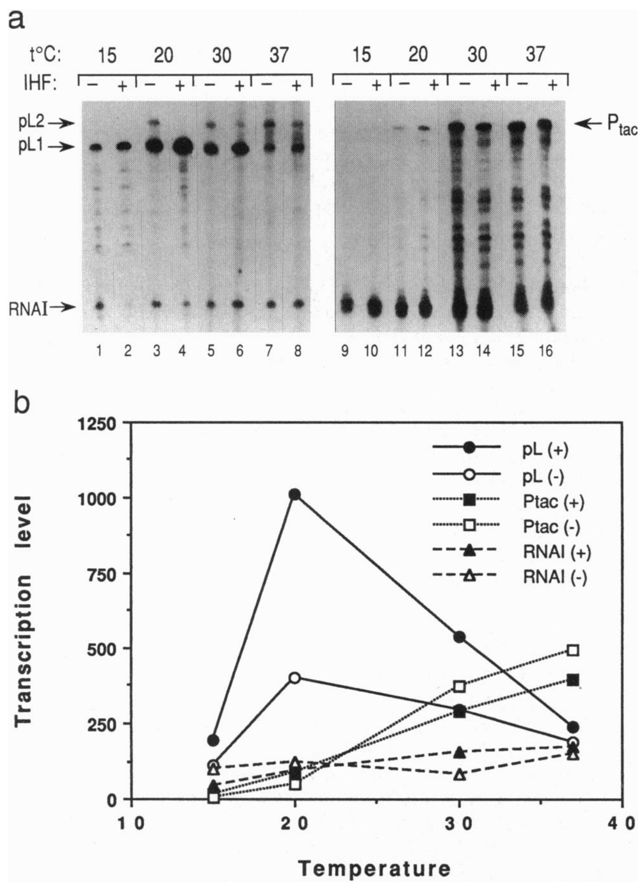

FIG. 4. The effect of temperature on transcription *in vitro* from the wild-type P_L and P_{tac} promoters. Transcription was carried out at 15°C, 20°C, 30°C, and 37°C in the presence (+) or absence (−) of 50 nM IHF. (*a*) Transcription from template plasmids pSE2, carrying the wild-type P_L promoter (lanes 1–8) and pKK233-3, carrying P_{tac} (lanes 9–16). (*b*) Graphic presentation of the transcription levels of the experiment shown in *a* of the P_L, P_{tac}, and P_{RNAI} promoters in the absence (−) or presence (+) of IHF. In the assays done with the pKK233-2 plasmid, there are additional unidentified bands in the region of the RNAI transcript.

a linear DNA template responds to temperature in a similar way to transcription on a supercoiled template.

The results presented above lead us to conclude that P_L shows, in a purified *in vitro* system, a promoter-specific temperature response, suggesting that specific cold-response factors are not required. Both the intrinsic activity of the P_L promoter and its activation by IHF are temperature-dependent.

Temperature Dependence of IHF–DNA Interactions. The temperature profile of P_L activation by IHF prompted us to test whether IHF is more active in DNA binding at lower temperatures. The affinity of IHF to DNA at different temperatures was tested by the gel mobility-shift assay. Fig. 5*a* shows that IHF bound to a P_L DNA fragment more efficiently at 5°C than at 37°C. The calculated apparent equilibrium binding constant of IHF protein bound simultaneously at L1 and L2 (the slowest migrating band) was ≈6 nM at 5°C and 24 nM at 37°C. Serial dilutions of preformed complexes between IHF and a DNA fragment carrying the H′ IHF site of the λ *att* region gave similar results (data not shown).

The affinity of IHF to DNA at different temperatures was also tested by dissociation kinetic experiments (Fig. 5*b*). IHF was first bound to P_L DNA at 5°C to form IHF–DNA complexes. These complexes were then challenged with excess DNA carrying the specific H′ IHF-binding site or with poly (dI-dC) at 37°C and 5°C. At 37°C, the specific competitor DNA chased all the radioactive probe from the IHF–DNA complexes to the nonbound form within 1 min. In contrast, at 5°C, a portion of the radioactive probe remained bound even after 10 min of incubation.

To rule out the possibility that the reduced DNA-binding activity of IHF at 37°C was due to protein inactivation, the P_L DNA fragment was first incubated with purified IHF at 37°C for 15 min, transferred to 5°C for additional 15 min, and then subjected to electrophoresis at 5°C. IHF was found to retain full activity upon transfer to the low temperature (data not shown).

DISCUSSION

This study demonstrates that the activity of the phage λ P_L promoter is inversely dependent on temperature, both *in vivo* and *in vitro*. The temperature response of a P_L–*lacZ* operon

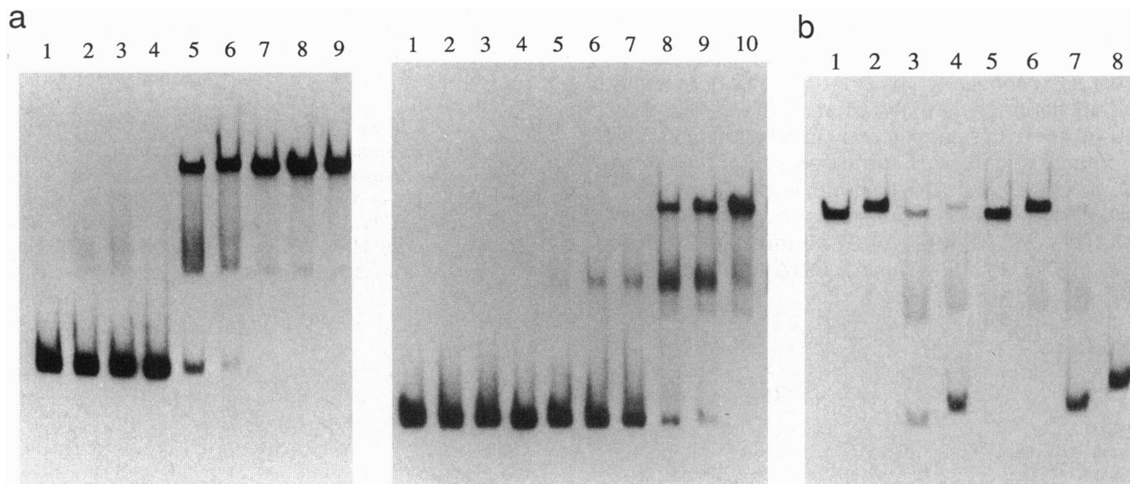

FIG. 5. Higher DNA-binding affinity of IHF at low temperature. (*a*) A 275-bp DNA fragment (100 pM) containing wild-type P_L carrying the L1 and L2 IHF-binding sites was bound to purified IHF protein at a final concentration of 0, 1, 2, 4, 6, 8, 16, 24, and 32 nM at 5°C (*Left*, lanes 1–9) or to 0, 1, 2, 4, 6, 8, 12, 16, 24, and 32 nM IHF at 37°C (*Right*, lanes 1–10) in the presence of 6-fold excess poly(dI-dC). After 15-min incubation the samples were electrophoresed at the same temperature of incubation. (*b*) Dissociation kinetics of IHF–DNA complexes at different temperatures in the presence of specific competitor. The same P_L DNA fragment as in *a* (100 pM) was incubated for 15 min at 5°C with purified IHF (16 nM) (lane 1) and then challenged with specific H′ competitor DNA at 50-fold molar excess (25 ng/ml) (lanes 3, 4, 7, 8) or with poly(dI-dC) at 25 ng/ml (lanes 5, 6) for 1 min (lanes 3, 5, 7) or 10 min (lanes 4, 6, 8) at 5°C (lanes 3, 4) or at 37°C (lanes 7, 8). As a control, 10 μl of buffer was added, and the sample was incubated for 10 min at 5°C before loading on the gel (lane 2).

fusion was observed at the exponential, as well as in the stationary, phase of cell growth. Our results also indicate that individual promoters have an inherent response to temperature that is embedded within the promoter DNA sequence. In addition, we found that IHF binds with higher affinity to DNA at lower temperatures.

Biochemical analysis of bacterial promoters whose activity is enhanced at low temperature is limited. Moreover, little is known about message stability at low temperature. One common element found in the promoter region of a number of cold-shock genes, including *cspA, hns*, and *gyrA*, is the Y-box sequence ATTGG (10, 16). The P_L promoter region contains two Y-box elements at −185 and +110. The role of these sequences for P_L activity is not known. However, removal of the Y box at −185 did not affect the cold response of the promoter (our unpublished data). Comparing the sequence of P_L with the *cspA* promoter, we found a homologous stretch of 8 bp (GCACATCA) located in the vicinity of the transcription start site (from −8 to −1 in P_{cspA} and from −4 to +4 in P_L). We tested the significance of this sequence by mutating 3 bp of the 8 (to GGACAAGA) by site-directed mutagenesis. These changes reduced significantly the *in vivo* cold response of P_L (our unpublished results). However, our preliminary experiments showed that additional promoter mutations, at the −10 region and around the promoter start site, also reduced the temperature response of P_L *in vivo.* For example, changing the −10 region from GATACT to the consensus sequence TATAAT or to GATGCT (mutation −9G) resulted in a greatly reduced temperature response. On the other hand, the weakest P_L promoter mutant, P_L-9G-50, exhibited an enhanced response to low temperature.

Thus, it seems that the general DNA structure of the promoter region, rather then a specific cold-responsive sequence, determines the temperature response of P_L. Low temperatures may favor the formation of a ternary complex at the P_L promoter region, involving RNA polymerase, IHF, and bent DNA. IHF, similarly to HU and the eukaryotic high-mobility-group proteins, may act as a DNA chaperone (17) to facilitate the formation of this complex. One requirement for chaperone function, besides changing the DNA conformation, is a transient association with the nucleoprotein complex. This idea is supported by our observation that IHF dissociates rapidly from DNA (Fig. 5).

We speculate that the temperature response of the P_L promoter facilitated by IHF plays an important regulatory role in phage λ development. It has been shown that after adsorption, the injection of phage λ DNA into the host is highly temperature dependent: instantaneous at 37°C and very slow at 23°C and lower (18). This phenomenon may be related to the fact that the optimal temperature for the growth of the *E. coli* host and, accordingly, for the λ lytic cycle, is at body temperature. Consistent with this idea, our preliminary experiments showed that at low temperature (20°C) an infecting λ phage (after 5 min at 37°C to allow phage DNA injection) was incapable of completing the lytic cycle, although it could lysogenize cells with a high efficiency (data not shown).

These findings are reminiscent of λ*cro*$^-$ phage infection in which overexpression from the P_L promoter aborts the lytic cycle. A number of leftward early genes probably participate in this inhibition (19, 20). It is thus possible that the high activity of the phage λ P_L promoter at low temperature results in a similar unbalanced gene expression that interferes with the phage lytic cycle.

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