5. A. P. Maloney and H. D. Van Etten, *J. Cell. Biochem.* **12C**, 284 (1988).
6. K. M. Weltring, B. G. Turgeon, R. C. Garber, unpublished data.
7. B. G. Turgeon, R. C. Garber, O. C. Yoder, *Mol. Cell. Biol.* 7, 3297 (1987).
8. M. M. Yelton, J. E. Hamer, W. E. Timberlake, *Proc. Natl. Acad. Sci. U.S.A.* **81**, 1470 (1984); A. Upshall *et al.*, *Mol. Gen. Genet.* **204**, 349 (1986).
9. *Cochliobolus heterostrophus* and *A. nidulans* mycelia were grown overnight (*10*), cell walls were removed, and protoplasts were transformed (*7, 8*) with 10 μg of plasmid DNA per 10^7 protoplasts. After treatment with polyethylene glycol, each suspension of transformed protoplasts was diluted to 2.5 ml with osmoticum (*8*) and 50-μl portions were plated as described previously (*7, 8*).
10. B. G. Turgeon, R. C. Garber, O. C. Yoder, *Mol. Gen. Genet.* **201**, 450 (1985).
11. *Cochliobolus heterostrophus* strain C2 and *A. nidulans* strain UCD1 were chosen for this study because they carry mutations that provide biological containment for recombinant DNA molecules. Strain UCD1 is auxotrophic for arginine, methionine, tryptophan, *p*-amino benzoate, and biotin, and has *yA*$^-$, an allele that prevents synthesis of the wild-type green pigment. For plant tests, inoculum was supplemented with the required nutrients. Strain C2 has *alb1*$^-$, an allele for albinism that causes reduced epidemiological fitness in the greenhouse (*12*) and failure to propagate in the field, although the strain is normally pathogenic to maize in a growth chamber (*13*); *alb1*$^-$ strains are very sensitive to ultraviolet radiation, which may explain their inability to survive in nature. In addition, since the sexual stage of *C. heterostrophus* has never been found in nature and there is an abundance of heterokaryon incompatibility alleles in the field population (*14*), it is highly unlikely that recombinant DNA molecules could be passed to other organisms in the event that a strain escaped and survived briefly in the field.
12. K. J. Leonard, *Phytopathology* **67**, 1273 (1977).
13. W. E. Fry, O. C. Yoder, A. E. Apple, *ibid.* **74**, 175 (1984).
14. J. Leach and O. C. Yoder, *J. Hered.* **74**, 149 (1983).
15. *Cochliobolus heterostrophus* transformants were selected on medium containing hygromycin B at 50 μg/ml. Two of these grew when exposed to hygromycin B at concentrations as high as 1 mg/ml, suggesting high copy numbers of inserted plasmids. DNA analysis (*16*) showed that strain C2-P had 18 copies of pUP1 and strain C2-V had 11 copies of pUCH1. *Aspergillus nidulans* transformants tend to have multiple copies of transforming plasmids (*17*); since strain UCD1-P was found (*16*) to have 12 copies, strain UCD1-V was assumed to have a comparable number, although copies were not actually counted.
16. Plasmid copy number was estimated by slot-blot analysis (*18*) and quantified by scintillation counting. For *C. heterostrophus*, serial dilutions of equal amounts of genomic DNA from strains C2-P, C2-V, and C2 were spotted on nitrocellulose membrane and hybridized against the *C. heterostrophus* promoter 1 sequence of pUCH1, known to be a single-copy sequence in the wild-type genome (*7*). For *A. nidulans*, serial dilutions of genomic transformant DNA and plasmid pD1 were spotted and hybridized to pD1. The most dilute concentration of plasmid was adjusted to represent one copy of *PDA1*/genome, given a genome size of 2.6×10^4 kb (*19*).
17. K. Wernars *et al.*, *Curr. Genet.* **9**, 361 (1985); J. M. Kelly and M. J. Hynes, *ibid.* **12**, 21 (1987).
18. F. C. Kafatos, C. W. Jones, A. Efstratiadis, *Nucleic Acids Res.* 7, 1541 (1979).
19. W. E. Timberlake, *Science* **202**, 973 (1978).
20. D. C. Straney and H. D. Van Etten, *J. Cell. Biochem.* **13E**, 49 (1989).
21. H. D. Van Etten *et al.*, *Physiol. Plant Pathol.* **16**, 257 (1980).
22. W. Schäfer and O. C. Yoder, unpublished data.
23. S. R. Long and D. W. Ehrhardt, *Nature* **338**, 545 (1989); C. L. Diaz, L. S. Melchers, P. J. J. Hooykaas, B. J. J. Lugtenberg, J. W. Kijne, *ibid.*, p. 579.
24. H. D. Van Etten and P. S. Matthews, *Physiol. Plant Pathol.* **25**, 149 (1984).
25. P. S. Matthews, unpublished data.
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Interleukin-1 Costimulatory Activity on the Interleukin-2 Promoter Via AP-1

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Interleukin-1 (IL-1) is a major regulator of inflammation and immunity. IL-1 induces T lymphocyte growth by acting as a second signal (together with antigen) in enhancing the production of interleukin-2 (IL-2). An IL-1–responsive element in the promoter region of the human IL-2 gene was similar to the binding site for the transcription factor AP-1. IL-1 enhanced expression of c-*jun* messenger RNA, whereas the antigenic signal enhanced messenger RNA expression of c-*fos*. Thus, the two components of the AP-1 factor are independently regulated and the AP-1 factor may serve as a nuclear mediator for the many actions of IL-1 on cells.

IL-1 AFFECTS MANY CELL TYPES; IT INduces the acute phase response of hepatocytes, fever mediated by the hypothalamus, and fibroblast and T lymphocyte growth [reviewed in (*1*)]. An IL-1 receptor has been cloned and characterized (*2*), but little is understood of the intracellular mechanisms mediating the action of IL-1 (*3*).

We examined the nuclear basis of IL-1 action on T cell activation. The mouse T lymphoma LBRM-331A5 (LBRM) reflects the two-signal requirement of normal T cells (*4*) in the competence phase of lymphocyte activation. One signal is derived from antigen engaging the T cell receptor [mimicked by phytohemagglutinin (PHA)], the second signal is provided by IL-1 via a high-affinity receptor (*2*). These two signals result in IL-2 production, which is regulated at the level of transcription (*5*).

Activation of the IL-2 gene appears to be mediated through a transcriptional enhancer region between −548 and +52 upstream of the transcription start site [reviewed in (*6*)]. The enhancer region of the human IL-2 gene is 85% homologous with the mouse IL-2 gene, which permits expression in murine cells. To look for an IL-1–responsive element on the IL-2 promoter, we used the −548- to +39-bp region of the human IL-2 gene linked to the chloramphenicol acetyltransferase (CAT) indicator gene (*7*); this construct, termed "IL-2–CAT," was transiently transfected into LBRM cells. The IL-2–CAT construct responded to IL-1 plus PHA six- to sevenfold above controls of PHA alone or IL-1 alone (Table 1). Control experiments with plasmids that contained enhancer elements of the Rous sarcoma virus upstream of reporter genes (pRSV-luciferase or pRSV-CAT (*9, 10*) did not increase luciferase or CAT activity in response to IL-1 (*8*); thus, IL-1 had no nonspecific effect on viability, transfection efficiency, or stability of CAT mRNA or protein.

To identify the IL-1–responsive element within the −548- to +39-bp enhancer region, we constructed 5′ deletion mutants of IL-2–CAT and transiently transfected LBRM cells. The −218 to +39 fragment retained full IL-1 responsiveness (about six to seven times background PHA stimulation) (Table 1A). Therefore, the region 3′ to −218, which must harbor an IL-1–responsive element, was examined with internal deletion mutants. The deletions −107 to −76 or −83 to −42 contained one of the two recently defined T cell receptor-responsive elements (−93 to −63) (*11*), and had about 50% of the CAT activity of the full-length IL-2–CAT, although an IL-1 response was maintained; this was expected, since PHA delivers its signal via T cell receptors, as verified by antibodies against the CD-3 complex, which mimic the PHA effect on this cell (*8*). The deletion −218 to −176 abrogated the IL-1 effect (Table 1B),

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indicating that this region contained an IL-1–responsive element.

Position −185 has the sequence TCAGTCAG in both human and murine IL-2 genes. This sequence is similar to the motif TGAGTCAG, which confers 12-*O*-tetradecanoyl phorbol-13-acetate (TPA) inducibility upon heterologous promoters and is recognized by the nuclear factor AP-1 (*12, 13*). We used gel mobility shift assays to determine whether PHA and IL-1–induced nuclear factors could bind to a double-stranded oligonucleotide (designated "A") that spanned −192 to −169 of the human IL-2 gene (including the AP-1–like binding site). One major complex in nuclear extracts prepared from PHA and IL-1–stimulated cells bound to the AP-1–like site (Fig. 1A) (the extract was prepared after 5 hours when maximal IL-2 gene expression occurred). Competition with a 5 to 40 molar excess of cold AP-1 oligonucleotide (designated "A") inhibited the binding of the nuclear factor, whereas a control sequence of the same length ("B") failed to compete; thus the binding was specific for the AP-1–like sequence.

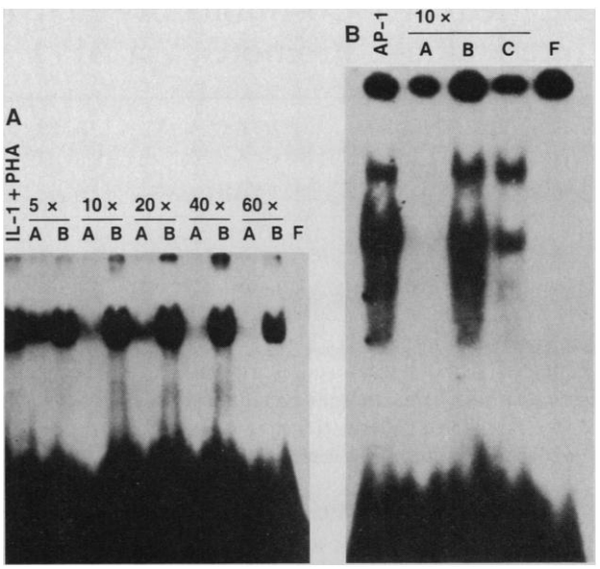

Fig. 1. Gel retardation assays of nuclear extracts from stimulated LBRM cells. (**A**) LBRM cells were stimulated for 5 hours with PHA and IL-1, then nuclear extracts were prepared (*24*). Gel retardation assays (*25, 26*) were done by incubating 2 μg of nuclear extracts for 30 min at 21°C in 20 μl of reaction buffer containing 3 μg of poly-d(I-C), 13% glycerol, 13 m*M* Hepes (*p*H 7.9), 0.6 m*M* dithiothreitol (DTT), 300 ng pBR322, 65 m*M* KCl and 1 ng of double-stranded synthetic 24-bp oligonucleotide (ON) that had been end-labeled with ^{32}P. This AP-1 ON spanned the region −192 to −169 of the human IL-2 enhancer region (which included the AP-1–like recognition site). Complexes were separated on polyacrylamide gels with 45 m*M* tris-borate, 1 m*M* EDTA, *p*H 8.0, buffer. Two unlabeled ON, designated "A" and "B," were used for competition: A was the same as the labeled ON and B was an unrelated 24-bp ON from the IL-2 promoter that spanned residues −81 to −68. Lane F contained free labeled probe. (**B**) Purified AP-1 factor from HeLa cells (*12*) was incubated with the same end-labeled probe and electrophoresed as in (A), except that no poly-d(I-C) or pBR322 were included. Three competing ON (A, B, and C) were used: A and B were the same as in (A), and C was an AP-1 point mutant (TCAGACAGA).

The AP-1–like site of the IL-2 gene was specifically recognized by an AP-1 factor that was derived from HeLa cells and purified using the AP-1 sequence from the collagenase gene (*12*) (Fig. 1B). Binding was specific: there was complete competition with the unlabeled IL-2 AP-1 sequence ("A"), reduced competition with a sequence that contained a point mutation ("C"), and no competition with an unrelated sequence ("B").

We then examined whether binding of IL-1–induced factors to the AP-1–like site resulted in gene activation. Three AP-1 binding sites (from the human collagenase gene) that were linked to a thymidine kinase minimal promoter and the CAT gene (*12*) were transiently transfected into LBRM cells. IL-1 acted on an AP-1 site; addition of IL-1 and PHA resulted in about three to four times the CAT activity compared to control levels induced by PHA alone, IL-1 alone, or medium (*8*) (Table 1C). The positive control, TPA stimulation, had a similar increase above background. As a negative control, a construct lacking the AP-1 site was not induced by IL-1.

The major protein component of the AP-1 factor is encoded by the c-*jun* proto-oncogene (*14*). The DNA-binding capacity of the c-*jun* protein is strongly enhanced by interaction (via a leucine "zipper") with the product of the c-*fos* proto-oncogene (*15*). Protein synthesis is required to induce the IL-2 gene in LBRM cells (*16*). We tested whether IL-1 and PHA induced either c-*fos* or c-*jun* mRNA before the earliest expression of the IL-2 gene occurs [about 45 min (*8*), see also (*6*)] (Fig. 2). IL-1 induced an increase in c-*jun* mRNA (2.7 kb) after 30 min of stimulation, but PHA did not. On the other hand, *jun-B*, another member of the *jun* family that can contribute to the AP-1 complex (*17*), was neither induced by IL-1 nor by PHA (*8*). In contrast, PHA strongly induced c-*fos* mRNA in 30 min, but IL-1 had a minimal effect. A TPA control enhanced c-*fos* but not c-*jun*. De novo protein synthesis was not required for increases in c-*fos* or c-*jun* mRNA, because cycloheximide treatment did not block the effect.

Table 1. IL-1 induces CAT activity in LBRM cells transfected with IL-2–CAT or AP-1–CAT. Cells were transiently transfected by electroporation (15 μg of DNA/1.5 × 10^7 cells, 500 μFd, and 0.2 V) with a Bio-Rad electroporation apparatus (*21*). Twenty-four hours later, viable cells were purified by Ficoll-hypaque. This pool of transfected cells was then divided into different groups and stimulated for 24 hours with PHA (0.05%), a combination of PHA and mouse recombinant IL-1α (Hoffmann–La Roche, 100 U/ml), or 12-*O*-tetradecanoyl phorbol-13-acetate (TPA, 30 ng/ml). The transfected constructs had portions of the IL-2 promoter linked to CAT (*7*). Full-length (−548 to +39), 5′, and internal deletion mutants are shown. In (**A**), SV-40 enhancer sequences were also present. In (**B**), no SV-40 sequences were present. In (**C**), a collagenase AP-1 trimer linked to a thymidine kinase promoter and CAT (*12*) was compared to a control lacking AP-1. CAT activity was measured in cell lysates incubated for 15 hours and expressed as percent acetylation (*22*). Data are the mean of three experiments in which CAT enzyme activity was normalized according to the amount of plasmid uptake [measured by blot analysis (*23*)]. Stimulatory index (Stim. index) is (PHA + IL-1)/PHA. Data are the mean and SD from four to eight independent experiments (indicated by "*N*"), some of which were normalized according to DNA uptake, others by luciferase activity (cotransfection with pRSV-luciferase).

Construct	Stimuli			Stim. index	*N*
	PHA	PHA + IL-1	TPA		
A					
Full	5.1	17.6	—	6.6 ± 2.9	8
d240–162	2.9	4.8	—	1.8 ± 0.4	4
d169–101	16.8	43.3	—	5.8 ± 1.7	3
d107–76	2.0	12.0	—	6.2 ± 1.3	4
d83–42	2.3	9.6	—	4.1 ± 1.0	4
d218′	7.0	42.0	—	6.5 ± 1.5	4
B					
Full	1.3	7.1	—	6.1 ± 4.2	4
d218–176	0.7	1.0	—	1.2 ± 0.6	4
C					
AP-1	0.8	2.7	3.0	3.4 ± 0.4	4
(control)	0.25	0.32	0.34	1.3 ± 0.05	4

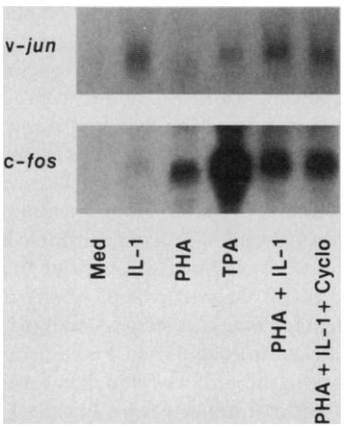

Fig. 2. IL-1 induces c-*jun* whereas PHA induces c-*fos*. LBRM cells were stimulated for 30 min as in Table 1. Cells were incubated in cycloheximide (30 μg/ml) for 30 min (this inhibited 95% of total protien synthesis measured by [^{35}S]methionine incorporation) and stimulated with PHA and IL-1 for another 30 min. Total cytoplasmic RNA was extracted (*27*) and fractionated on a 1% agarose gel (*28*). Blots were hybridized with ^{32}P-labeled mouse c-*fos* or v-*jun* probes. Hybridization with human actin cDNA probes verified uniformity of loaded RNA.

Multiple functional sequences in the IL-2 enhancer have been defined in the past, some specifically responsive to T cell receptor triggering or to TPA stimulation (*6, 11, 18*). We now define a sequence element similar to the AP-1 binding site of the collagenase gene and recognized by a purified AP-1 factor that is responsible for mediating the IL-1 effect on the IL-2 promoter. This element has only weak activity (*7*) in Jurkat cells, a T cell leukemia line that does not require IL-1 to produce IL-2 (it lacks IL-1 receptors). Although the AP-1 site appears to be necessary in LBRM cells, it is probably not sufficient for IL-2 gene expression; TPA alone, like PHA and IL-1, can activate the AP-1 factor but cannot induce the IL-2 gene; this may be because it fails to stimulate the T cell receptor element (−93 to −63) that responds to PHA as a costimulus. In a study of a different cell line, the EL4 thymoma, TPA induced an AP-1 factor that preferentially bound to a different AP-1 binding site on the murine IL-2 promoter (at −150) (*18*). This site, however was not required in LBRM cells, since we deleted the segment from −169 to −101 and retained the IL-1 effect (Table 1A).

TPA and IL-1 have similar actions (*1*) and we show now that IL-1 can act through AP-1 recognition sites. However, there are clearly differences in mode of action between TPA and IL-1. Whereas TPA activates protein kinase C, IL-1 does not (*3*). TPA alone activated the AP-1 trimer, whereas IL-1 required PHA as a costimulus (Table 1C). TPA mainly induced c-*fos*, not c-*jun*, whereas IL-1 induced c-*jun*, not c-*fos* (Fig. 2). TPA can activate the AP-1 factor post-translationally (*19*), and from the current study, it is likely that IL-1 activates AP-1 through de novo synthesis of c-*jun*. Finally, TPA is much less effective than IL-1 as a costimulus for the IL-2 promoter (*4, 8*).

Tumor necrosis factor–α (TNF-α), a cytokine that has actions similar to IL-1, acts via the AP-1 site on the collagenase promoter (*20*). AP-1–like sites occur in many IL-1–inducible genes, including IL-1 itself, IL-6, colony-stimulating factor 1, TNF-α, ornithine decarboxylase, transforming growth factor–β, β nerve growth factor, serum amyloid A, and serum albumin. The AP-1 factor can be produced by most cell types, and thus may provide a general mechanism for the multiple effects of IL-1.

REFERENCES AND NOTES

1. J. J. Oppenheim, E. J. Kovacs, K. Matsushima, S. K. Durum, *Immunol. Today* **7**, 45 (1985).
2. J. E. Sims *et al.*, *Science* **241**, 548 (1988); S. K. Dower *et al.*, *J. Exp. Med.* **162**, 501 (1985).
3. R. T. Abraham, S. N. Ho, T. J. Barna, D. J. McKean, *J. Biol. Chem.* **262**, 2719 (1987).
4. S. Gillis and S. B. Mizel, *Proc. Natl. Acad. Sci. U.S.A.* **78**, 1133 (1981).
5. S. K. Arya and R. C. Gallo, *Biochemistry* **23**, 6685 (1984).
6. G. R. Crabtree, *Science* **243**, 355 (1989).
7. T. M. Williams *et al.*, *J. Immunol.* **141**, 662 (1988).
8. K. Muegge, T. Williams, S. Durum, unpublished data.
9. C. Gorman, G. Merlino, M. Wiooingham, I. Pastan, B. Howard, *Proc. Natl. Acad. Sci. U.S.A.* **79**, 6777 (1982).
10. J. de Wet, K. V. Wood, M. DeLuca, D. R. Helinski, S. Subramani, *Mol. Cell. Biol.* **7**, 725 (1987).
11. D. B. Durand *et al.*, *ibid.* **8**, 1715 (1988).
12. P. Angel *et al.*, *Cell* **49**, 729 (1987).
13. W. Lee, P. Mitchell, R. Tjian, *ibid.*, p. 741.
14. D. Bohmann *et al.*, *Science* **238**, 1386 (1987); P. Angel, *Nature* **332**, 166 (1988).
15. E. Ziff and T. Kouzarides, *Nature* **336**, 646 (1988); P. Sassone-Corsi, L. J. Ransone, W. W. Lamph, I. M. Verma, *ibid.*, p. 692.
16. H. Hagiwara *et al.*, *J. Immunol.* **138**, 2514 (1987).
17. Y. Nakabeppu, K. Ryder, D. Nathans, *Cell* **55**, 907 (1988).
18. E. Serfling *et al.*, *EMBO J.* **8**, 465 (1989).
19. P. Angel, K. Hattori, T. Smeal, M. Karin, *Cell* **55**, 875 (1988).
20. D. A. Brenner, M. O'Hara, P. Angel, M. Chojkier, M. Karin, *Nature* **337**, 661 (1989).
21. M. A. McNally, J. S. Lebkowski, T. B. Okarma, B. L. Lerch, *Biotechniques* **6**, 882 (1988).
22. C. M. Gorman, L. F. Moffat, B. H. Howard, *Mol. Cell. Biol.* **2**, 1044 (1982).
23. P. McIntyre and G. Stark, *Anal. Biochem.* **174**, 209 (1988).
24. D. J. Shapiro, P. A. Sharp, W. W. Wahli, M. J. Keller, *DNA* **7**, 47 (1988).
25. M. Fried and D. M. Crothers, *Nucleic Acids Res.* **9**, 6505 (1981).
26. F. Strauss and A. Varshavsky, *Cell* **37**, 889 (1984).
27. J. M. Chirgwin, A. E. Przybyla, R. J. Macdonald, W. J. Rutler, *Biochemistry* **18**, 5294 (1979).
28. T. Maniatis, E. F. Fritsch, J. Sambrook, *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1982).
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Human Genotoxicity: Pesticide Applicators and Phosphine

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Fumigant applicators who, 6 weeks to 3 months earlier, were exposed to phosphine, a common grain fumigant, or to phosphine and other pesticides had significantly increased stable chromosome rearrangements, primarily translocations in G-banded lymphocytes. Less stable aberrations including chromatid deletions and gaps were significantly increased only during the application season, but not at this later time point. During fumigant application, measured exposure to phosphine exceeds accepted national standards. Because phosphine is also used as a dopant in the microchip industry and is generated in waste treatment, the possibility of more widespread exposure and long-term health sequelae must be considered.

GLOBAL DEMAND FOR GRAIN AND grain products requires a complex system of transport, storage, and processing before final distribution to the consumer. To maintain the product in a usable state, fumigants and other pesticides are routinely applied by licensed specialists at one or more points from the time of harvest to final processing and use. Before

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