Proc. Natl. Acad. Sci. USA
Vol. 85, pp. 4672–4676, July 1988
Biochemistry

Transcriptional activation of *HLA-DRα* by interferon γ requires a trans-acting protein

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(HeLa/major histocompatibility complex/cycloheximide/nuclear runoff)

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Communicated by Sherman M. Weissman, March 17, 1988

ABSTRACT **Stimulation of the human epithelial-like cell line, HeLa, with interferon γ (IFN-γ) induces steady-state levels of HLA-DRα mRNA. Using a sensitive RNase-mapping procedure, we detect induced HLA-DRα mRNA as early as 8 hr after IFN-γ treatment; maximal accumulation occurs by 48 hr. Treatment with the protein synthesis inhibitor, cycloheximide, abolishes the IFN-γ-induced accumulation of HLA-DRα mRNA, indicating that *de novo* synthesis of a trans-acting protein factor is required for induction of this major histocompatibility complex class II gene. Nuclear run-off transcription assays revealed that IFN-γ acts by directly stimulating the transcription rate of *HLA-DRα*. Similarly, IFN-γ increased the transcription rate of the class I HLA-A2-encoding gene as well as that of the human invariant chain gene. IFN-γ-induced transcription of *HLA-DRα* and of the invariant chain gene was blocked by treatment with cycloheximide, but IFN-γ-induced transcription of *HLA-A2* was unaffected. Our findings show that transcriptional induction of *HLA-DRα* and the invariant chain gene by IFN-γ requires the action of an unidentified trans-acting protein.**

The class II genes of the major histocompatibility complex (MHC) encode heterodimeric cell-surface glycoproteins that are critical in immune response. Genes for the three principal human class II antigens—DR, DP, and DQ—have been mapped to the *HLA-D* region on chromosome 6. These molecules are expressed primarily in cells of lymphoid lineage—namely, B cells, macrophages/monocytes, and some activated T cells (1). In addition, many nonlymphoid cell types can express class II antigens (2–8). Reports indicate that these cells, once induced, can present antigen to T-helper cells (6, 9).

Treatment of cells with interferon γ (IFN-γ) increases steady-state levels of class II mRNA, which then leads to increase in cell-surface expression of protein (5, 10–12). IFN-γ-treatment also increases class I mRNA levels (5, 10, 13) as well as mRNA levels for invariant chain protein (14), a protein associated with expression of class II protein and which is encoded by a gene unlinked to the MHC.

Thus far, evidence suggests that induction of class II expression by IFN-γ is regulated at the transcription level (8, 15, 16). Furthermore, studies have described protein factors that bind at or near different human class II promoters (17, 18). No direct evidence shows that class II genes respond to IFN-γ by increasing their rates of transcription or whether induction requires the *de novo* production of a protein or merely modification of a preexisting factor. We have studied induction of *HLA-DRα* in a genetically and biochemically well-defined cell line, HeLa, so as to facilitate identification and subsequent isolation of any factor responsible for IFN-γ induction of MHC class II gene expression.

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MATERIALS AND METHODS

Cell Culture. The human epitheloid adenocarcinoma cell line, HeLa, was obtained from A. Baldwin and P. Sharp [Massachusetts Institute of Technology (MIT) Cancer Center]. Cells were grown in Dulbecco's modified Eagle's medium (GIBCO) supplemented with 10% fetal bovine serum and glutamine. Cells were treated with IFN-γ (Immuneron, Biogen, Cambridge, MA) at 1×10^3 units/ml for the indicated times. Cycloheximide was used at a final concentration of 10 μg/ml.

Fluorescence Flow Cytometry. Cells were harvested by treatment with Dulbecco's phosphate-buffered saline (lacking both Mg^{2+} and Ca^{2+}) supplemented with 1 mM EDTA. Cell-surface HLA-DR antigen was measured by staining with murine monoclonal antibody L243, directly coupled to phycoerythrin (Becton Dickinson); nonspecific staining was determined by use of phycoerythrin-coupled clone X39 (Becton Dickinson), an isotype-matched monoclonal antibody specific for keyhole limpet hemocyanin. Direct immunofluorescence staining was done according to the procedure of the supplier. Fluorescently stained cell populations were analyzed with a FACStar cell sorter (Becton Dickinson).

RNA Protection Analysis. Total cellular RNA was prepared from trypsinized cells as described (19). Synthesis of ^{32}P-radiolabeled antisense RNA probes and protection analysis were done according to Melton *et al.* (20). Forty micrograms of RNA was used in each analysis. Incubations with RNase A (12.5 μg/ml) and RNase T1 (1 unit/ml) were done at 37°C for 30 min, followed by further incubation with 0.5% $NaDodSO_4$ and proteinase K at 125 μg/ml for 15 min. The template used to generate antisense transcripts for RNA protection analysis was prepared by subcloning a 134-base pair (bp) *Bal* I restriction fragment containing sequences −66 to +68 of *DRα* (21, 22) in the antisense orientation into *Hin*cII-digested pSP64 (20). Template plasmid (pSP64-DRα) was digested with *Eco*RI before synthesis of the antisense transcript; total length of the input antisense transcript was ≈190 nucleotides (nt), and length of the protected, *DRα*-specific fragment was ≈66 nt.

Nuclear Run-Off Transcription Assay. The method used was essentially that of Greenberg and Ziff (23) with some modifications as suggested by B. Cochran (MIT Cancer Center) and by J. Woodward (University of Kentucky School of Medicine). Five micrograms of linearized, alkali-treated plasmid DNA was immobilized on nitrocellulose and hybridized in 2 ml with $\approx 1 \times 10^8$ cpm of run-off RNA for 60 hr at 65°C. The plasmid probes used were: (*i*) pβ2[#3], a 2.2-kilobase (kb) β_2-tubulin (genomic) *Sst* I restriction fragment derived from pUC(L1.) [provided by N. Cowan (New York University School of Medicine) (24)], subcloned into *Sst* I-digested pSP64; (*ii*) pDRα-15, a 1.1-kb DRα cDNA in the

Abbreviations: MHC, major histocompatibility complex; IFN-γ, interferon γ.

Pst I site of pBR322 (25); (*iii*) pγ2, a 1.3-kb invariant chain cDNA in the *Pst* I site of pBR322 [provided by V. Quaranta (Scripps Clinic and Research Foundation) (26)]; (*iv*) pHLA-2a, a 5.1-kb genomic clone of the *HLA-A2* gene in the *Hin*dIII site of pUC9 [provided by H. Orr (University of Minnesota School of Medicine) (27)]; (*v*) pBR322; and (*vi*) pSP64. Densitometric measurement was done from autoradiograms of various exposures with a Joyce–Loebl Chromoscan 3 densitometer.

RESULTS

Induction of HLA-DR Antigen in HeLa Cells by IFN-γ. Many nonlymphoid-cell types can express MHC class II genes upon treatment with IFN-γ. The epithelial-like cell line, HeLa, generally has been assumed not to express MHC class II genes. Fig. 1 shows that treatment of HLA-DR-negative HeLa cells with IFN-γ for 72 hr causes a uniform induction of cell-surface HLA-DR antigen expression in >90% of cells. We characterized this IFN-γ-stimulated induction of HLA-DR antigen in greater detail by using a sensitive RNase-mapping procedure (20) to follow IFN-γ-induced accumulation of HLA-DRα mRNA. Fig. 2*A* shows that DRα mRNA is neither found in untreated cells nor in cells exposed to IFN-γ for <8 hr. HLA-DRα transcripts are detectable first at 8 hr after addition of IFN-γ; steady-state DRα message accumulates to maximum levels by 48 hr. Between 12 and 24 hr of treatment, DRα mRNA levels increase 10-fold. Although most induced transcripts are initiated at the expected position [≈60 nt 5′ of the initiation codon (21, 22)], some DRα-specific transcripts appear to be initiated farther 5′ and 3′ of the presumed cap site (Fig. 2 *A* and *B*). This apparent multiplicity of start sites has already been shown for *DRα* and its murine homologue, *I-E*$_\alpha$ (21, 22, 28).

Cycloheximide Abolishes IFN-γ-Induced DRα mRNA Accumulation. To determine whether protein synthesis is required for IFN-γ induction of *HLA-DRα*, we treated HeLa cells with IFN-γ in the presence of cycloheximide (10 μg/ml), an inhibitor of protein synthesis. At the concentration used,

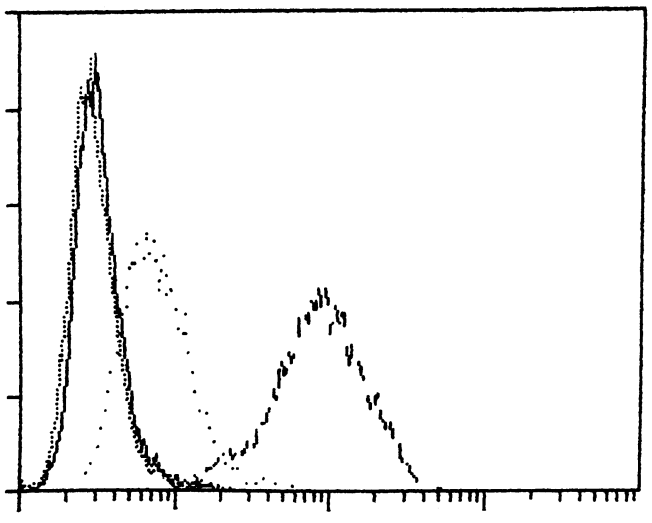

FIG. 1. Cell number (*y* axis) vs. logarithm of fluorescence intensity (*x* axis) for HeLa cells, either control cells or cells treated with IFN-γ at 1000 μ/ml for 72 hr. Closely spaced dots represent control cells stained with a control antibody; the interrupted solid line represents control cells stained for HLA-DR antigen; widely spaced dots represent IFN-γ-induced cells stained with a control antibody (for cell-surface Fc receptor expression); and the broken curve to the right represents IFN-γ-induced cells stained for HLA-DR antigen. More than 90% of the IFN-γ-treated cells express cell-surface HLA-DR antigens.

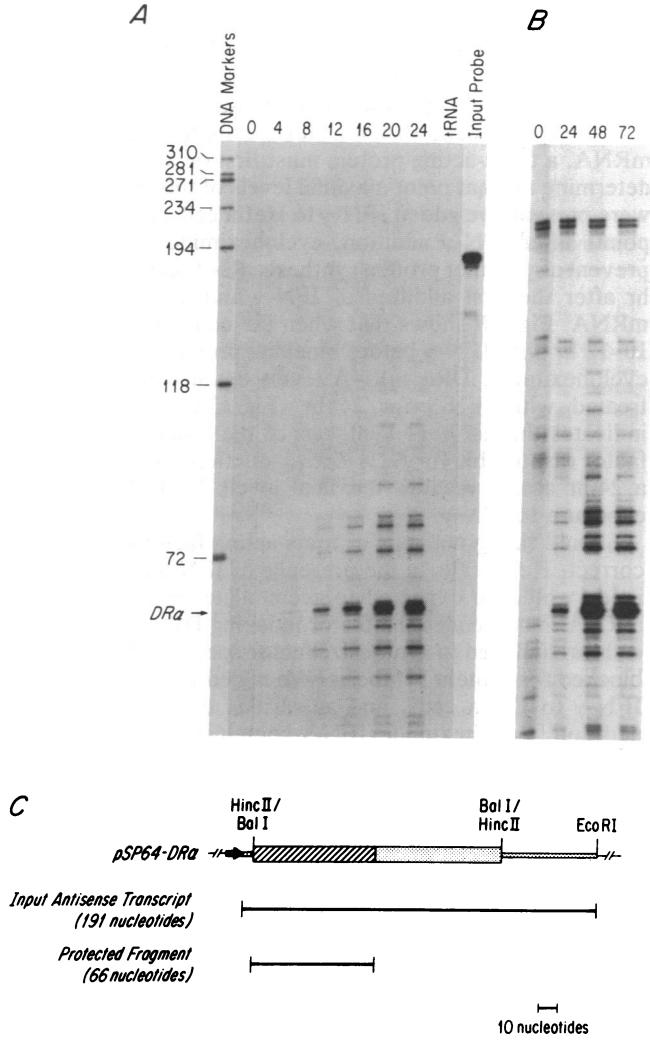

FIG. 2. IFN-γ-induced accumulation of HLA-DRα mRNA in HeLa cells. (*A*) Time course of 24 hr. Forty micrograms of total cellular RNA from cells exposed to IFN-γ for the indicated times were hybridized with the input antisense transcript, treated with RNases, and electrophoresed on an 8% acrylamide/7 M urea gel. M_r markers were derived from *Hae* III-digested φX174 DNA. Arrow indicates position of the major species of DRα mRNA. (*B*) Time course of 72 hr. (*C*) HLA-DRα probe. Arrow represents SP6 promoter in pSP64 plasmid vector; narrow line denotes linker piece in the vector; cross-hatched box, transcribed region 5′ of the DRα structural gene; stippled box, region located 5′ of the initiation of *DRα* gene transcription. Sizes of the full-length input antisense transcript and of the RNase-protected DRα mRNA fragment are indicated below.

protein synthesis (measured by [^{35}S]cysteine incorporation) was reduced ≈95% (data not shown). Cells were treated for 24 hr, after which total cellular RNA was isolated and analyzed by DRα-specific RNase-mapping. Fig. 3*A* shows that cycloheximide prevents the accumulation of DRα mRNA levels as compared to that induced by IFN-γ alone, whether added simultaneously with IFN-γ or 2 hr before IFN-γ addition. Reproducibly, a low level of DRα mRNA was detected (≈7% of the 24-hr level) in cycloheximide/IFN-γ-treated cells. Because cycloheximide itself did not increase DRα mRNA levels, the low level of residual DRα expression observed probably results from some protein synthesis not blocked by cycloheximide. All RNA samples were analyzed for α-tubulin mRNA levels to ascertain whether lack of DRα mRNA could be due to an increased rate of nonspecific mRNA degradation in cycloheximide-treated cells. α-

Tubulin mRNA was not significantly affected in treated-vs.-control HeLa cells (data not shown). Therefore, a general effect on mRNA stability does not account for the DRα mRNA decrease in cycloheximide-treated HeLa cells.

We thus conclude that for HeLa cells to induce class II mRNA, a trans-acting protein must first be synthesized. To determine at what point maximal levels of this protein factor were present, we added IFN-γ to HeLa cells, and at various points after IFN-γ addition, cycloheximide was added to prevent any further protein synthesis. RNA was isolated at 24 hr after the first addition of IFN-γ and assayed for DRα mRNA. Fig. 3*B* shows that when the cells are treated only 10–12 hr with IFN-γ before blocking protein synthesis with cycloheximide, DRα mRNA levels equaled levels in cells treated with IFN-γ for 24 hr (Fig. 3*A*). This evidence indicates that *de novo* synthesis of the trans-acting protein factor responsible for *HLA-DRα* induction, as determined in a 24-hr assay, reaches maximal levels 10–12 hr after cell exposure to IFN-γ.

Should the hypothesis of trans-acting factor synthesis be correct, HeLa cells in the presence of IFN-γ and cycloheximide would be expected to accumulate maximal levels of mRNA that encodes the factor inducing DRα; that is, these cells are induced to synthesize factor-specific mRNA but are blocked for protein synthesis. We added cycloheximide and IFN-γ to HeLa cells, and at various times after the first addition, cycloheximide/IFN-γ-containing medium was removed and replaced with IFN-γ-containing medium for a total elapsed time (cycloheximide/IFN-γ plus IFN-γ) of 24 hr. Fig. 3*C* shows that maximal levels of DRα mRNA (equal to levels seen in cells exposed for 24 hr to IFN-γ only) are seen even when protein synthesis is blocked for 12 hr after the first addition of IFN-γ. Submaximal levels of DRα mRNA accumulation are seen when cycloheximide is present initially for >12 hr. Therefore, protein synthesis can be blocked for up to 12 hr after the first IFN-γ addition without affecting the subsequent accumulation of DRα mRNA to levels equal to those in cells exposed to IFN-γ for 24 hr.

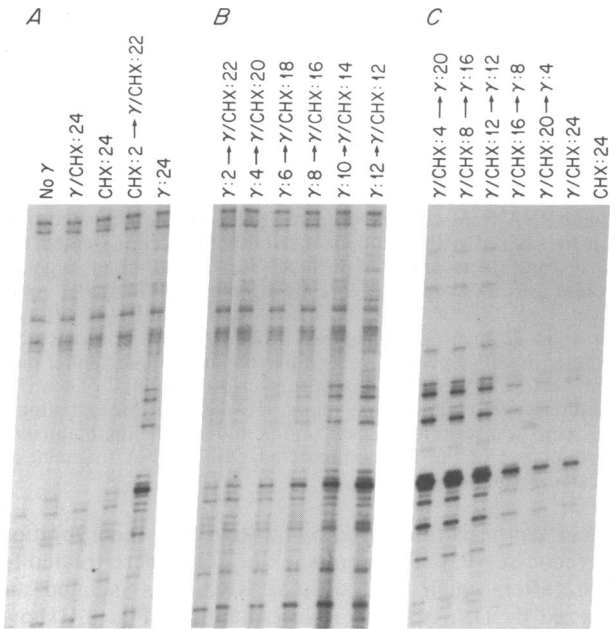

FIG. 3. Effect of cycloheximide (CHX) on IFN-γ-induced (γ) accumulation of HLA-DRα mRNA. (*A*) Cells were treated for a total of 24 hr without additions, with IFN-γ and cycloheximide, with cycloheximide alone, with cycloheximide for 2 hr followed by 22 hr with cycloheximide and IFN-γ, or with IFN-γ alone. (*B*) Cells were incubated first with IFN-γ. At the indicated times, cells were exposed to cycloheximide and IFN-γ for a total period (IFN-γ plus IFN-γ/cycloheximide) of 24 hr. (*C*) Cells were incubated first with IFN-γ and cycloheximide after which they were washed and incubated with only IFN-γ for a total period (IFN-γ/cycloheximide plus IFN-γ) of 24 hr. γ/CHX:24, IFN-γ with cycloheximide for 24 hr; CHX:24, cycloheximide alone for 24 hr. Arrow, position of the major species of DRα mRNA.

Transcriptional Induction of DRα by IFN-γ Is Inhibited by Cycloheximide Treatment. We compared the transcription of genes encoding HLA-DRα, HLA-A2, invariant chain, and β_2-tubulin in nuclei isolated from untreated HeLa cells as well as those exposed to various IFN-γ and cycloheximide treatments. Labeled RNA from *in vitro* nuclear transcription assays was hybridized as described. The *in vitro*-labeled RNA from nuclei of cells treated with IFN-γ for either 24 or 48 hr, with cycloheximide for 24 hr, with IFN-γ/cycloheximide for 24 hr, or with IFN-γ for 24 hr followed with IFN-γ/cycloheximide for 24 hr, all gave β_2-tubulin-specific hybridization equivalent to that seen in the control (untreated) sample (Fig. 4). Treatment with IFN-γ for 24 or for 48 hr increased *DRα* transcription from undetectable mRNA levels seen in untreated HeLa cells. Transcription of the invariant chain gene, although present in the uninduced state, was induced 3- to 5-fold by IFN-γ, and the rate of *HLA-A2* transcription increased 10- to 20-fold over untreated HeLa nuclei. These findings show that IFN-γ-induced DRα mRNA accumulation acts directly through activation of HLA-DRα gene transcription. Similarly, IFN-γ stimulates transcription of the invariant chain gene and of the class I gene, *HLA-A2*.

We then determined whether *de novo* synthesis of protein was required for IFN-γ-induction of *HLA-DRα*. Cycloheximide, when added simultaneously with IFN-γ, reduced the level of *DRα* transcription to that of the untreated control (Fig. 4). Treatment of cells with IFN-γ for 24 hr followed with IFN-γ/cycloheximide for an additional 24 hr similarly reduced the level of DRα transcription. Cycloheximide alone had no effect on the transcription rate of *DRα*. Transcription of the invariant chain gene was reduced to levels about equal to that seen in untreated cells when treated with IFN-γ/cycloheximide. In addition, treatment with cycloheximide

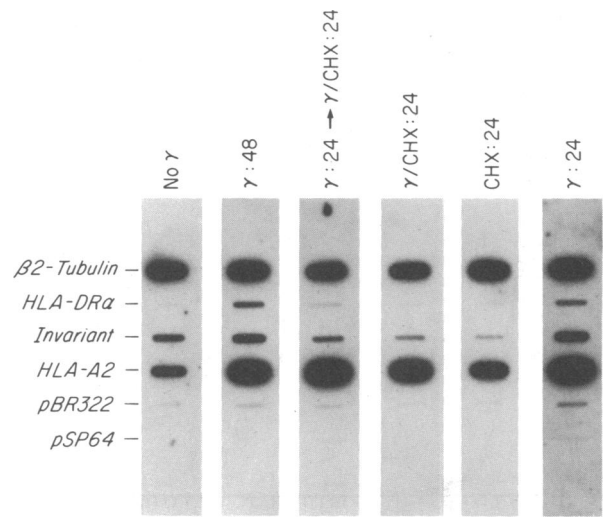

FIG. 4. Effect of IFN-γ (γ) and cycloheximide (CHX) on *HLA-DRα*, *HLA-A2*, and invariant chain gene transcription. Nuclear run-off transcription assays and the gene-specific probes are described elsewhere. Labeled RNA from the transcription assays was hybridized to immobilized DNA probes (5 μg per slot). Run-off assays were done on nuclei isolated from HeLa cells with no treatment, with IFN-γ alone for 48 hr, with IFN-γ for 24 hr followed by IFN-γ and cycloheximide for an additional 24 hr, with IFN-γ and cycloheximide for 24 hr, with cycloheximide alone for 24 hr, or with IFN-γ alone for 24 hr.

alone for 24 hr reproducibly decreased the uninduced basal transcription rate of the invariant chain gene by ≈50%.

When added with IFN-γ, cycloheximide appeared not to affect the transcription rate of the MHC class I gene, *HLA-A2*. Transcription levels of IFN-γ/cycloheximide-treated HeLa nuclei were about equal to those levels seen with IFN-γ treatment for 24 or 48 hr (Fig. 4). Surprisingly, however, cycloheximide treatment alone induced transcription of HLA-A2 to increase approximately 5- to 10-fold.

DISCUSSION

The capacity of HeLa cells to respond to IFN-γ has been documented for other gene products (29, 30). For example, within 8 hr after stimulation with interferon γ, HeLa cells induce maximal mRNA and protein levels of (2′-5′)oligoadenylate synthetase, an enzyme synthesized in response to all interferons and believed to help establish an antiviral state. More recently, murine MHC class I promoter constructs, when transfected into HeLa cells, have been shown to respond to treatment with interferon (31). We showed that HeLa cells, upon stimulation with IFN-γ, can induce cell-surface expression of the endogenous human MHC class II antigen, HLA-DR. IFN-γ apparently induces *de novo* synthesis of DRα mRNA.

The time course of DRα mRNA accumulation in HeLa cells resembles the time course found in other cells that express MHC class II genes only upon induction (5, 10, 11, 32). Interferon-induced mRNA appears at ≈8 hr after addition of the lymphokine and increases linearly until reaching maximal levels of accumulation, usually within 48–72 hr. Cell-surface antigen roughly parallels the appearance of class II-specific message (5, 10, 12, 32).

In contrast with our results, induction of class II mRNA accumulation by IFN-γ in human dermal fibroblasts is not inhibited by high concentrations of cycloheximide (33). The reason for this difference is unknown, but the difference may reflect cell-specific mechanisms of MHC class II gene regulation. Interestingly, IFN-γ-induction of a 56-kDa protein (C56) and of (2′5′)oligoadenylate synthetase mRNA in HeLa cells is also blocked by treatment with cycloheximide (29, 30). It is tempting to speculate that these genes may share a common regulatory element.

Only suggestive and indirect evidence exists for the induction of class II mRNA accumulation by IFN-γ as a direct result of transcriptional induction of the class II genes. Hypomethylation of genomic DNA has been shown to be required, in combination with IFN-γ-treatment, to induce the accumulation of DRα mRNA in one subline of the human monocytoid cell line U937 (15). More recently, 5′ deletion analyses of *DRα* in human glioblastoma *multiforme* cell lines (8) and of *DQ2β* in xeroderma pigmentosa and osteosarcoma fibroblast cell lines (16) showed that DNA sequences 5′ of the class II structural genes are important for IFN-γ-induced accumulation of chloramphenicol actyltransferase activity and DQ2β mRNA, respectively. We showed, by nuclear run-off transcription assays, that IFN-γ acts by increasing the transcription rate of *HLA-DRα*. Furthermore, because the IFN-γ-induced transcriptional activity of *DRα* is sensitive to cycloheximide, we conclude that synthesis of a specific trans-acting protein factor is required for the transcriptional induction.

Evidence that at least one trans-acting gene product is necessary for class II expression has been derived from cell fusions of mutant B-lymphoblastoid cell lines, in which expression of all MHC class II genes was lost, with several class II-positive cell lines (34, 35). Similarly, genetic analysis of patients with severe-combined immunodeficiency (SCID), in whom all cells normally expressing class II genes are negative, has indicated that at least one gene, unlinked to the human MHC, controls class II expression (36).

The nonpolymorphic invariant chain protein occurs only intracellularly in association with several other proteins, in addition to class II α and β chains. In many cell lines, IFN-γ induces invariant chain mRNA over a time course identical to class II mRNA accumulation (5, 11, 14). Moreover, in murine pre-B cells and in human simian virus 40-transformed fibroblasts, IFN-γ-induced invariant chain mRNA accumulation can be blocked by cycloheximide treatment (14). We showed that HeLa cells stimulate transcription of invariant chain and DRα mRNA in response to IFN-γ and that this induction depends on production of at least one unidentified protein factor.

In many different cell types, addition of IFN-γ stimulates class I mRNA accumulation between 4- to 10-fold (5, 10, 13, 33). In human dermal fibroblasts, cycloheximide when added with IFN-γ has no effect on HLA-A,B mRNA induction; cycloheximide alone has no effect on steady-state levels of class I mRNA (33). We showed, by nuclear run-off transcription assays of HeLa nuclei, that IFN-γ directly stimulates the *HLA-A2* transcription rate; cycloheximide, when added with IFN-γ, neither inhibited nor superinduced this transcriptional stimulation. Unexpectedly, cycloheximide by itself significantly induced the transcription rate of HLA-A2 mRNA.

The transcription rate of several genes increases upon cycloheximide treatment. Cycloheximide induces the transcription rate of the interferon β gene in Chinese hamster ovary cells by 3- to 4-fold (37). In HeLa cells, cycloheximide has been shown to increase the transcription rate of histone and various nonhistone genes (38). One mechanism by which cycloheximide may exert its stimulatory effect on the transcription rate of these genes and of the MHC class I gene, *HLA-A2*, is through inhibition of a labile regulatory protein that controls either the level or duration of transcription. Alternatively, no labile repressor exists, but rather inhibition of protein synthesis has consequences (such as protein kinase C activation) that activates a trans-acting factor. For example, treatment of murine pre-B cells with cycloheximide causes a 5-fold increase in the transcription rate of the κ light-chain gene (39). A cycloheximide-induced, posttranslational modification has been proposed to result in the induction of the activity of the protein NF-κB, implicated in κ light-chain gene expression (40). Interestingly, NF-κB activity can be induced in HeLa cells, as in pre-B cells, by phorbol esters (activators of protein kinase C). Because MHC genes and the immunoglobulin genes are comembers of the immunoglobulin supergene family (41, 42) it seems reasonable to suggest that some members of this supergene family are regulated similarly and share regulatory factors.

This paper resulted from an equal contribution by the first two authors. We thank Drs. Brent Cochran and Jerry Woodward for their nuclear transcription assay protocols; Drs. Nicholas Cowan, Vito Quaranta, and Harry Orr for providing plasmids in a timely fashion; Drs. Hamish Allen, Al Baldwin, Linda Burkly, and Gerry Waneck for stimulating discussions and for comments on the manuscript; Dr. Eric Long for suggesting originally that we try HeLa cells; and Ms. Geraldine Kemske for final preparation of the manuscript. M.A.B. is a recipient of an Arthritis Foundation Postdoctoral Fellowship. E.C.B. is a recipient of a Deutsche Forschungsgemeinschaft Postdoctoral Fellowship. This work was funded in part by Biogen N.V.

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