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Regulation of Gene Expression by Interferons: Control of H-2 Promoter Responses

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The magnitude of the response to interferons and the requirement for individual elements in the promoter of the H-2D^d gene were shown to be cell-specific and dependent on the type of interferon used. Three DNA sequences in the promoter were found to bind murine nuclear factors. Two of these sequences are in functionally defined enhancer regions and also bind to the transcription factor AP-1. The third sequence is part of the region involved in interferon regulation and is homologous to the enhancer element of the interferon β gene. A model for interferon regulation of H-2 promoters is discussed.

INTERFERONS (IFNs) AFFECT SUCH cellular processes as anti-viral responses, cell growth, differentiation, and gene regulation. Type I IFNs (α and β) and type II IFN (γ) are synthesized by different cells, and it is likely that they use different mechanisms to elicit their different cellular responses (*1–3*).

Major histocompatibility (H-2) class I genes are regulated by type I and type II IFNs (*4*). The murine H-2 class I proteins are highly polymorphic, 45-kD polypeptides that function during antigen presentation as cell-surface recognition molecules for cytotoxic T lymphocytes (*5*) and are expressed on most somatic tissues at tissue-specific levels (*6*). Early embryonic tissues do not express H-2 antigens, although expression can be induced by IFNs (*7*). Adult brain cells respond to IFN γ with a 30-fold increase in the expression of H-2 antigens; heart and kidney cells with a 13- to 17-fold increase; and the tissues that have the highest basal levels of H-2 antigens, like the spleen and lymph nodes, show the least change in expression (*8*).

IFN regulation of class I gene expression involves at least two mechanisms, one dependent on sequences upstream and the other on sequences downstream of the H-2 promoter region (*9–12*). IFN induction utilizing the upstream sequences accounts for less than a twofold enhancement of class I gene expression, corresponding to less than 40% of the entire IFN response in L cells (*10*). It is dependent on the presence of a 37-bp region, designated the IRS (IFN response sequence) (*11*) that lies between nucleotides 159 and 122 upstream of the transcription initiation site of the H-2D^d gene (*10*). Induction of class I expression by type I IFNs in L cells requires the concomitant presence of a second sequence located just upstream of the IRS (*10, 11*); this sequence is not required in NIH 3T3 cells (*12*). These conclusions were based on cells that express high endogenous levels of H-2 antigens that are only moderately inducible (2.5- to 8-fold) by IFNs. We have assessed the relative activities of the upstream regulatory sequences in cells in which H-2 antigen expression is highly inducible by IFNs.

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Table 1. Induction of CAT constructs in different cell types by murine type I and type II IFNs. The enhancement factor is a ratio of specific CAT activity (CAT activity per protein concentration) in cells treated with IFN to that in untreated cells. It is independent of the cotransfection efficiency in the individual pools of cells transformed with a mixture of *neo* gene and CAT constructs in all cell lines used. Ten cloned cell lines from a BL5 transfected cell pool were analyzed as controls; all were positive for CAT expression. The basal levels of CAT gene expression and their ability to respond to IFNs varied moderately between different cloned transfectants. Clone data confirmed results observed on pooled transfectants. Levels of class I antigens on different cell lines were measured with different antibodies by radioimmunoassay (*32*), and compared on a per cell basis with the levels of the class I antigen expression on spleen cells of the same haplotype. L cells expressed 5- to 10-fold higher levels than H-2^k C3H spleen cells, BLK SV cells expressed 3- to 6-fold more than H-2^b C57BL/6 spleen cells, MPC 11 cells expressed 1.5- to 6-fold higher levels than H-2^{d}Balb/c spleen cells, and BL5 cells expressed lower levels of H-2^b antigens than C57BL/6 spleen cells (ranging from 0.25-fold to comparable levels of expression).

Construct	Enhancement factor							
	BL5 + IFN γ		BL5 + IFN (α + β)		BLK SV + IFN γ		BLK SV + IFN (α + β)	
	Range	$\bar{x}$ ± SD (*n*)	Range	$\bar{x}$ ± SD (*n*)	Range	$\bar{x}$ ± SD (*n*)	Range	$\bar{x}$ ± SD (*n*)
L^{d}CAT	3.0– 5.2	4.5 ± 0.7 (3)	2.5– 6.6	4.5 ± 1.1 (4)	5.3– 9.9	8.0 ± 0.9 (5)	4.7–8.0	6.6 ± 0.8 (4)
D^{d}CAT	9.7–10.4	10.1 ± 0.2 (3)	9.9–16.7	12.3 ± 1.5 (4)	5.5–11.8	7.9 ± 0.9 (6)	3.8–8.3	5.8 ± 1.0 (4)
Δ-385CAT	5.2– 6.1	5.5 ± 0.2 (3)	4.9– 6.2	5.6 ± 0.4 (3)				
Δ-236CAT	1.9– 4.9	2.9 ± 1.0 (3)	3.4– 5.6	4.5 ± 0.7 (3)				
Δ-159CAT	2.0– 6.4	4.1 ± 0.5 (9)	4.9– 6.3	5.8 ± 0.2 (6)	1.9– 7.0	3.2 ± 0.6 (8)	0.7–1.4	1.1 ± 0.1 (7)
Δ-122CAT*	0.7– 1.1	1.0 ± 0.1 (3)	0.7– 1.2	1.0 ± 0.1 (4)	0.8– 1.6	1.1 ± 0.1 (5)	1.1–1.2	1.2 ± 0.1 (4)

*Endogenous levels of H-2 antigens in Δ-122CAT transfectants were inducible by IFNs to the same degree as in the untransfected cells. The ability of these molecules to respond to IFN served as an internal control for the noninducible phenotype of Δ-122CAT gene.

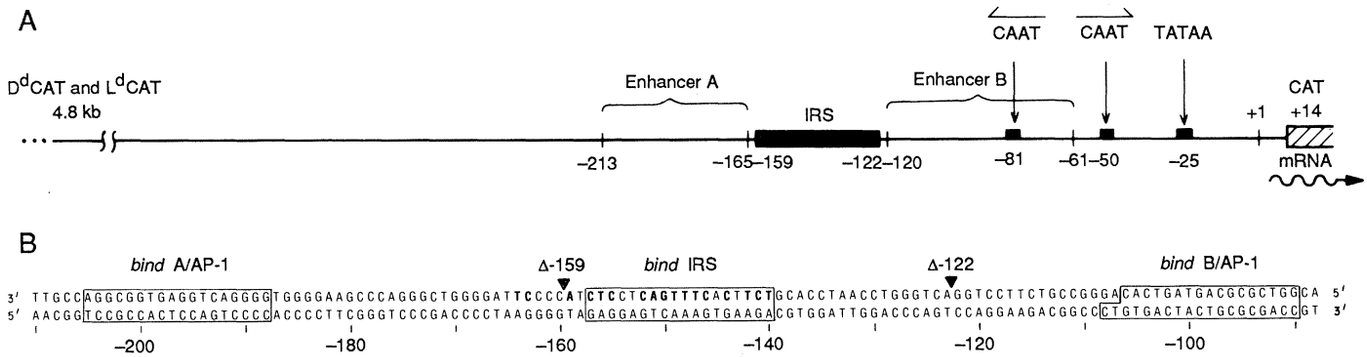

Fig. 1. Regulatory elements in class I promoter regions. (**A**) The D^{d}CAT and L^{d}CAT constructs contain 4.8-kb-long restriction fragments derived from the 5′ flanking regions of the H-2L^d or H-2D^d genes, fused 15 nucleotides downstream from their putative transcription start site to the CAT structural gene. The numbering is shown relative to the cap site that is defined by analogy to the mapped H-2K^d gene transcription start site (*33*). The transcription start sites for D^{d}CAT, Δ-385CAT, and Δ-159CAT have been mapped by ribonuclease I protection in noninduced and induced L cells. In these transfectants transcription initiated at the predicted site; in Δ-122 CAT transfectant ~12% of transcription was initiated at a cryptic pBR322 site. In BL5 cells transcription initiated on Δ-122 CAT template at the predicted site. (**B**) DNase I footprinting. Regions protected by MPC 11 nuclear extracts are boxed. Locations of the homologies between the H-2D^d protected regions and the binding sites of the positive and negative acting proteins of the IRE (*25*) are indicated. The AP-1 core recognition site (*21*) is also shown. Bold letters in the H-2D^d sequence correspond to the homology with the Friedman and Stark consensus sequence (*24*). The locations of the deletion endpoints of Δ-159 and Δ-122 are shown. (**C**) Presence of the enhancer motif of the IFN β gene IRE in the 5′ flanking region of H-2D^d and IFN α and γ genes. The sequence of the IFN α consensus is derived from Ryals *et al.* (*27*), and of the IFN γ gene promoter from Gray and Goeddel (*28*). Homologous bases are indicated by dots. (**D**) Comparison of the DNA sequence homology between the negative regulatory element of the IFN β gene IRE and the H-2D^d IRS. (**E**) Comparison of the DNA sequence homology between the AP-1 core recognition motif and the AP-1/MPC 11 binding sites *bind* A and *bind* B. NC, noncoding; C, coding.

The chloramphenicol acetyltransferase (CAT) gene was fused to H-2 class I promoters and IFN enhancement of CAT activity was studied in transfected cell lines derived from C57BL/6 mouse embryos (H-2^b haplotype). BL5 is a primary fibroblast line derived from 14- to 16-day-old embryos (*13*). Its basal level of endogenous H-2^b antigens is low compared to L cells (Table 1) and it can be induced 15- to 30-fold by IFNs. BLK SV (*14*) is an embryonic fibroblast line immortalized by SV40 transformation. IFN enhances H-2 messenger RNA (mRNA) levels of BLK SV cells at least 15-fold although they express basal levels of endogenous H-2^b antigens similar to H-2 K^k levels on uninduced L cells (*15*).

CAT constructs carrying deletions of the promoter region of the H-2D^d gene were selected from the original deletion set used to transfect L cells (Fig. 1) (*10*). The construct Δ-159CAT contains an IRS sufficient for the induction of the class I promoter by type II IFN in L cells, but the region necessary for a type I IFN response has been deleted; Δ-122CAT lacks the IRS and upstream regions and is not inducible by either type of IFN. Two additional deletion constructs, Δ-385 and Δ-236, which contain all of the known IFN regulatory elements of H-2D^d, were studied in BL5 cells. The deletion constructs and the parental D^{d}CAT plasmid carrying a 4.8-kb fragment from 5′ flanking region of H-2D^d gene were transfected stably into the two embryonal cell lines. The L^{d}CAT plasmid derived from 5′ flanking region of H-2L^d gene was also tested (*16*).

In BL5 and BLK SV cells the type II IFN enhancement factor for D^{d}CAT and L^{d}CAT was up to tenfold higher than it is in L cells (Table 1). The deletion constructs (except Δ-122CAT) were also inducible although their response was reduced compared to the parental D^{d}CAT. The reduction may reflect a partial requirement for sequences in the deleted region or may be due to the presence of the pBR322 sequences fused upstream of the deletion junctions.

When type I IFN was used the magnitude of the response in BL5 and BLK SV cells was similarly high (Table 1). In agreement with previous data for L cells (*10, 11*), in BLK SV cells Δ-159CAT was not induced by a mixture of murine α + β IFN or by a purified human recombinant IFN α, while D^{d}CAT and L^{d}CAT constructs were inducible. In contrast, in BL5 cells the expression of Δ-159CAT was inducible to an equal degree by both types of IFNs (Table 1). The simplest interpretation of this result is that the IFN regulation of H-2 D^d promoter in BL5 cells is independent of the upstream regulatory element; this may be a consequence of differences between the cell lines, such as the availability of trans-acting factors.

To determine whether trans-acting factors can interact with the functionally defined regulatory regions of the H-2D^d promoter,

deoxyribonuclease I (DNase I) protection analyses (*17*) were done. Since BL5, BLK SV, and L cells did not yield sufficient quantities of active extracts, most studies were carried out with nuclear factors derived from the mouse myeloma cell line MPC 11 (*18*). It expresses high levels of endogenous H-2 antigens (comparable to L cells) and it can be easily grown in suspension to a high density. H-2 antigens on MPC 11 cells are weakly inducible (< twofold) by IFNs. One or more nuclear factors from MPC 11 cells bound to sequences −157 to −140, within the IRS region (Figs. 1 and 2). This site was designated ***bind*** IRS for the IFN response sequence binding region. In addition, protection was observed at sites designated ***bind*** A (−205 to −188) and ***bind*** B (−108 to −90), which are located within the H-2D^d promoter regions homologous to the class I enhancer fragments A and B of the H-2K^b gene (*19*) (Figs. 1 and 2). The DNase I footprint pattern is specific by the following criteria: the protection of all the sites was observed on both strands of the H-2D^d template, and the MPC 11 extracts used in our studies did not footprint randomly chosen fragments of bacterial plasmid DNA.

The presence of IRS-binding activity in untreated extracts from MPC 11 cells suggests that IFN-regulated transcriptional enhancement may involve activation of an IRS-specific factor present in cells in a latent form prior to IFN exposure. Alternatively, MPC 11 cells may constitutively express endogenous IFNs that induce DNA binding factors involved in IFN regulation. The latter hypothesis is plausible because constitutive production of autogenous type I IFNs was reported in several lymphoma cell lines in which H-2 antigen expression was elevated (*20*).

Next, we determined if the mammalian transcription factor AP-1 (*21*) binds to the same regions as the MPC 11 nuclear extracts. Purified AP-1 was incubated with the H-2D^d promoter in DNase I protection experiments (Fig. 2). Two strongly protected regions are apparent: one of them coincides with the ***bind*** A and the other with the ***bind*** B site. The footprinted regions appear to be identical as determined with either crude MPC 11 extracts or purified AP-1. Two additional points strengthen this suggestion. First, there is significant homology between ***bind*** A, ***bind*** B, and the AP-1 core consensus sequence (Fig. 2). In addition, the ***bind*** A and ***bind*** B sites lie within regions with demonstrated enhancer activity, as would be predicted for AP-1 binding sites (*22*). AP-1 also protects four other lower affinity sites (Fig. 2); the significance of this binding remains to be established.

The differences observed in the magnitude of response in different cell lines may reflect availability and inducibility of transacting transcription factors, IFN receptor expression and signal transduction, or the proportion of cells responding to IFN in a population. In both embryonal fibroblastic lines, the magnitude of the promoter-controlled response was up to ten times greater than in L cells. Since the uninduced levels of endogenous H-2 gene expression in BLK SV cells are approximately tenfold higher than in BL5 cells, it is apparent that a low basal level of promoter activity alone does not dictate high IFN inducibility. Consistent with this conclusion is the finding (*12*) that the H-2L^d promoter is inducible in NIH 3T3 cells to the same degree as in BL5 or BLK SV cells in our study.

The cis-acting sequences of the H-2D^d promoter necessary to elicit an IFN response vary among different cell lines. The action of type I IFN in L and BLK SV cells required the presence of a sequence upstream of the IRS region, while in BL5 cells the IRS region was sufficient for induction. The action of type II IFN on H-2 promoters was

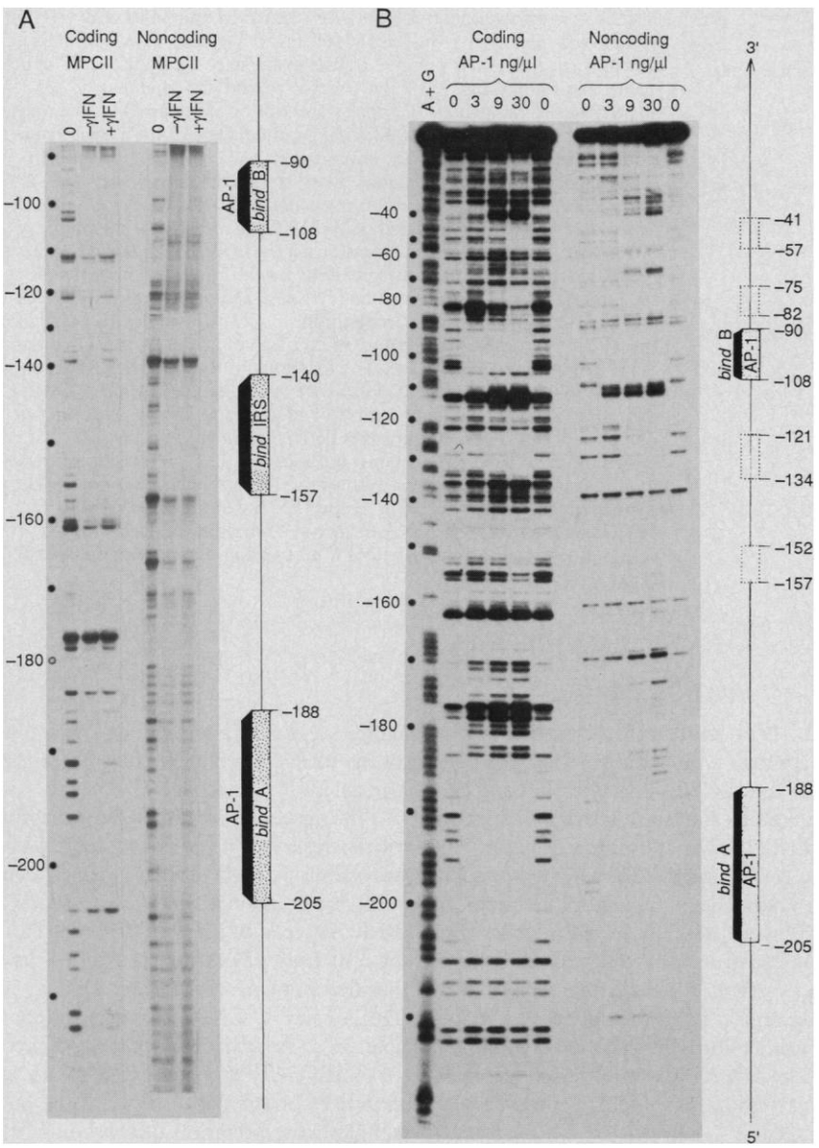

Fig. 2. DNase I protection analysis of the H-2D^d flanking sequences with MPC 11 nuclear extracts (**A**) or AP-1 transcription factor purified from HeLa cells (**B**) (*35*). The coding and noncoding DNA strands were labeled by T4 DNA kinase or by DNA polymerase (Klenow fragment) end-filling at an Eco RI site within the pBR322 vector, ~260 bp upstream from the start point of transcription. For reference the kinase-treated strand was sequenced by means of G+A chemical cleavage reactions. Each labeled strand was digested by DNase I (0 lanes) or incubated with 100 to 150 μg of MPC11 extracts made from uninduced cells (−γIFN) or IFN γ–induced cells (+γIFN). AP-1 concentrations used in footprint analysis in (B) are indicated above the gel lanes. The positions of strongly protected sites are indicated by continuous lines, positions of weakly protected sites by dotted lines.

independent of the upstream sequence in all three cell lines tested. These findings lend support to the seemingly conflicting reports on *cis*-acting elements required for H-2 promoters' responses to IFNs (*10–12*).

While the extensive circumstantial evidence points to the *bind* IRS being involved in IFN regulation our data did not demonstrate that the MPC11 factor that binds to this region controls H-2 promoter expression (*23*). The IRS sequence that interacts with the MPC11 binding factor is contained within the 30-bp IFN consensus sequence (*24*) identified by a homology search of the 5′ flanking regions of genes inducible by type I/type II IFN. We have found striking homology between the region protected in our footprinting assay and the inducible response element (IRE) of the human IFN β gene (*25*) (Fig. 1). This element, which is active in either orientation, and controls the induction of the IFN β gene by virus or double-stranded RNA, consists of an enhancer and a repressor motif. Induction of the IRE is thought to involve the release of repressor molecules, thus permitting the binding of a positive-acting protein to the enhancer motif (*25*). The IRS/IRE homology includes the hexamer AAGTGA, present in multiple copies in the 5′ flanking region of the IFN β gene (*26*). The hexamer repeated in tandem was reported to function as a virus-inducible enhancer in the absence of the IRE repressor motif and was proposed to bind a positive regulatory factor (*26*).

The enhancer motif of the IFN β gene IRE is present in the class I IRS on the noncoding strand. The same IRS/IRE homology was found in the promoters of the IFN α (*27*) and γ (*28*) genes (Fig. 1), and in all genes containing Friedman and Stark's IFN consensus sequence (*24*). Thus, IFN enhancement of class I gene expression (and of other IRS/IRE containing genes) may be controlled in part by an IFN-mediated induction of the IRE enhancer-binding transcription factor. This idea is particularly appealing considering that IFN can positively influence its own regulation (*29*).

Another level of control in the IFN regulation of class I genes may be exerted by the dissociation of specific repressors in a situation analogous to IFN β gene IRE induction. The differential response to type I and type II IFNs could then be explained by the use of the IRS enhancer in conjunction with different negative elements responsive to a specific IFN. One interpretation of the requirement for two cis-acting elements in the action of type I IFN on class I genes is that the upstream element (located 5′ of −159) binds to a specific repressor which dissociates after type I IFN induction. There is evidence that a repressor motif exists in this region because the sequence of the H-2L^d promoter from −195 to −159 can act as a negative element in some cell types (*30*). In BL5 cells the repressor-binding factor may be absent and so the induction of class I genes might directly reflect the induction of the IRE-enhancer-binding factor—hence the observed independence of the upstream region (*31*).

Other models may explain the requirement for two cis-acting elements in a response to type I IFN. For example, Israel *et al.* (*11*) proposed that type I IFN regulation of H-2 class I promoters involves a potentiating effect of the IRS on transcription controlled from enhancer A. It is possible that different mechanisms are used to control the IFN response of H-2 promoters in different cells.

Previous and present studies suggest that the transcriptional regulation and IFN induction of H-2 antigen expression is complex. The multiplicity of cis-acting elements which can contribute to the regulation of these genes in a cell-specific fashion suggests that combinatorial effects of trans-acting factors determine the ultimate level of expression. Since quantitative variation in H-2 class I antigen expression influences the acquisition of tolerance, major histocompatibility complex restriction, and the efficiency of cell-mediated immune responses, class I promoters may have evolved multiple cis-acting elements responsive to different regulatory pathways to ensure their specific and sensitive regulation.

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16. The 5′ flanking regions of the H-2L^d and H-2D^d genes differ from each other in several positions (*10*) [A. Kimura, A. Israël, O. LeBail, P. Kourilsky, *Cell* **44**, 261 (1986)], but the DNA sequence of the IFN regulatory elements (IRS and upstream region) is identical.
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22. Additional studies are necessary to determine if AP-1 protein functions as a transcription initiation factor for H-2 genes. Since AP-1 expression can be modulated by tumor promoter 12-O-tetradecanoylphorbol-13 acetate (TPA) it would be important to test if H-2 promoters are sensitive to TPA.
23. The *bind* IRS is the only sequence within the functionally defined IRS yet shown to interact with nuclear factors. No additional binding sites were detected when extracts from IFN γ–treated MPC 11 cells were used to footprint the H-2D^d promoter (Fig. 1). A region homologous to the *bind* IRS is not always essential for IFN γ responsiveness. For example, the *bind* IRS homology in the 5′ flanking regions of H-2 class II genes can be deleted without loss of the promoter's ability to respond to IFN γ. [J. Boss and J. Strominger, *Proc. Natl. Acad. Sci. U.S.A.* **83**, 9139 (1986)]. This may reflect a requirement for different IFN regulatory elements in different genes or cell types.
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29. In some cases induction of IFN gene expression by viral or double-stranded RNA can be augmented by prior exposure of cells to IFN [called IFN priming, T. Fujita and S. Kohno, *Virology* **112**, 62 (1981); T. Enoch, K. Zinn, T. Maniatis, *Mol. Cell. Biol.* **6**, 801 (1986)]. This phenomenon is dependent on protein synthesis and requires an IFN-inducible factor. We speculate that priming may be due to IFN mediated induction of a positive IRS/IRE binding factor.

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31. The functional significance of a partial homology between the IRS region and the repressor region of the IRE (Fig. 1) remains to be evaluated, since dissociation of the repressor from the IFN β IRE is controlled by virus or double-stranded RNA, not by IFN itself.
32. Radioimmunoassays were performed as described (*10*) with the antigen-specific monoclonal antibodies 28-14-8, (αH-2D^b); 20-8-4, (αH-2K^b); or 11-4-1, (αH-2K^k).
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34. Time course studies determined the appropriate time lengths for IFN incubations. Endogenous H-2 antigen expression on BL5 and BLK SV cells was fully induced in 48 hours by type I and 60 hours by type II IFN (determined by radioimmunoassay). CAT gene expression driven by the H-2 class I promoter was fully induced in 12 hours by type I IFN, and 36 hours by type II IFN. All inductions were stable for up to 5 days. Therefore 3-day IFN incubation periods were chosen for these experiments. IFN α + β (1000 unit/ml), and IFN γ (20 unit/ml), were fully saturating and used for all experiments described. Murine IFN α + β was purchased from LEE BioMolecular. CAT assays were performed as described (*10*).
35. MPC11 nuclear extracts and footprinting reactions were performed as described in B. Korber, L. Hood, and I. Stroynowski [in *Major Histocompatibility Genes and Their Role in Immune Function*, C. S. David, Ed. (Plenum, New York, in press)]. AP-1 purification and footprinting reactions were performed as described (*21*).
36. We thank K. Blackburn and M. Krempin for superb professional assistance with tissue culture, and D. Bohmann for advice during AP-1 purification. We thank M. Shepard of Genentech for providing the recombinant murine IFN γ, D. Vapnek of AMGen for human α consensus IFN, and T. Hansen for monoclonal antibodies. Supported by a National Research Service Award (T32GM7616) from the National Institute of General Medical Sciences and by an NIH grant AI 19624.

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Salivary Gland Lysates from the Sand Fly *Lutzomyia longipalpis* Enhance *Leishmania* Infectivity

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Leishmaniasis is a parasitic disease transmitted by phlebotomine sand flies. The role of sand fly saliva in transmission of the disease was investigated by injecting mice with *Leishmania major* parasites in the presence of homogenized salivary glands from *Lutzomyia longipalpis*. This procedure resulted in cutaneous lesions of *Leishmania major* that were routinely five to ten times as large and contained as much as 5000 times as many parasites as controls. With inocula consisting of low numbers of *Leishmania major*, parasites were detected at the site of injection only when the inoculum also contained salivary gland material. This enhancing effect of sand fly salivary glands on cutaneous leishmaniasis occurred with as little as 10 percent of the contents of one salivary gland of one fly. Material obtained from other bloodsucking arthropods could not mediate the phenomenon.

PROTOZOAN PARASITES OF THE GENUS *Leishmania* are transmitted through the bite of infected phlebotomine sand flies. The sand fly can transmit several different species of *Leishmania*, causing different forms of the disease in the infected mammalian host. Cutaneous forms are characterized by the development of lesions at sites in the skin where the infected sand fly has probed for a blood meal (*1*). In a manner analogous to other bloodsucking arthropods, sand flies salivate into the skin of the host in the course of obtaining a blood meal. The saliva of arthropods in general (*2*), and sand flies in particular (*3*), has several potent pharmacological activities, including apyrase activity and factors that inhibit platelet aggregation and induce erythema. We therefore investigated the possibility that these activities, or others not yet described, in the salivary glands of the sand fly might affect transmission of *Leishmania*.

We worked with the sand fly *Lutzomyia longipalpis*, a known vector for visceral leishmaniasis in South America and the only sand fly for which pharmacological data are available for its salivary secretion (*3*). The parasite we used was *Leishmania major*, the causative agent of cutaneous leishmaniasis in the Old World and a parasite for which there is an established murine model of infection. Promastigotes of *Le. major* were mixed with lysates of the salivary glands of *Lu. longipalpis*. An inoculum consisting of 10^5 parasites and 0.5 of one salivary gland was injected into the footpads of either CBA mice [animals genetically resistant to infection with *Le. major* (*4*)] or into BALB/c mice [animals genetically susceptible to the disease (*4*)]. Lesions on mice that received a mixture of *Le. major* promastigotes and lysates from the sand fly salivary gland were substantially more severe than lesions on control animals both in CBA and in BALB/c mice (Fig. 1).

We then examined the effect that lysates of the salivary glands of *Lu. longipalpis* would have on the course of lesions induced in mice injected with decreasing numbers of *Le. major* promastigotes. To this end, CBA mice were injected in the footpad with inocula consisting of 10^4, 10^3, 10^2, or 10^1 *Le. major* promastigotes admixed with the lysate of 0.5 of one salivary gland. We found (Fig. 2) that the effect on the course of cutaneous leishmaniasis was especially pronounced when the infecting inoculum contained low numbers of parasites. Lesions on mice receiving 10^2 or 10^1 *Le. major* promastigotes mixed with salivary gland lysates were often five to ten times as large as lesions on control mice receiving parasites alone.

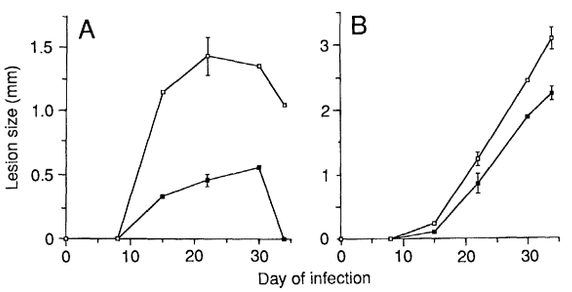

Fig. 1. Enhancement of the infectivity of *Le. major* by lysates of the salivary glands of *Lu. longipalpis*. Groups of five (**A**) CBA/T6 or (**B**) BALB/c mice (Jackson Laboratory) were injected subcutaneously in one hind footpad with either 10^5 *Le. major* promastigotes in 20 μl of 0.1% bovine serum albumin, *p*H 7.0 (BSA) (Sigma) in phosphate-buffered saline (PBS) (■) or 10^5 promastigotes in 20 μl of BSA/PBS containing 0.5 of one gland of *Lu. longipalpis* (□). *Leishmania major* promastigotes (LV 39) were maintained as described (*7*); parasites from stationary phase (*6*) cultures were used in all the experiments. *Lutzomyia longipalpis* were reared by the procedure of Modi and Tesh (*8*). Salivary glands from mated, non–blood-fed, 5- to 7-day-old adult female sand flies were dissected, placed in 0.1% BSA in water (*p*H 7.0), and frozen to achieve complete disruption (*3*). The glands were stored at −70°C until use, at which time they were brought to isotonicity by the addition of 10× PBS. We determined lesion size by measuring, with a vernier caliper, the thickness of the infected footpad compared to the thickness of the contralateral uninfected footpad. The values given are means ± SEM.

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