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Overlapping positive and negative regulatory domains of the human β-interferon gene

(interferon induction/inducible enhancer/point-mutation analysis/negative regulation)

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ABSTRACT **Virus or poly(I)·poly(C) induction of human β-interferon gene expression requires a 40-base-pair DNA sequence designated the interferon gene regulatory element (*IRE*). Previous studies have shown that the *IRE* contains both positive and negative regulatory DNA sequences. To localize these sequences and study their interactions, we have examined the effects of a large number of single-base mutations within the *IRE* on β-interferon gene regulation. We find that the *IRE* consists of two genetically separable positive regulatory domains and an overlapping negative control sequence. We propose that the β-interferon gene is switched off in uninduced cells by a repressor that blocks the interaction between one of the two positive regulatory sequences and a specific transcription factor. Induction would then lead to inactivation or displacement of the repressor and binding of transcription factors to both positive regulatory domains.**

β-Interferon gene expression is induced in many cell lines by either viral infection or exposure to double-stranded RNA. Transcriptional activation is a major component of induction (1–3), and cis-acting sequences that mediate this process have been identified (4–8). The sequence requirements for efficient induction of the human β-interferon gene differ between cell lines (for discussion, see refs. 6 and 8), but a 40-base-pair (bp) element, the interferon gene regulatory element (*IRE*), is both necessary and sufficient for induction in mouse C127 cells (6). In addition, this element is an inducible enhancer capable of acting on a heterologous promoter (6). Analyses of deletion mutants and promoter fusions have indicated that the *IRE* contains a constitutive transcription element that is prevented from functioning in uninduced C127 cells by an adjacent or overlapping negative regulatory element (7). Induction may involve both the derepression of the constitutive activity of the transcription element (7) and further stimulation by positive regulatory factors (6, 8). Changes in the genomic DNase protection pattern provide additional evidence for a role of negative control in β-interferon gene expression (9). In our previous studies (7), it was not possible to determine whether the negative regulatory region physically overlaps with the sequences required for the transcriptional activation. Here we present evidence that strongly suggests this is the case, and we show that the positive transcription element is comprised of two genetically separable domains, both of which are necessary for maximal induction by virus or poly(I)·poly(C).

MATERIALS AND METHODS

Generation and Subcloning of Single-Base Mutations. Single-base substitutions in the human β-interferon gene promoter were generated and isolated as described by Myers *et al.* (10). The G-C clamp sequences used to ensure that mutations could be isolated throughout the promoter region were found to inhibit transcription from the adjacent β-interferon gene promoter, both in transient and stable expression systems. As a result of this, β-interferon genes containing either wild-type or mutant promoter regions were transferred from the G-C clamp vector to the bovine papilloma virus-based vector pBPV-BV1 (4) as described (6).

Cell Culture and Induction Procedure. Recombinant plasmids were transfected into mouse C127 cells, and duplicate pools of transformed foci were isolated and propagated as described (6). Pools of foci were maintained for at least 35 days posttransfection before inducing with poly(I)·poly(C) and harvesting cellular RNA. Some mutants reproducibly gave low pool sizes, although the expression levels in duplicate pools was similar. All of these mutants fall within the putative negative regulatory region. To obtain further data on the phenotypes of these mutants, multiple single foci were isolated and amplified for analysis in parallel to foci bearing a wild-type β-interferon gene promoter. In contrast to the wild-type promoter, some of the mutants showed considerable variation in expression between individual foci and, in some cases, even displayed an alteration in the behavior of the endogenous mouse β-interferon gene upon induction. However, when the expression was averaged between independent foci, the results were similar to that obtained from duplicate pools. We do not understand the basis of these anomalies.

Analysis of Cellular RNA. Twenty micrograms of total cellular RNA was analyzed by a quantitative RNase mapping procedure (4). Each sample was hybridized to a mixture of three probes; the probes for the 5′ end of human βinterferon (5′IF) and for mouse β interferon are described in Goodbourn *et al.* (6), while that for human γ-actin is described in Enoch *et al.* (11). To ensure that the three protected species could be quantitated on the same autoradiographic exposures, the 5′IF probe was prepared at a 10-fold higher specific activity than were the mouse β-interferon and actin probes. Prolonged RNase treatment of hybrids causes the 5′IF signal to form a doublet and, therefore, is difficult to quantitate. To avoid this problem, RNase digestion conditions were adjusted to give a single protected fragment. As a result, incomplete digestion products of the human actin probe and mouse actin mRNA were frequently observed. However, this did not interfere with the normalization of the 5′IF signal to the actin control. After electrophoresis, each gel was autoradiographed for several exposures with preflashed film, and the exposed films were scanned with a Joyce–Loebl densitometer. The intensity of 5′IF signals was corrected for variations in the

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Abbreviations: RTL, relative transcription level; RIR, relative induction ratio; PRD I and II, positive regulatory domains I and II; NRD I, negative regulatory domain I.

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amounts of RNA as determined by the intensity of the actin signal. The levels of induced β-interferon mRNA were also corrected for variations in the mouse β-interferon signal, which monitors the extent of induction. The ratio of the corrected 5'IF signals from the test genes and wild-type β-interferon gene was determined in each case and presented as the relative transcription level (RTL). Induction ratios were compared to wild-type and expressed as a relative induction ratio (RIR).

RESULTS AND DISCUSSION

The positive transcription element within the *IRE* is located between positions −77 and −55 and appears to contain two separate domains (7). The first domain is located between −77 and −64 and has been defined on the basis of the following observations. First, the 5' boundary of the sequence required for efficient induction is between −77 and −73 (6). Second, the poor inducibility of the −73 deletion can be restored to levels approaching that of the −77 deletion by placing a −77 to −64 fragment 100 bp upstream from −73 (6). Third, it has been shown that the −77 to −64 region (7) or closely related fragments present in multiple copies (8) can confer inducibility to heterologous promoters. We refer to this region as the positive regulatory domain I (PRD I) and have previously noted that this sequence is similar to a region from within the adenovirus *E1a* gene enhancer (7). The presence of a second domain (PRD II) within the positive transcription element is suggested by the observation that removal of the sequences immediately downstream of PRD I (between −64 and −55) dramatically decreases the level of transcription before and after induction (7). This region is related to the simian virus 40 enhancer core sequence. Further evidence that PRD II is essential for promoter function is provided by experiments in which the *IRE* was placed 100 bp upstream from a 5' deletion series of the β-interferon gene promoter. The inducibility of a mutant promoter with a deletion at position −66 can be fully restored by the *IRE*, whereas a mutant promoter with a deletion at position −58 is inactive in such an assay (Fig. 1). Thus, the 5' boundary of an element essential to respond to the *IRE* lies between positions −66 and −58. We regard an element (PRD II) that has outer boundaries of −66 (5') and −55 (3') as being a separate and distinct component of the positive transcription element of the *IRE*.

We have investigated the interactions between PRD I, PRD II, and the negative regulatory region by analyzing the effects of single-base mutations on the expression of the cloned β-interferon gene stably introduced into mouse C127 cells in a bovine papilloma virus vector (4). Pools of several hundred independent transformants were analyzed for β-interferon mRNA production before and after induction with poly(I)·poly(C). A representative example of this analysis is shown in Fig. 2. Relatively low levels of human β-interferon mRNA were observed prior to induction of the wild-type gene and a gene with a mutation at position −38, while a high level was observed after induction in both cases (Fig. 2). In contrast, single-base mutations within the "TATA" box (at positions −23 and −27) caused a significant decrease in the level of transcription before and after induction. In another example, a mutation at −43 caused an increase in the level of transcription prior to induction but a decrease relative to wild type after induction. The results of analyzing the phenotypes of 50 mutants with 42 point mutations, 6 double-base substitutions, and 2 single-base deletions are presented in Table 1 and Fig. 3 and summarized below.

The TATA Box. Mutations in the TATA-box region result in a significant decrease in the RTL (see Table 1) before and after induction. The RIR (see Table 1) of these mutants is

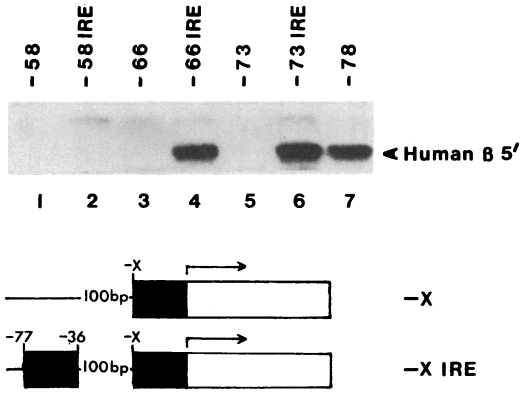

FIG. 1. Determination of β-interferon gene 5' sequence requirements for interaction with the *IRE*. The 5' deletion mutants of the β-interferon gene with or without the *IRE* placed 100 bp upstream of the deletion breakpoint (−x IRE and −x, respectively) were introduced into mouse C127 cells by using the vector pBPV-BV1 (4) and analyzed as described. A mapping experiment on RNA from induced cells is shown. Transcripts that are correctly initiated from the human β-interferon gene promoter are indicated (human β 5'). In contrast to the −78 deletion, deletions at −73, −66, and −58 produce low levels of β-interferon mRNA in induced cells. When the *IRE* is positioned 100 bp upstream from the deletion breakpoint, a high level of transcription can be seen from the −73 and −66 deletion mutants of the β-interferon gene after induction. The extent of induction as determined by the intensity of the mouse β-interferon signal is equivalent for each cell line (data not shown). In uninduced cells, the level of expression from each construction is also equivalent (data not shown).

≈1.0, indicating that the TATA box is not involved in induction. A similar phenotype is observed with mutations at several positions that flank the TATA-box region (−18, −19, −20, and −31), suggesting that these bases also interact with transcription factors whose activities do not change upon induction.

The Negative Regulatory Domain I (NRD I). This region was previously identified by deletion of *IRE* sequences between −36 and −55 (7). Since deletion of sequences upstream of −55 destroys PRD II, the 5' boundary of the NRD I could not be determined. Point mutations that fall within the negative element should cause an increase in the RTL in uninduced cells. This phenotype is observed with mutations at −63, −62, −61, −54, −52, and −43. The changes at positions −63, −62, and −61 all fall within PRD II, suggesting that the NRD I physically overlaps the positive transcription element. An alternative explanation, that these single-base changes are up mutations for PRD II function, seems unlikely, since the same mutations lead to a significant reduction in the RTLs after induction (see below). The changes at −54 and −52 fall within the same half of a dyad symmetry as the −63, −62, and −61 cluster, and we have previously proposed that this dyad may be involved in negative regulation (7). The −43 change falls within the downstream half of this dyad sequence.

The mutants with single-base changes that cause an increase in the RTL prior to induction display significant differences in their phenotypes after induction. The most common phenotype (seen with mutations at −63, −62G→A, −61A→T, −52, and −43; see Table 1) is a decrease by a factor of ≈2 in the RTL after induction. Since the basal levels are higher than wild-type levels, these mutants have significantly lowered induction ratios. These differences in mutant phenotypes before and after induction suggest that these mutations decrease the affinity of a repressor protein prior to induction and of a positive transcription factor after induction. The up mutations at −52 and −43 do not overlap PRD II, but they still cause a

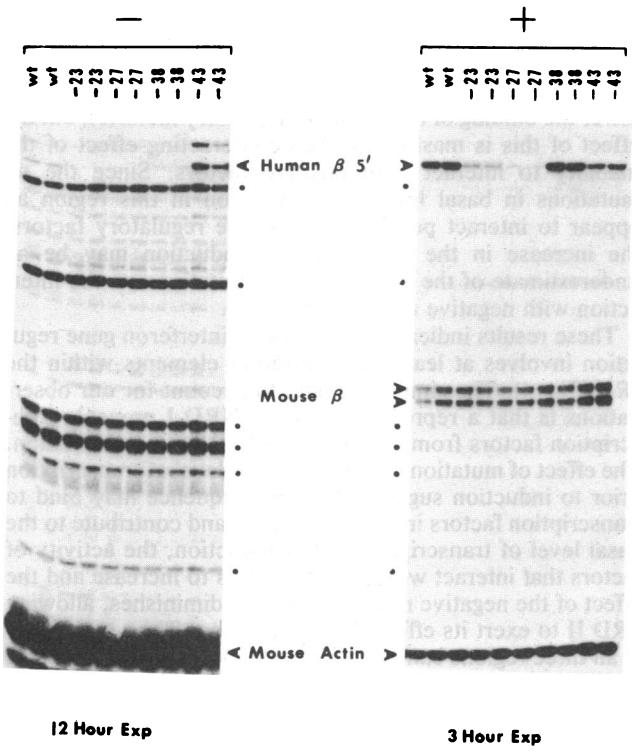

FIG. 2. Analysis of the effects of single-base substitutions on human β-interferon gene expression. The autoradiograph shows a typical mapping analysis of cellular RNA prepared from mouse C127 cell lines stably transformed with a bovine papilloma virus-based vector carrying wild-type or mutant β-interferon genes. Duplicate pools of stable transformants were isolated and analyzed in parallel. RNA analyses of uninduced (*Left*) and induced (*Right*) cells are shown. The expression of a gene with a wild-type promoter is indicated above gel lanes as wt, while mutants are indicated by the number corresponding to the single-base change. The specific changes are listed in Table 1. The actin probe signals (see *Materials and Methods*) are indicated by the side of the gel. Human β 5′, as in Fig. 1; mouse β, mouse β interferon.

decrease in the RTL after induction. Thus, the region between −43 and −52 may contain an additional positive promoter element.

A different phenotype is observed with the mutant with a single-base change at position −54 and also with the mutants with single-base changes at −62G→C and −61A→G. In these cases the induced RTL is higher than the wild-type RTL, although the relative induction ratios are still less than 1.0. These mutations thus affect induction but do not appear to interfere with positive regulatory elements to the same extent as the first class of negative regulatory mutants.

The Positive Transcriptional Domain. Many mutations in PRD I (−75, −72, −71, −70, −68, and −67) cause a decrease in expression in uninduced cells, although mutations at the extremities (−76 and −65) do not have a significant effect. These data suggest that PRD I may be smaller than the −77/−64 region defined by deletion analysis (6). As discussed above, PRD I retains residual inducibility, therefore, we expected to see that these mutations would also have induced RTLs lower than wild type and that the relative RIRs would be less than 1.0. Changes at −75, −70, −68, and −67 lower the induced RTL, and mutations at −70, −68, and −67 cause a lowered ratio of induction relative to wild type, confirming that this region is involved in induction.

Table 1. Tabulation of the relative transcription levels from mutant human β-interferon promoters in mouse C127 cells

Mutation	Uninduced RTL	Induced RTL	RIR
−76A→G	0.95	1.42	1.49
−75G→A	0.49	0.62	1.26
−72G→A	0.54	1.23	2.28
−71T→C	0.76	1.21	1.59
−70G→A	0.37	0.08	0.22
−68A→G	0.37	0.05	0.13
−67A→G	0.40	0.08	0.20
−65T→C	0.79	0.49	0.62
−64G→A	1.05	0.08	0.08
−63G→A	3.11	0.54	0.17
−62G→A	4.42	0.49	0.11
−62G→C	2.31	1.05	0.45
−61A→G	6.86	1.76	0.26
−61A→T	1.61	0.35	0.22
−60A→G	0.44	0.05	0.11
−57T→C	0.91	0.08	0.09
−56C→T	0.41	0.05	0.12
−55C→T	0.82	0.38	0.46
−54T→C	3.84	1.96	0.51
−52T→C	3.63	0.61	0.17
−52ΔT	1.37	0.42	0.31
−51G→A	0.51	0.53	1.04
−51ΔG	1.00	1.10	1.10
−50A→G	1.04	0.70	0.67
−49A→G	0.73	0.67	0.92
−48T→C	1.17	1.07	0.91
−46G→A	0.78	0.78	1.00
−44G→A	1.15	0.71	0.62
−43A→G	2.79	0.35	0.12
−42G→A	0.84	0.89	1.06
−41A→G	0.98	0.62	0.63
−38A→G	0.49	0.88	1.80
−34T→C	0.94	0.82	0.87
−32T→C	1.33	0.80	0.60
−31C→T	0.59	0.47	0.80
−29T→C	0.67	0.67	1.00
−28A→G	0.04	0.05	1.25
−27T→C	0.10	0.06	0.60
−26A→G	0.05	0.05	1.00
−23T→C	0.20	0.23	1.15
−20G→A	0.84	0.66	0.78
−19C→T	0.74	1.12	1.51
−18C→T	0.65	0.37	0.57
−15A→G	0.90	1.09	1.21
−23C/−65C	0.12	0.15	1.25
−42A/−48C	0.63	0.78	1.24
−44A/−46A	0.39	0.43	1.10
−46A/−70A	0.47	0.13	0.27
−49A/−51A	0.33	0.74	2.24
−63A/−65C	0.82	0.71	0.87
−61GΔ[−60/−57]	0.74	0.10	0.13

The data presented in the table are averages from two or more pools of transformed foci. The mutations are listed with the correct nucleotide first, followed by the nucleotide present after the mutation. Thus, −67A→G indicates that the nucleotide at −67 is normally an adenosine but that this has been changed to a guanosine by mutation. For brevity, the double mutations are only indicated by the new nucleotide. Two single-base deletions (at −51 and −52) and a complex mutation (a −61A→G transition coupled to a deletion of nucleotides from −60 to −57) were also analyzed. Two of the mutants (italicized) were generated in a promoter extending to −91 and are compared to a wild-type −91 promoter. The induction ratio of the wild-type gene was about 30-fold in these experiments.

Mutants with single-base changes in the PRD II region might be expected to have a complex phenotype, since the mutations can potentially affect binding to both negative and

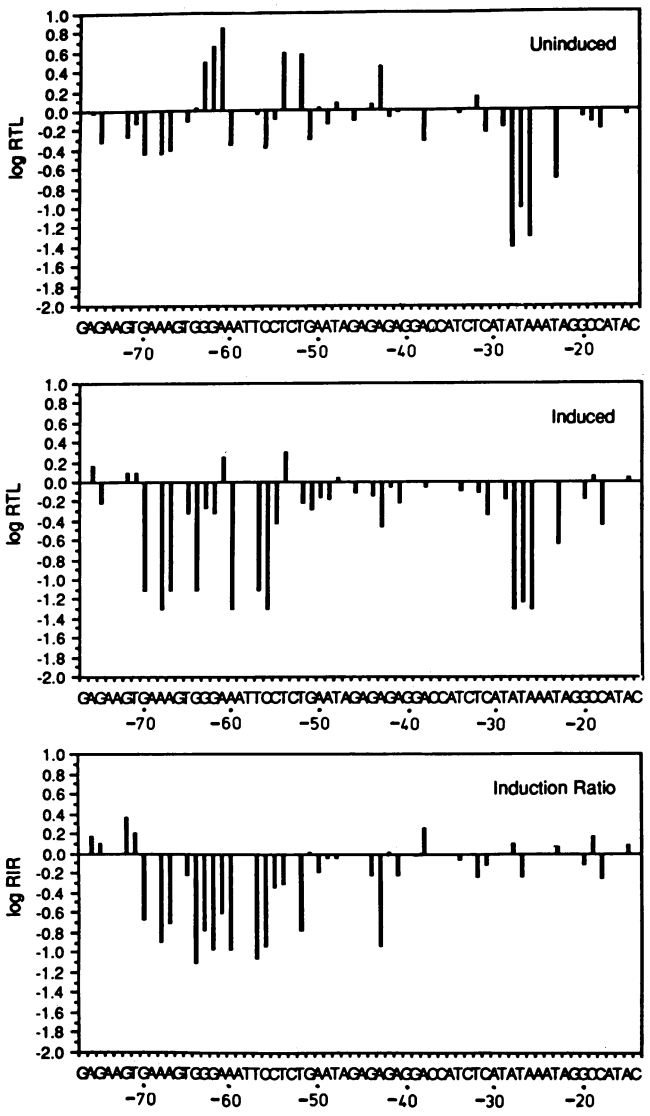

FIG. 3. Histogram of the RTLs and RIRs of mutant β-interferon promoters. The nucleotide sequence of the human β-interferon promoter region mutated in these experiments is shown on the abscissa in each panel, and RTL or RIR is plotted along the ordinate. (*Top*) Expression in uninduced cells. (*Middle*) Expression in induced cells. (*Bottom*) Comparison of RIR. For both RTL and RIR, the wild-type promoter gives a value equivalent to 0.0 (log of 1.0). As a result of using log scales, "up" mutants appear above and "down" mutants appear below the center line. Where data were obtained on more than one base substitution at a given position, only the mutation giving the higher basal level of transcription is shown.

positive regulatory factors. In induced cells, mutations at −64, −63, −62, −61, −60, −57, −56, and −55 are all down relative to wild type, indicating that this region is crucial for expression. These mutations presumably have their effect by lowering the interaction of PRD II with a positive transcription factor. Since this element contributes substantially to the high basal level of expression observed when the negative regulatory element is deleted (7), the failure to see down effects in uninduced cells with many of the mutations in this region indicates that access of transcription factors to this element may be blocked by the negative regulatory factor(s). Mutants with single-base changes at −60 and −56 have a particularly low RTL after induction, suggesting that these mutations severely affect interactions with positive regulatory factors. The observation that these mutants also have a lowered RTL after induction suggests that PRD II contributes to the level of transcription prior to induction. Thus, PRD II must not be fully repressed by negative regulatory factors in uninduced cells. Alternatively, the mutations at −60 and −50 may also lower the binding of the negative regulatory factor(s), but the effect of this is masked by the counteracting effect of the inability to interact with positive factors. Since the up mutations in basal levels of expression in this region all appear to interact poorly with positive regulatory factors, the increase in the RTL prior to induction may be an underestimate of the effect of these mutations on the interaction with negative regulatory factors.

These results indicate that human β-interferon gene regulation involves at least three different elements within the *IRE* (Fig. 4). The simplest model to account for our observations is that a repressor bound to NRD I prevents transcription factors from binding to PRD II prior to induction. The effect of mutations in PRD I on the level of transcription prior to induction suggests that this sequence may bind to transcription factors in uninduced cells and contribute to the basal level of transcription. After induction, the activity of factors that interact with PRD I appears to increase and the effect of the negative regulatory region diminishes, allowing PRD II to exert its effect on transcription. Thus, mutations in all three regions can affect the induction ratios. We do not yet know if the activity of the negative regulatory factor(s) are altered by the induction process. One interesting possibility is that the repressor is displaced from its binding site by an increase in the activity of factors that interact with PRD I. This displacement could result from direct competition or by interactions between factors. Evidence for the existence of cellular factors that specifically interact with PRD I and PRD II has recently been obtained (A. Keller and T.M., unpublished data). The binding activity of a factor that specifically recognizes PRD I is inducible by virus or poly(I)·poly(C).

In summary, the activation of the human β-interferon gene by virus or poly(I)·poly(C) appears to involve specific interactions between both positive and negative regulatory factors and DNA recognition sequences within the *IRE*. Based on the observation that induction occurs in the absence of protein synthesis, it is likely that the activities of these factors require some type of posttranslational modification. Inducible binding activities and negative regulatory DNA

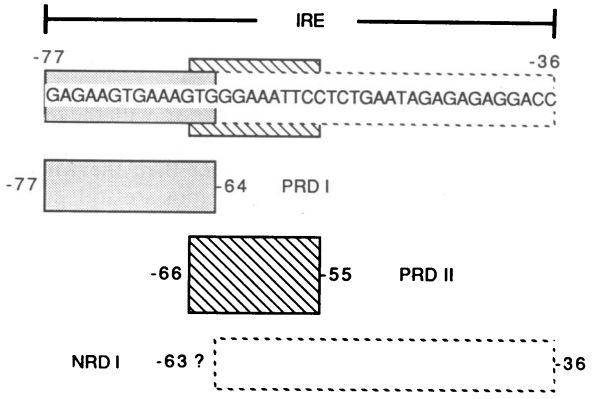

FIG. 4. Positive and negative regulatory domains within the *IRE*. The three domains within the *IRE* that have been shown to be involved in β-interferon gene regulation are outlined as boxed areas. The 5′ and 3′ boundaries of PRD I and PRD II are discussed in the text. The 3′ boundary of NRD I has been delineated by deletion and fusion experiments (7); the 5′ boundary is indicated as −63, since this is the most 5′ point mutation that causes an increase in the level of transcription before induction. However, it is possible that positions 5′ to this are still within NRD I, but these remain undetected.

sequences have also been implicated in the control of other eukaryotic genes (see ref. 12 for a recent review and references). The mechanisms by which these DNA–protein interactions control initiation of transcription are not understood at the present time.

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