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Posttranscriptional Control of Human Gamma Interferon Gene Expression in Transfected Mouse Fibroblasts

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Human gamma interferon genomic DNA was introduced into NIH 3T3 fibroblasts by calcium phosphate precipitation and was not expressed in these cells at the cytoplasmic mRNA or protein level. Treatment of the transfected cells with cycloheximide (1 μg/ml) induced the accumulation of cytoplasmic gamma interferon mRNA and biologically active human gamma interferon. Analysis of the nuclear enriched RNA from untreated cells indicated that human gamma interferon mRNA was present, suggesting that cycloheximide may act by inhibiting a specific nuclease or may enhance the processing or transport of the RNA from the nucleus to the cytoplasm.

Gamma interferon (IFN-γ) is a unique lymphokine which has been reported to have a large number of immunoregulatory functions (for a recent review, see reference 26). Synthesis of this lymphokine appears to be limited to relatively few cell types, namely T cells and large granular lymphocytes (LGL) (5, 25). Workers in this laboratory have analyzed the expression of the transfected human IFN-γ genomic DNA in both a mouse T cell line and NIH 3T3 fibroblasts. It has been found that human IFN-γ can be efficiently produced in mouse T cells but is not expressed in transfected mouse fibroblasts at the cytoplasmic RNA or protein level (H. A. Young, J. F. Dray, and W. L. Farrar, J. Immunol., in press), suggesting cell specificity for transcriptional regulation. Since it has been reported that cycloheximide treatment of transfected mouse fibroblasts permits the expression of genes not normally expressed in fibroblasts (11), the effects of this inhibitor on mouse fibroblasts containing the human IFN-γ genomic DNA were tested. Here we report that untreated transfected mouse fibroblasts contain human IFN-γ RNA in the nucleus and that cycloheximide treatment of these cells results in accumulation of cytoplasmic RNA and production of human IFN-γ. Furthermore, brief treatment of the cells with actinomycin D at the end of the cycloheximide treatment enhances human IFN-γ production 5- to 10-fold.

Human IFN-γ genomic DNA was isolated as previously described (8; Young et al., in press), and the 8.6-kilobase (kb) *Bam*HI fragment, containing almost 3 kb of 5′ noncoding sequences and approximately 1 kb of 3′ noncoding sequences, was ligated to the vector pSV2-neo (20). The *Bam*HI site in this vector is 3′ to the termination site for *neo* transcription, minimizing the influence of simian virus 40 regulatory sequences on the inserted DNA. This construct was then introduced into NIH 3T3 cells by calcium phosphate precipitation in the presence of chloroquine (7, 12), and G418-resistant (Geneticin; Gibco Laboratories) colonies were isolated after 5 to 7 days of selection. Initial attempts to induce IFN-γ from mouse fibroblasts containing the transfected human gene were unsuccessful (Young et al., in press), as neither phorbol myristate acetate, the calcium ionophore A23187, or the beta interferon inducer polyinosinic acid-polycytidylic acid [poly (I:C)] was able to stimulate IFN-γ production. However, treatment of the cells (Clone [CL] 2C) with 10 μg of cycloheximide per ml for 4 h resulted in the detection, in a slot-blot analysis, of IFN-γ mRNA in total cytoplasmic RNA following hybridization with human IFN-γ ^{32}P-labeled cDNA (Fig. 1). This human IFN-γ cDNA probe had no homology with mouse IFN-γ under the hybridization conditions used (8; Young et al., in press). To determine the kinetics of appearance of this mRNA, we treated the cells with cycloheximide for 1, 2, 3, or 4 h and analyzed total cytoplasmic RNA by Northern blot analysis following glyoxal denaturation (24). The 1.3-kb IFN-γ mRNA could be detected as early as 1 h (Fig. 2, lane 3) after cycloheximide treatment and reached a maximum approximately 3 to 4 h after treatment.

To investigate whether cycloheximide induced transcription of the human IFN-γ gene or instead enhanced processing or transport of the RNA from the nucleus to the cytoplasm, RNA was extracted from the nuclear pellet of untreated cells by guanidium isothiocyanate-cesium chloride ultracentrifugation and analyzed for hybridization to a human IFN-γ cDNA probe. Significant hybridization to the nuclear enriched RNA but not to the cytoplasmic RNA from untreated cells was observed (Fig. 3). This RNA was then denatured with glyoxal (24) and analyzed after electrophoresis on a 1% agarose gel and transfer to a nylon membrane (Nytran; Schleicher & Schuell). No hybridizing RNA was found in the cytoplasmic RNA from untreated transfected fibroblasts (Fig. 4, lanes 1 and 2), while the expected 1.3-kb mRNA was seen in the cytoplasmic RNA extracted from cycloheximide-treated cells (lanes 5 and 6), cycloheximide- and actinomycin D-treated cells (lanes 7 and 8), and the transfected T cells treated with phorbol myristate acetate (lane 10) (Young et al., in press). A smear of larger hybridizing RNA occurred in the nuclear enriched RNA extracted from untreated transfected cells (lanes 3 and 4). In addition, two hybridizing bands of approximately 2.5 and 3.7 kb were observed in the nuclear enriched RNA. The size of these bands was consistent with the expected RNA size if either the first or third introns had been spliced out of the primary

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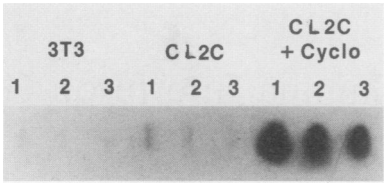

FIG. 1. Slot-blot analysis of cycloheximide (Cyclo)-treated transfected mouse fibroblasts. Procedures for isolation and analysis of cytoplasmic RNA have been described elsewhere (6, 17). All blots were hybridized for 48 to 72 h in 50% formamide–5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–1% sodium dodecyl sulfate (SDS) at 42°C and washed in 2× SSC–1% SDS at room temperature for 15 min and in 0.2× SSC–1% SDS at 60°C twice for 30 min. Filters were exposed to Kodak XOMAT X-ray film with Cronex (Du Pont) intensifying screens for 24 h at −70°C. Lanes 1, 2, and 3 contain 10, 5 and 2.5 μg of total cytoplasmic RNA, respectively.

5.0-kb transcript. Similar minor bands were also faintly visible in the cycloheximide-treated cytoplasmic RNA preparations, indicating that a slight leakage of nuclear RNA occurred during the extraction process.

The culture supernatants from transfected mouse fibroblasts treated with cycloheximide were then analyzed for production of human IFN-γ protein. Following treatment with the appropriate inducers for the times indicated, the cells were washed twice with phosphate-buffered saline, fresh medium was added, and the cultures were incubated an additional 18 to 24 h. The culture supernatants were then harvested, cells were removed by centrifugation, and the supernatants were assayed by radioimmunoassay. At a concentration of 10 μg of cycloheximide per ml, human IFN-γ was not detected in the culture medium following cycloheximide treatment (data not shown). However, by lowering the cycloheximide concentration 5- to 20-fold, a different set of results was obtained (Table 1). Treating cells

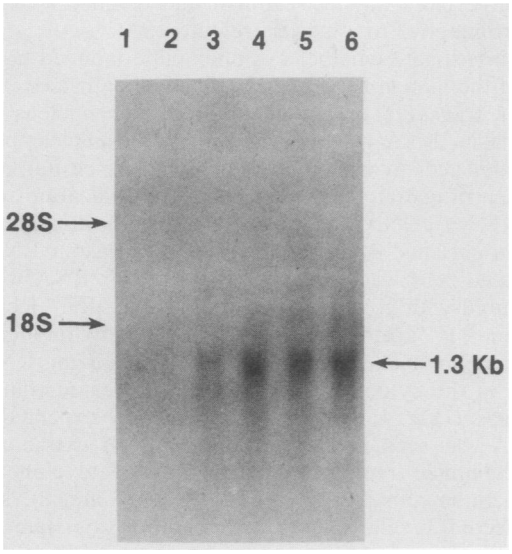

FIG. 2. Northern analysis of cycloheximide-treated transfected mouse fibroblasts. Procedures for the isolation, separation, and analysis of total cytoplasmic RNA have been described elsewhere (6, 17, 24). Each lane contained 10 μg of total cytoplasmic RNA. Lanes: 1, NIH 3T3 cells, 4-h cycloheximide treatment; 2 through 6, CL 2C cells, untreated (lane 2) and treated for 1, 2, 3, or 4 h, respectively, with cycloheximide.

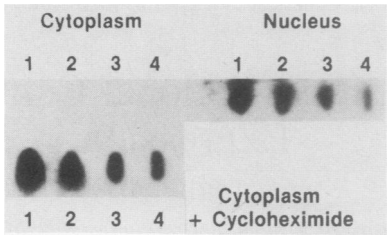

FIG. 3. Slot-blot analysis of untreated transfected mouse fibroblasts. Procedures for the isolation, separation, and analysis of total cytoplasmic and nuclear enriched RNA are described elsewhere (6, 17). Lanes 1, 2, 3, and 4 contain 10, 5, 2.5, and 1.25 μg of RNA, respectively.

with lower concentrations of cycloheximide resulted in the production of significant levels of IFN-γ in the culutre supernatants, and increasing the time of cycloheximide treatment also resulted in increased IFN-γ synthesis. To determine whether IFN-γ production could be superinduced by actinomycin D after cycloheximide treatment as had been observed for human beta interferon (18, 23), cultures were treated with 0.5 μg of actinomycin D per ml for 30 min at the end of cycloheximide treatment, and IFN-γ levels were measured. This treatment was found to enhance IFN-γ production 10-fold, whereas actinomycin D alone had no effect (Table 1). To further determine whether actinomycin

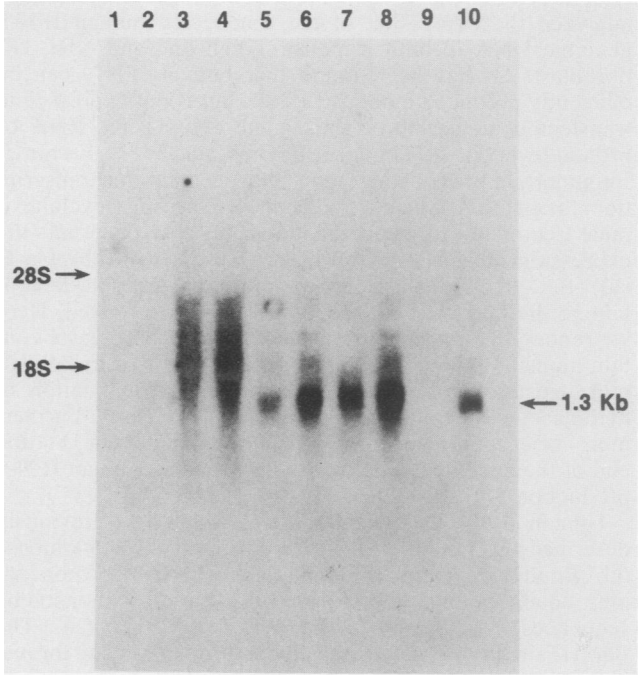

FIG. 4. Northern analysis of nuclear enriched and cytoplasmic RNA. Procedures for isolation, separation, and analysis of nuclear enriched and cytoplasmic RNA have been described elsewhere (6, 17, 24). Hybridization and washing conditions are as described in the legend to Fig. 1. Lanes: 1 and 2, cytoplasmic RNA from untreated cells; 3 and 4, nuclear enriched RNA from untreated cells; 5 and 6, cytoplasmic RNA from cycloheximide (1 μg/ml)-treated cells; 7 and 8, cytoplasmic RNA from cycloheximide (1 μg/ml)-and actinomycin D-treated cells. For each pair, the first lane contained 5 μg and the second lane contained 10 μg. Lanes 9 and 10, Cytoplasmic RNA (10 μg) from nontransfected (lane 9) and transfected (lane 10) mouse T cells treated with phorbol myristate acetate.

TABLE 1. Human IFN-γ production in mouse fibroblasts[a]

Cells	Inhibitor (μg/ml)		Time (h)	Radioactivity in RIA supernatant (cpm)	IFN-γ (U/ml)
	Cycloheximide	Actinomycin D			
3T3	0	−	4	503 ± 75	0
	0.5	−	4	505 ± 75	0
	1.0	−	4	502 ± 75	0
	2.0	−	4	428 ± 64	0
	0	+	4	448 ± 67	0
	0.5	+	4	493 ± 74	0
	1.0	+	4	496 ± 74	0
	2.0	+	4	480 ± 72	0
CL 2C	0	−	4	519 ± 77	0
	0.5	−	4	2,120 ± 318	3.8
	1.0	−	4	2,371 ± 355	4.4
	2.0	−	4	2,492 ± 374	4.8
	0	+	4	480 ± 72	0
	0.5	+	4	18,588 ± 2,784	48.9
	1.0	+	4	20,990 ± 3,148	>50
	2.0	+	4	15,278 ± 2,292	39.9
	0.5	−	7	4,979 ± 747	11.7
	1.0	−	7	7,742 ± 1,161	19.5
	2.0	−	7	6,773 ± 1,016	16.5
	0.5	+	7	27,998 ± 4,200	>50
	1.0	+	7	35,576 ± 5,336	>50
	2.0	+	7	34,067 ± 5,110	>50

[a] Procedures for collection of culture medium are described in the text. Interferon units were determined by radioimmunoassay (Centocor, New Malvern, Pa.). The assay is linear up to 50 U/ml and has no cross-reactivity with mouse IFN-γ (Young et al., in press).

D enhanced IFN-γ mRNA stability, IFN-γ cytoplasmic RNA levels were analyzed immediately and 18 h after cycloheximide or cycloheximide-plus-actinomycin D treatment. Similar levels of cytoplasmic mRNA remained after either treatment, suggesting that actinomycin D did not enhance IFN-γ cytoplasmic mRNA stability (Fig. 3). Furthermore, treatment of the cells with phorbol myristate acetate, poly(I:C), or platelet-derived growth factor during and following cycloheximide treatment did not result in enhanced IFN-γ production or mRNA accumulation (data not shown), indicating that these compounds were not effective in inducing increased transcription even after the processing or transport block was overcome.

The human IFN-γ produced by the mouse fibroblasts was next tested for biological activity by its ability to protect WISH cells against vesicular stomatitis virus challenge (1). After treatment with cycloheximide (1 μg/ml) or cycloheximide plus actinomycin D for 7 h, IFN-γ activity was 120 and 1,600 IU/ml, respectively (values corrected to reference units per NIAID standards).

Expression of IFN-γ is restricted to a few specific cell types, although a wide variety of agents can affect the expression of this protein in these cells (5, 26). Furthermore, the controlling mechanisms behind this restricted expression are largely unknown. The ability to induce expression of other species of interferon, e.g., beta interferons, with cycloheximide has been reported by a number of laboratories (2, 22, 27). Early studies showed that human beta interferon could be induced from human fibroblasts with cycloheximide and that this induction could be enhanced with actinomycin D. In more recent studies, it has been demonstrated that after transfection of human beta interferon genomic DNA into mouse (14) or hamster cells (16), expression of this gene could also be induced with cycloheximide. In all cases, the expression of the beta interferon genes occurred in the cells normally observed to express this gene (i.e., fibroblasts). However, when promoter sequences upstream from the TATA box of the human beta interferon DNA were removed and this construct was introduced into mouse cells as part of a bovine papilloma virus episome. Nir and co-workers observed that the interferon mRNA remained in the nucleus until the cells were treated with double-stranded RNA or cycloheximide (15).

The use of protein synthesis inhibitors to enhance expression of specific genes is not limited to the interferon family. Similar results have been obtained when a human immunoglobulin heavy-chain genomic DNA clone was transfected into mouse L cells (11), indicating that treatment of mouse fibroblasts with protein synthesis inhibitors can overcome tissue-specific restriction of gene expression. In addition, cycloheximide has been shown to stimulate early adenovirus transcription (3), c-*myc* transcription (13), histone mRNA synthesis (21), and the synthesis of new proteins in mouse fibroblasts (10, 19). We have now observed that the restriction of the protein expression of the human IFN-γ gene observed after introduction of this T-cell- and large-granular-lymphocyte-specific gene into mouse fibroblasts can be overcome with protein synthesis inhibitors. Furthermore, we found that brief treatment of the transfected cells with actinomycin D at the end of the cycloheximide treatment enhanced production of IFN-γ 10-fold above that with cycloheximide alone.

The specific mechanism by which cycloheximide and actinomycin D permit the expression of exogenously introduced genes remains unknown. One interpretation often stated is that cycloheximide blocks the synthesis of a repressor protein and that actinomycin D blocks synthesis of the repressor mRNA, which has a very short half-life. Although this hypothesis is plausible, a putative repressor protein has not yet been identified, and our observation of IFN-γ mRNA in the nuclear fraction would rule out this possibility. Another possible explanation is that cycloheximide blocks the synthesis of a short-lived nuclease which rapidly degrades the mRNA. Evidence for the presence of specific nucleases has been supported by the observations of Gurney (9), who recently found that low levels of cycloheximide inhibit specific steps in rRNA processing, suggesting that cycloheximide may block specific nuclease action by binding directly to ribosomal proteins. A third possibility is that cycloheximide can enhance the processing and transfer of nuclear RNA to cytoplasmic mRNA (4). This would lead to a greater accumulation of cytoplasmic mRNA without enhanced transcription of a specific gene and is most consistent with our observed results. Furthermore, secondary folding of the RNA could permit actinomycin D to bind to a double-stranded RNA region, which might then accelerate RNA processing or block the action of a specific nuclease. Nevertheless, the enhancement of human IFN-γ gene expression by protein and RNA synthesis inhibitors suggests that the regulation of this gene expression may depend on both negative and positive controlling DNA sequences or specific protein factors. Analysis of the mechanisms involved in the regulation of expression of the transfected human IFN-γ gene in both permissive T cells and nonpermissive fibroblasts should provide a novel experimental system for more precisely defining these controlling elements.

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