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Interferon-induced transcription of a major histocompatibility class I gene accompanies binding of inducible nuclear factors to the interferon consensus sequence

(cis-acting element/DNA-binding proteins)

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ABSTRACT **Interferon (IFN) induces transcription of major histocompatibility class I genes by way of the conserved cis-acting regulatory element, termed the IFN consensus sequence (ICS). Binding of nuclear factors to the ICS was studied in gel mobility shift assays with the 5′ upstream region of the murine *H-2L*^{d} gene. We found that the ICS binds a constitutive nuclear factor present in lymphocytes and fibroblasts regardless of IFN treatment. Within 1 hr after IFN treatment, new ICS binding activity was induced, which consisted of at least two binding activities distinguished by their requirement for *de novo* protein synthesis. Methylation interference and competition experiments showed that both constitutive and induced factors bind to the same ≈10-base-pair binding site within the ICS. Site-directed mutagenesis of *H-2L*^{d}–chloramphenicol acetyltransferase fusion genes showed that mutations in the binding site, but not in other regions of the ICS, abolish transcriptional activation of class I genes by IFN, providing evidence that specific binding of nuclear factors to the ICS is an essential requirement for transcriptional induction. Finally, we show that IFN-inducible genes of various species share a sequence motif that is capable of competing for the nuclear factors identified here. We propose that specific protein binding to the conserved motif represents a basic mechanism of IFN-mediated transcriptional induction of a number of genes.**

Interferons (IFN) α, β, and γ (1, 2) induce the major histocompatibility complex (MHC) class I genes that encode transplantation antigens (3–5), in addition to other genes (3, 6). Previous studies indicated (7, 8) that there is a regulatory element responsible for the enhanced gene expression induced by IFN and that the element is in close proximity to the coding region of the genes. We (9) and others (10, 11) have reported evidence that the IFN-induced transcription of the MHC class I genes is mediated by the conserved IFN consensus sequence (ICS) present in the 5′ upstream region. The ICS was originally described by Friedman and Stark (12) as a homologous region among IFN-inducible genes. It is thus anticipated that trans-acting factors exist that bind to the ICS and are involved in IFN-mediated transcriptional enhancement of MHC class I and other genes. Trans-acting proteins have been identified that are induced in response to steroids and to other agents that induce transcription of target genes (13–16).

MATERIALS AND METHODS

Gel Mobility Shift Assay and Methylation Interference Experiments. Nuclear extracts were prepared from a T-cell line, LH8, and fibroblasts Ltk$^-$ as described by Dignam *et al.* (17) with modifications (18). Aprotinin (Sigma) was added to all buffers, and extraction was performed with five volumes (packed cells) of buffer C (17). A mixture of natural murine IFN-α and IFN-β (IFN-α/β) (Lee Biomolecular Laboratories, San Diego, CA) was added to the culture at 800 units/ml (9, 19) and incubated for the time indicated. To inhibit protein synthesis, cell cultures were treated with cycloheximide (CHX) (35 μg/ml) (Sigma) for 20–30 min (19), prior to addition of and during treatment with IFN-α/β. Oligonucleotides were synthesized with an automated synthesizer, Vega Coder 300, and purified by HPLC. Duplex oligonucleotides that contained the ICS of the *H-2L*^{d} gene (from position −137 to position −167; see Fig. 1*C* and Table 1) and linker sequences at each end were subcloned into the *Bam*HI and *Sph* I site of pUC19. The *Eco*RI–*Hin*dIII fragment of this clone was end-labeled with ^{32}P (18). Nuclear extract protein (12–20 μg) was incubated with the end-labeled probe (5000 cpm, 0.1–0.5 ng) and 2.5 μg of poly(dI-dC)·poly(dI-dC) (Pharmacia) in 20–30 μl of a solution of 10 mM Tris·HCl (pH 7.5), 50 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, and 10% (vol/vol) glycerol for 15–20 min at room temperature. The mixtures were electrophoresed in a 4% polyacrylamide gel in 0.4× Tris/EDTA/borate buffer (18) at 150 V for 2 hr. Methylation interference experiments were performed as described (18, 20).

Site-Directed Mutagenesis and Chloramphenicol Acetyltransferase (CAT) Assay. Site-directed mutagenesis was performed as described by Taylor *et al.* (21) by using the ≈400-base-pair (bp) *Xba* I–*Hin*dIII fragment of the *H-2L*^{d} gene containing the ICS and the class I regulatory element (CRE) (22, 23) (see Fig. 1*C*) that was cloned into phage M13mp19. To confirm accurate mutagenesis, each mutant clone was sequenced by the Sanger method. Mutant ICS CAT fusion genes were constructed by replacing the *Xba* I–*Hin*dIII fragment of the wild-type pL^{d}CAT1.4K (23) with the mutant fragments (see Table 3). Mutant CAT genes were introduced transiently into Ltk$^-$ cells (9) by adding 4 μg of CAT gene and 16 μg of carrier pUC19 to 5 × 10^6 cells in Bes (*N*,*N*-bis[2-hydroxyethyl]-2-aminoethanesulfonic acid) buffer (23). About 16 hr after transfection, cells were treated with IFN-α/β at 800 units/ml for 12–14 hr, and CAT activity of each culture was measured.

RESULTS

Binding of Constitutive and Inducible Nuclear Factors to the ICS. The IFN-enhanced transcription of murine MHC class I genes is controlled at least in part by the ICS (9–11) that is

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Abbreviations: IFN, interferon; MHC, major histocompatibility complex; ICS, IFN consensus sequence; CHX, cycloheximide; CAT, chloramphenicol acetyltransferase; CRE, class I regulatory element.

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homologous to the Friedman–Stark ICS found in several IFN-inducible genes (12). The ICS in the MHC class I genes partially overlaps the CRE (22) that controls developmental expression of MHC class I genes (Fig. 1*C*) (10, 23). The binding of nuclear factors to the ICS was studied by using a ^{32}P-labeled probe corresponding to the ICS in a gel mobility shift assay. As shown in Fig. 1*A*, a specific retarded band, designated B1, in addition to a faster migrating nonspecific band were identified by using untreated LH8 extract. The B1 band (but not the nonspecific band) was eliminated by a competitor, ICSW3, that encompassed the entire 29-bp ICS and by a shorter competitor, ICSW103 (Table 1 and Fig. 1*B*). No competition was seen by oligonucleotides corresponding to the CRE (Fig. 1*B*). Increasing the amounts of nuclear extract (Fig. 1*A*) or prolonged exposure did not reveal additional retarded bands. The retarded bands seen before and after IFN treatment (see below) were the results of protein binding to the ICS, since they were completely abrogated upon treatment of the extracts with proteases (data not shown). A new slower-migrating band, B2 (Fig. 1*A*), was detected in extracts from IFN-treated cells that was not detectable in untreated cells, even with the highest amount of extracts tested (22 μg). In addition, IFN treatment increased the intensity of the B1 band by ≈5-fold. The induction of B2 and the increased intensity of B1 were observed within 1 hr (earliest time tested) after IFN treatment and lasted up to 8 hr (the latest time tested). Formation of both the B1 and B2 complexes from IFN-treated cells and the B1 complex from untreated cells was competed completely by the entire ICS and by the shorter competitor (Fig. 1*B*). Since no alterations were noted in the gel mobility shift pattern with the CRE probe after IFN treatment (Fig. 1*A*, lanes 7 and 8), the inducible binding activity described above was not due to a nonspecific change that occurred in nuclei after IFN treatment. Analyses of IFN-treated extracts on a heparin-agarose column yielded two separate peaks for B1, whereas untreated extracts yielded only one of the two peaks (data not shown). This result and data presented below suggest that the B1 formed in IFN-treated extracts has two overlapping protein components, the constitutive binding component and the new binding component induced by IFN. The inducible component is designated B3 in Fig. 1.

Effect of Protein Synthesis Inhibitor. We tested the effect of CHX on induction of the ICS-binding proteins to examine the requirement of *de novo* protein synthesis. LH8 cells were preincubated with CHX at 35 μg/ml for 30 min and were further treated with IFN in the presence of CHX. As shown in Fig. 2, binding of the constitutive factor (B1) in untreated cells was not influenced by CHX treatment (lanes 1 and 3). Moreover, IFN-induced B2 was also CHX resistant (lane 7), suggesting that induction of B2 is largely independent of new protein synthesis (Fig. 2). In contrast, the increased intensity of B1 (i.e., the B3 complex) seen with IFN was no longer detected after CHX treatment (lane 7). Results indicate that B1 from IFN-treated cells consists of two components and that the INF-induced component is sensitive to CHX.

Binding Site Within the ICS Assigned by Methylation Interference Analysis and by Competition. To determine the precise binding site for the constitutive and inducible nuclear factors, we performed methylation interference experiments. As shown in Fig. 3, methylation of the probe at guanosines −148 and −146 in the noncoding strand strongly interfered with its ability to form the B1 complex in untreated extracts. Methylation of the probe at guanosine −143 was often noted to weakly interfere with B1 complex formation. In contrast, clear interference by methylation was not readily detected in the coding strand of the probe derived from the B1 complex. The identical interference pattern was found with the mixture of B1 and B3 from extracts obtained after IFN treatment (Fig. 3). Likewise, the probe that formed the B2 complex after IFN treatment showed the same interference as B1 in the noncoding strand, showing methylation interference strongly at residues −148 and −146 and weakly at residue −143. In the coding strand, this probe reproducibly elicited interference at position −152. Guanosine residues that showed interference are indicated by asterisks in Table 1. These results indicate that constitutive and inducible factors bind roughly to the same region, the right-center part of the ICS, although the binding property of the B2 complex appears to be subtly different from that of B1 complex. To further examine the protein-binding site, we tested a series of mutant ICS oligonucleotides for their

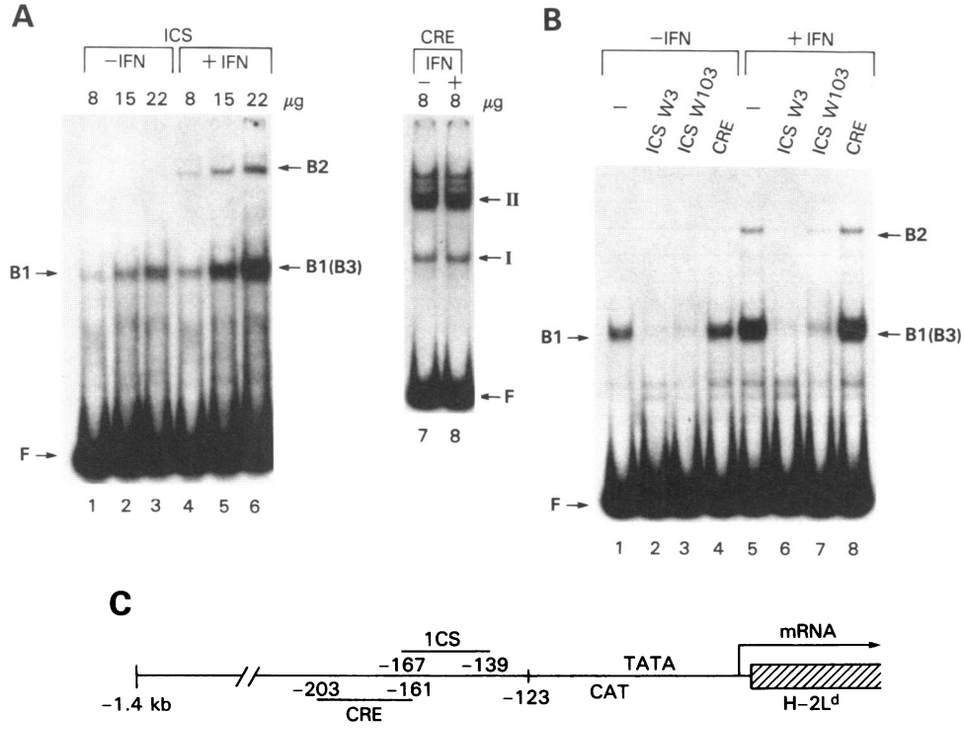

FIG. 1. Binding of nuclear factors to the ICS. (*A*) Binding of nuclear factors was analyzed by gel mobility shift assay with LH8 nuclear extracts either untreated (lanes 1–3 and 7) or treated (lanes 4–6 and 8) with IFN (800 units/ml) for 3 hr. The end-labeled ICS probe (lanes 1–6) and CRE probe (lanes 7 and 8) (see *C*) were incubated with 8 μg (lanes 1, 4, 7, and 8), 15 μg (lanes 2 and 5), and 22 μg (lanes 3 and 6) of LH8 nuclear extracts and electrophoresed. (*B*) Specificity of ICS-binding factors. The end-labeled ICS probe was incubated with LH8 nuclear extracts in the absence (lanes 1 and 5) or presence of 20 ng of competitor oligonucleotides, ICSW3 (lanes 2 and 6), ICSW103 (lanes 3 and 7), and CRE (lanes 4 and 8) (see Table 1 and *C*) and electrophoresed. (*C*) Diagram of the 5′ upstream region of the *H-2L^d* gene.

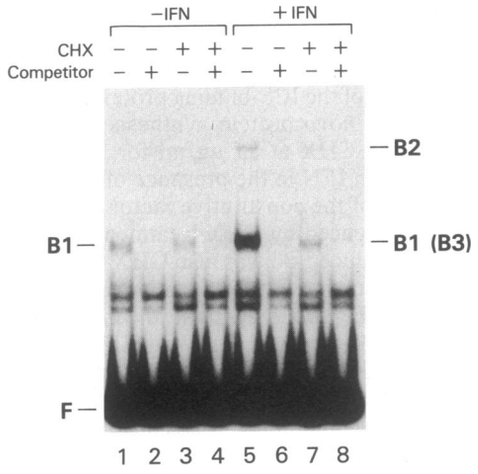

FIG. 2. Effect of CHX on the IFN-inducible protein binding to the ICS. The end-labeled ICS probe was incubated with either untreated (lanes 1–4) or IFN-treated (lanes 5–8) LH8 nuclear extracts in the absence (lanes 1, 3, 5, and 7) or presence (lanes 2, 4, 6, and 8) of 20 ng of ICSW3 competitor oligonucleotides and electrophoresed. LH8 cells were preincubated with CHX for 30 min and then cultured with or without IFN for 2 hr in the presence of CHX.

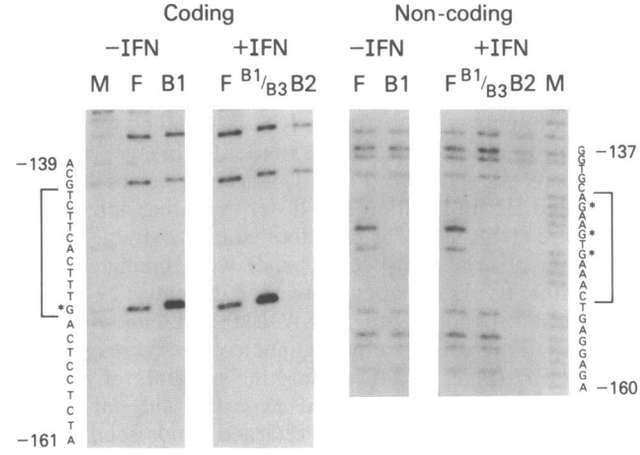

FIG. 3. Methylation interference experiments. The coding and noncoding strands of the probe were end-labeled with polynucleotide kinase and the Klenow fragment of DNA polymerase I, respectively. The probe was partially methylated and incubated with either IFN-treated or untreated LH8 nuclear extracts. DNA–protein complexes corresponding to B1, B1/B3, and B2 bands were separated from free probes by gel electrophoresis. Guanosine residues showing methylation interference are indicated by asterisks. Lane M is the G+A chemical cleavage sequencing ladder. Lane F represents free probe.

ability to compete with the wild-type probe for protein binding. As shown in Table 1, competitor oligomers had several substitutions in the ICS. Gel mobility shift experiments were performed in the presence of a 20- to 50-molar excess of unlabeled mutant competitor with ^{32}P-labeled wild-type probe and nuclear extracts from LH8 and Ltk$^-$ cells. Results of competition are shown in Table 1. Oligonucleotides with substitutions upstream from residue −153 (mutant competitors M11, M25, and M96) competed, as did mutations in the downstream sequence from position −140 (M19 and M98). In contrast, oligomers containing mutations in the region between residues −152 and −143 failed to compete for protein binding (M9, M23, M93, and M97), indicating that the binding site resides in this area. The constitutive and induced factors evidently bound to the same region, as the competition pattern for all oligomers was identical in extracts before and after IFN treatment. Competitors M7 and M21 that had the same substitutions from residues −144 to −146 competed only

Table 1. Summary of competition experiments

Oligonucleotide	Sequence	Competition		
			+ IFN	
		− IFN (B1)	B1/B3	B2
	−160 −150 −140			
Native ICS (*H-2L^d*)	GATTCCCCATCTCCTCAGTTTCACTTCTGCACCTA			
	CTAAGGGGTAGAGGAGTCAAAGTGAAGACGTGGAT			
Competitor				
W3	GATTCCCCATCTCCTCAGTTTCACTTCTGCACC	+	+	+
M7	——————————GAA—AGC—	±	±	±
M9	————————CAG—GAC————	−	−	−
M11	—CAG——GCA——————————	+	+	+
M19	——————————————AGC—	+	+	+
M21	——————————GAA———	±	±	±
M23	——————————GAC———	−	−	−
M25	————————CAG———————	+	+	+
M93	——————————GA————	−	−	−
M96	——————AG——————————	+	+	+
M97	——————————TG———————	−	−	−
M98	——————————————TA——	+	+	+
W103	————————————	+	+	+
ISG15	————————GG—TCC—T	+	+	+
(2′-5′)oligoadenylate synthetase	—TG———GT——CT—	+	+	+

Asterisks in the native *H-2L^d* ICS indicate the position of methylation interference. The binding domain assigned by competition tests is boxed. Methylation interference at position −152 of the coding strand was seen only with the B2 complex. Mutant competitors (double strand) had base substitutions at the indicated positions. A dash indicates the same base as the native ICS. All competitors except for W103, *ISG15* (24) and (2′-5′)oligoadenylate synthetase (25) had linker sequences, GATCT and GCATG, at the 5′ and 3′ ends of the coding strand, respectively. Competition was tested in gel mobility shift experiments by using 20 ng of the mutant oligomers. +, Complete competition; ±, partial competition; −, no competition.

Table 2. Effect of ICS mutations on transcriptional induction of MHC class I gene by IFN

CAT construct	Enhanced CAT activity I	II	III	IV	V	VI	Mean ratio	*P*
pL^{d}CAT123	1	1	1	1	1	1	1	—
pL^{d}CAT1.4K	8.7	2.2	1.6	5.1	4.0	2.6	4.0	—
pL^{d}CAT1.4K(M93)	1.6	1.6	1.0	3.4	1.2	1.7	1.6	<0.016
pL^{d}CAT1.4K(M96)	4.6	3.3	1.9	8.4	2.4	2.1	3.8	<0.656
pL^{d}CAT1.4K(M97)	1.6	1.4	1.1	1.4	0.9	1.3	1.3	<0.016
pL^{d}CAT1.4K(M98)	7.4	5.9	2.7	4.0	3.9	1.6	4.3	<0.656

ICS mutants corresponding to competitors (M93–M98) in Table 1 were tested for the ability to enhance CAT transcription in response to IFN in transient CAT assay in Ltk$^-$ cells. The level of CAT enhancement is shown as the ratio of CAT activity normalized to pL^{d}CAT123 before and after IFN treatment in six experiments (I–VI). The *P* value was calculated from the one-tailed signs test by using the binomial distribution (26). Mutant constructs are compared to wild type based on the null hypothesis where $P = 0.50$ and $n = 6$.

partially for the protein binding. This may suggest a certain level of flexibility in nucleotide requirement at these positions. Ltk$^-$ cell extracts manifested retarded bands essentially identical to those of LH8 extracts before and after IFN addition. Competition by the mutant oligomers found by Ltk$^-$ nuclear extract was also the same as that seen with LH8 cell extracts (data not shown).

Functional Properties of the Binding Site. To evaluate the functional significance of the binding activity observed above, we constructed a series of mutant CAT constructs in which CAT transcription was directed by the *H-2L^d* promoter containing the mutated ICS and tested their ability to enhance CAT activity in response to IFN. The wild-type CAT construct pL^{d}CAT1.4K (23) has a 1.4-kilobase 5′ flanking region of the *H-2L^d* gene that contains the ICS, CRE, and the 123-bp promoter region (Fig. 1*C*). Two-base substitutions that corresponded to the mutant ICS competitors described in the preceding section (M93, M96, M97, and M98 in Table 1) were introduced into pL^{d}CAT1.4K by site-directed mutagenesis (21) [e.g., pL^{d}CAT1.4K(M93) contained the same substitutions as those in M93]. No other alterations were introduced in the remaining sequences. As a negative control, pL^{d}CAT123 containing the 123-bp promoter region without the ICS and unresponsive to IFN (9) was also tested. pL^{d}CAT1.4K and pL^{d}CAT123 were shown to initiate transcription (ref. 9 and data not shown) at the standard MHC class I initiation site, regardless of IFN treatment. CAT activity of these mutants was assayed after transient transfection of Ltk$^-$ cells after IFN treatment for 12–14 hr. Results are summarized in Table 2. Because IFN treatment caused ≈10% nonspecific reduction in CAT activity in pL^{d}CAT123 and other CAT contructs that do not respond to IFN (9), the level of transcriptional enhancement was estimated after normalizing the raw counts to the values obtained by pL^{d}CAT123. As expected, the wild-type pL^{d}CAT1.4K showed enhanced CAT activity, an average of a 4-fold increase in six independent experiments. Mutants pL^{d}CAT-1.4K(M93) and pL^{d}CAT1.4K(M97) that had mutations within the binding site failed to elevate CAT activity in response to IFN. Conversely, pL^{d}CAT1.4K(M96) and pL^{d}CAT1.4K-(M98) with mutations outside the binding site showed enhanced CAT activity after IFN treatment that was not significantly different from the wild-type pL^{d}CAT1.4K (Table 2). A CRE mutant in which positions −173 and −174 were changed from TC to AA was not affected in responsiveness to IFN (data not shown). Thus, mutations (M93 and M97) that abolished the binding of all three factors also abrogated the ability to respond to IFN *in vivo*. These results demonstrate a strong correlation between protein binding and functional activity of the ICS, indicating that the induced protein binding after IFN treatment is directly responsible for transcriptional activation of the MHC genes.

DISCUSSION

We have identified nuclear proteins that specifically interact with the ICS. Two types of binding activity were detected,

Table 3. Conserved binding motif present in IFN-inducible genes

Gene	Ref(s).	Position of 5′ end	Sequence	Position of 3′ end
Entire ICS	12		TTCN NACCTCNGCAGTTTCTCCTCTCT	
Mouse				
H-2L^d	9,23	−167	TTCCCCATCTCCTCAGTTTCACTTCTGCA	−139
H-2K^b	22	−164	TTCCCCATCTCCACAGTTTCACTTCTGCA	−135
H-2D^d	11	−165	TTCCCCATCTCCTCAGTTTCACTTCTGCA	−136
β_2m	22	−120	ACTTTCCCATTTTCAGTTTCATGTTCTTA	−148
Human				
HLA-DR	12	−591	TGTTGAACCTCAGAGTTTCTCCTCTCAT	−564
HLA-A3*	12		TTCCCCAACTCCGCAGTTTCTTTTCTCTC	
MT-II	12	−631	CTCTGGACCTGCAGTTTCTCCTCTCTA	−603
Factor B	32	−154	GGTGGGACTTCTGCAGTTTCTGTTTCCTT	−126
ISG15	24	−81	CCAAATTTGGCTTCAGTTTCGGTTTCCCT	−109
ISG54	24	−113	TTCACTTTCTAGTTTCACTTTCCC	−90
6-16	31	−85	TTTCTGCACTCTGCAGTTTCATTTTCCCC	−113
(2′-5′)oligoadenylate synthetase	25	−74	CTTGGACTGCTGTTGGTTTCGTTTCCTCA	−102
Consensus			AGTTTCNNCTCC T TT	

The binding motif is boxed. β_2m, β_2-microglobulin.
*The cap site of this gene has not been determined.

binding by a constitutive factor and binding inducible by IFN treatment. Thus, this work presents an additional system of inducible trans-acting factors similar to those described in other eukaryotic genes (14–16, 27–29). Many of these factors—e.g., the heat shock activator protein (27), NF-κB (28, 29), and AP-1 and AP-2 (14, 15)—show binding to DNA sequences that are not occupied prior to induction. Binding of the induced factors to the same region of the ICS where the constitutive factor is already bound distinguishes this induction system from the above examples. The induction of B2 complex by IFN was insensitive to CHX treatment, indicating that this binding is induced as a result of post-translational modification of a preexisting protein. It may be envisaged that the preexisting protein that forms B1 before IFN treatment undergoes a structural modification such as phosphorylation (30) to generate B2 after IFN treatment. Alternatively, B2 may represent a protein distinct from B1 that replaces the constitutive protein after IFN stimulation. In either case, formation of B2 may be relevant to MHC class I gene induction by IFN, since class I mRNA induction is also resistant to CHX (3, 19). The other inducible binding activity, designated B3 (Figs. 1 and 2), was sensitive to CHX, indicating dependence on *de novo* protein synthesis.

Studying a series of mutant ICS sequences and the corresponding CAT constructs, we show complete concordance between binding of nuclear factors and elicitation of the function. These results provide strong evidence that the inducible protein binding to the ICS, presumably the CHX-resistant component, is directly responsible for increased transcription of MHC class I genes. The observation that the gel shift pattern of the CRE is not altered by IFN treatment is consistent with the previous finding that the ICS alone is capable of acting as an inducible enhancer (9). Nevertheless, sequences other than the ICS could affect IFN regulation; for example, the CRE appears to modulate responsiveness to IFN (10, 11). A similar interplay of two regulatory sequences was noted for induction of (2′-5′)oligoadenylate synthetase (25).

We determined the binding site of the ICS to be from position −152 to position −142 of the ICS. Both constitutive and induced factors bound roughly the same 10-bp sequence, a relatively short segment of the ICS. Even though the entire ICS of the MHC class I genes shows extensive sequence homology, both protein binding and functional elicitation require only a small subregion of the ICS.

IFN response elements of additional IFN-inducible genes, namely human (2′-5′)oligoadenylate synthetase (25), *ISG15* and *ISG54* (24), 6-16 gene (31), and complement factor B (32) have been elucidated. The sequences of these genes are not similar to the sequence of the entire ICS. In light of the limited sequence requirement for protein binding found in this study, we reexamined upstream sequences of these and other IFN-inducible genes to look for a conserved motif. As shown in Table 3, a clear motif, AGTTTCNNT(C)TC(T)CT, emerges from the functional regions of these genes. It is of note that in some genes no similarity is found on either side of the flanking sequence. As shown in Table 1, we found that the binding motif of the human *ISG15* and (2′-5′)oligoadenylate synthetase gene is capable of competing for murine factors that bind to the ICS of the MHC class I gene. From these observations we suggest that this motif signifies the basic structural requirement for specific binding of nuclear factors and for transcriptional induction of not only MHC but also other IFN-inducible genes. Our results also indicate that the ICS-binding proteins share structural similarity among various species, in concert with the previous transfection studies with MHC class I genes of various species (7, 8).

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