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Regulation of Nuclear Gamma Interferon Gene Expression by Interleukin 12 (IL-12) and IL-2 Represents a Novel Form of Posttranscriptional Control

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Posttranscriptional control of gamma interferon (IFN-γ) gene expression has not been extensively studied and is poorly understood. Our work describes a posttranscriptional mechanism that modulates IFN-γ mRNA expression in stimulated natural killer (NK) cells through nuclear retention of the IFN-γ mRNA. This is evidenced by the elevated and sustained nuclear accumulation of both precursor and processed IFN-γ mRNAs in NK cells stimulated with interleukin-12 (IL-12). The elevated nuclear mRNA accumulation persists long after transcriptional activity has subsided and the rate of cytoplasmic IFN-γ mRNA accumulation has dropped. The IL-12-induced nuclear retention of the IFN-γ mRNA prevails until a secondary cytokine stimulus is received. The secondary stimulus, which is initiated by IL-2, mediates transcription-independent movement of the nuclear IFN-γ mRNA. Concurrent with the nucleocytoplasmic movement of the IFN-γ mRNA, we have observed increases in the amount of processed nuclear IFN-γ mRNA that are greater than that seen for the unprocessed IFN-γ mRNA. The increase in processed IFN-γ mRNA appears to be due to increased mRNA stability which then promotes increased nucleocytoplasmic shuttling of the mature IFN-γ mRNA. These data support a model whereby mobilization of nuclear IFN-γ mRNA stores allows NK cells to rapidly and robustly respond to secondary cytokine activators in a transcription-independent manner, thus shortening the time for overall cellular response to inflammatory signals.

Gamma interferon (IFN-γ) is a type II IFN that was initially discovered more than 30 years ago (55). Since that time IFN-γ has been extensively studied and has been found to profoundly affect a variety of immune responses, which include upregulation of major histocompatibility complex class I and II expression (19, 49), proliferation and differentiation of lymphocyte populations (5, 54), and induction of genes that code for immunomodulatory proteins such as tumor necrosis factor alpha (11, 52) and nitric oxide synthase (24). The primary cellular producers of IFN-γ are T and NK cells. In T cells, the T-cell receptor–CD3 complex mediates IFN-γ production. In NK cells, IFN-γ production is triggered by the interaction of NK cells with target cells (e.g., tumor or virally infected cells) or by a variety of cytokines.

The structure of the human IFN-γ gene was first reported in 1982 (17, 51). Shortly after this discovery, DNase I hypersensitivity studies identified several putative transcriptional regulatory regions in the promoter and first intron of the IFN-γ gene (20, 21). The IFN-γ promoter contains multiple *cis*-acting regulatory elements that may aid in tissue-specific expression of IFN-γ. An abbreviated list of nuclear factors that interact with these elements include GATA-3, CREB-ATF, NF-κB, NFAT, and YY-1 (10, 41, 48, 58). The abundance of regulatory sites in the IFN-γ promoter and the complexities associated with the number of DNA binding proteins have blocked full understanding of tissue-specific transcriptional control of IFN-γ. This understanding is further complicated in that multiple extracellular factors interact with NK or T cells to influence the expression of IFN-γ.

Two cytokine factors that strongly affect IFN-γ expression are interleukin-2 (IL-2) and IL-12. These cytokines can act independently to induce IFN-γ expression; however, together they act synergistically to induce large amounts of IFN-γ (25). Increases in the amount of secreted IFN-γ protein are preceded by increases in mRNA accumulation and protein synthesis (9, 32, 34, 53, 57, 60). The increased IFN-γ mRNA accumulation is at least partially due to increased transcriptional activity of the IFN-γ gene (1, 8, 57, 59); however, mRNA stabilization has been observed in phytohemagglutin-activated T cells in response to IL-12 alone (8, 35) and in NK cells costimulated by IL-2 and IL-12 (57).

Although many studies have partially elucidated the molecular mechanisms involved in transcriptional regulation of the IFN-γ gene, posttranscriptional control has not been widely studied. To better define the mechanism involved in stabilization of the IFN-γ mRNA, we conducted a series of experiments using human natural killer cell line NK 92 as a model system. This cell line was chosen because it parallels primary NK cells with respect to changes in IFN-γ production in response to IL-2 and IL-12. While it has been reported that both IL-2 and IL-12 are required for posttranscriptional control of mRNA expression in NK cells (57), we find that IL-12 acts alone and in a novel fashion to mediate posttranscriptional control of IFN-γ expression. In contrast to cytoplasmic stabilization by IL-2 plus IL-12, IL-12 effects are nuclear and result

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in increased and sustained accumulation of both the precursor and processed forms of IFN-γ mRNA. The increase in nuclear mRNA accumulation appears to be caused by retention and not synthesis of new IFN-γ mRNA, as nuclear IFN-γ mRNA accumulation remains elevated at times when transcriptional activity of the gene and cytoplasmic mRNA accumulation are greatly diminished. Nuclear retention of the IFN-γ mRNA suggests that the nucleus may function as a repository for IFN-γ mRNA. This is supported by our observation that IL-2 addition to NK 92 cells overcomes the IL-12-induced nuclear retention of the IFN-γ mRNA and stimulates rapid nucleocytoplasmic transport of the IFN-γ mRNA. Ultimately, the increase in cytoplasmic IFN-γ mRNA results in enhanced IFN-γ protein synthesis and release from NK 92 cells.

MATERIALS AND METHODS

Cell culture. NK 92 cells were maintained in RPMI 1640 medium (Bio Whittaker, Walkersville, Md.), supplemented with 10% fetal calf serum, 2 mM L-glutamine, and 100 U of penicillin, 100 μg of streptomycin, 200 U of recombinant human IL-2, and 10 ng of recombinant human IL-15/ml. Cells were cultured to a density of $\leq 5 \times 10^5$/ml in a 37°C incubator with 5% CO_2. For all experiments, cells were maintained at a density of 10^6/ml in medium lacking IL-2 and IL-15 for 12 h prior to cytokine stimulation. Cytokines were used at the following concentrations for stimulation of IFN-γ gene expression: IL-2, 100 U/ml, IL-12, 10 U/ml.

Nucleus isolation. NK 92 cells were pelleted by centrifugation at 1,200 rpm in a Sorvall H1000B rotor, washed once in phosphate-buffered saline (PBS), resuspended in 3 ml of homogenization buffer (15 mM HEPES [pH 7.4], 0.3 M sucrose, 60 mM KCl, 15 mM NaCl, 2 mM EDTA, 0.5 mM EGTA, 0.15 mM spermine, 0.5 mM spermidine, 1 mM phenylmethylsulfonylfluoride, 0.6% NP-40), and lysed by incubation on ice for 5 min. The homogenate was centrifuged at $800 \times g$ for 5 min at 4°C. The pellet was washed twice in 2 ml of nucleus wash buffer (homogenization buffer without detergent) and centrifuged at $800 \times g$ for 5 min at 4°C. The final nuclear pellet was resuspended in 100 μl of nucleus storage buffer (50 mM Tris-HCl[pH 8.3], 40% glycerol, 5 mM $MgCl_2$, 0.1 mM EDTA) and stored at −70°C or used immediately for nuclear RNA isolation.

RNA isolation. Nuclear and cytoplasmic RNAs were isolated from cells by using Trizol and Trizol LS (Life Technologies, Bethesda, Md.) extraction procedures, respectively. Approximately 10^8 cells were lysed according to the nucleus isolation protocol described above. Cytoplasmic and nuclear RNAs were prepared immediately following cell lysis and centrifugation steps. Following centrifugation, cytoplasmic RNA extraction was performed on the supernatant and nuclear RNA was extracted from the remaining pellet.

Probe design for RPA and in situ hybridization. For human IFN-γ RNase protection assays (RPAs), probes were designed to cross exon 1-intron 1 (E1-I1) and exon 3-intron 3 (E3-I3) boundary regions of the gene. The E1-I1 probe protects a 320-nucleotide (nt) exonic-intronic fragment and also a 113-nt fragment that spans all of exon 1. The E3-I3 probe protects a 433-nt exon-intron fragment and also all 182 nt of exon 3. Probe fragments were generated by PCR from plasmids containing human IFN-γ genomic DNA. *Eco*RI and *Bam*HI restriction enzyme sites were placed at the 5′ ends of the PCR primers for directional cloning of the PCR products into pGEM3Z (Promega, Madison, Wis.). Subclones derived from pGEM3Z that contained the IFN-γ exon-intron sequences were linearized with *Eco*RI and used as templates in in vitro transcription reactions. A eukaryotic 18S rRNA probe (Ambion, Austin, Tex.) was used as an internal control to assay for sample-to-sample variation in the RPA.

For in situ hybridization experiments, two IFN-γ sequences were used. An IFN-γ intron 1-specific probe used to detect unspliced forms of the IFN-γ mRNA was generated by cloning an intron 1-specific 259-bp *Xba*I-*Hin*dIII digestion fragment into pGEM3Z. The plasmid was linearized with *Xba*I for the generation of antisense RNA in in vitro transcription reactions. The cDNA-specific probe was generated by PCR amplification of a plasmid that contained the full IFN-γ cDNA sequence with primers 5′-*GTTAAC*CTTAATTCTCTCG GAAACGATG-3′ and 5′-*GTTAAC*CAAATATTGCAGGCAGGACAAC-3′. The primers were designed with *Hpa*I restriction sites (italicized) at the 5′ ends to allow for cloning into pGEM3Z. The recombinant pGEM3Z plasmid was linearized with *Ava*I for generation of an antisense IFN-γ-specific cDNA probe.

In vitro transcription reactions. All DNA fragments used in in vitro transcription reactions for IFN-γ-specific RPAs were gel purified prior to usage. In vitro transcription assays were performed according to the manufacturer's protocols using SP6 MAXIscript (Ambion) or Pharmingen (Sun Diego, Calif.) in vitro transcription kits. For IFN-γ-specific RPAs, the newly synthesized [α-^{33}P]UTP-labeled RNAs were loaded onto a 6% denaturing polyacrylamide gel and full-length probes were excised and eluted from the gel by overnight incubation at 37°C in gel elution buffer (supplied in RPA II kit; Ambion). To measure IFN-γ mRNA abundance, approximately 100×10^4 and 50×10^4 cpm of each exon-intron probe and 18S rRNA probe, respectively, were added to each RPA reaction. For Pharmingen multiprobe RPAs, α-^{33}P-labeled probes were purified from unincorporated nucleotides by size elusion chromatography with a Sephadex G-25 MicroSpin column (Amersham Pharmacia, Piscataway, N.J.). Approximately 0.5×10^6 to 1.0×10^6 cpm was added to each RNA in a final hybridization volume of 10 to 20 μl (at least 50% Pharmingen hybridization buffer). For in situ hybridization, the IFN-γ intron 1 and cDNA probes were synthesized in the presence of biotin-UTP and fluorescein-UTP (Roche Biochemicals, Indianapolis, Ind.), respectively. Probes were purified using Sephadex G-25 MicroSpin columns.

In situ hybridization. NK 92 cells were cytospun onto poly-L-lysine-coated slides and fixed in 4% paraformaldehyde–PBS for 10 min at room temperature (RT). The fixed cells were placed into PBS and stored at 4°C until used. For hybridization, probes were added to the slides at a final concentration of 2.5 ng/μl in PerfectHyb Plus hybridization buffer (Sigma, St. Louis, Mo.) and incubated overnight at 42°C in a humidity chamber. Slides were washed four times for 5 min in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% sodium dodecyl sulfate (SDS) at RT and then twice for 10 min in 0.1× SSC–0.1% SDS at 42°C and rinsed twice in 2× SSC. The slides were treated for 15 min in 2× SSC with 10 μg of RNase A/ml and then rinsed twice in 2× SSC and washed once for 15 min in PBS. Anti-fluorescein rabbit immunoglobulin G Alexa 488 (Molecular Probes, Eugene, Oreg.) and Rhodamine Red-X-conjugated steptavidin (Jackson ImmunoResearch, West Grove, Pa.) were diluted 1:200 in PBS and added to slides. Slides were incubated for 1 h in the dark, washed twice for 5 min in PBS, and then stained with DAPI (4′,6′-diamidino-2-phenylindole) (Molecular Probes). The cells were observed by confocal microscopy with a Carl Zeiss model 510 laser scanning microscope equipped with four lasers.

RPAs. IFN-γ-specific RPAs were performed according to the manufacturer's protocol using an RPA kit (RPA II) (Ambion) with 5 to 10 μg of input RNA per reaction. The multiprobe RPAs were performed according to the manufacturer's (Pharmingen) directions with the following modifications. RNase inactivation and precipitation were performed with a master cocktail containing 200 μl of Ambion RNase inactivation reagent, 50 μl of ethanol, 5 μg of yeast tRNA, and 1μl of Ambion GycoBlue coprecipitate per RNA sample. After the individual RNase-treated samples were added to 250 μl of the inactivation/precipitation cocktail, the samples were mixed well, placed at −70C for 30 min, and subjected to centrifugation at $20{,}800 \times g$ for 15 min in an RT microcentrifuge. The supernatants were decanted, a sterile cotton swap was used to remove excess liquid, and the pellet was resuspended in 3 μl of Pharmingen sample buffer. For all RPAs, RNA products were separated by size on a 6% denaturing polyacrylamide gel. Gels were dried under vacuum at 80°C for approximately 2 h and placed into storage phosphor cassettes and exposed for 16 to 24 h. The images were visualized and quantitated using PhosphorImager SI analysis and ImageQuant (Molecular Dynamics, Sunnyvale, Calif.).

Nuclear run-on assay. Nuclear run-on assays were performed as previously described (33) with the exception that, following UTP incorporation, the nuclei were digested in 1× SET buffer (10 mM Tris [pH 7.5], 5 mM EDTA, 1% SDS) with proteinase K at a final concentration of 1 mg/ml. A DE81 filter assay (47) was performed on the nuclear lysates to determine the percent incorporation of radioactive nucleotide into the newly synthesized transcripts. RNA was prepared from the nuclear lysate according to the manufacturer's protocol using Trizol LS. The amount of radioactivity in each sample was calculated, and approximately 20×10^6 cpm of sample RNA was hybridized to each filter, containing immobilized cDNAs for human IFN-γ, human β-actin, and PUC 8 vector DNA (control for nonspecific hybridization). Filters were hybridized for 48 to 72 h at 42°C. Filters were washed and placed into a PhosphorImager cassette for overnight exposure. Images were visualized and quantitated using PhosphorImager SI analysis and ImageQuant.

Reverse transcription and TaqMan PCR analysis. Nuclear RNA for reverse transcription was prepared by the Trizol purification procedure. Reverse transcription reactions were performed in a total volume of 20 μl. Each reaction mixture contained 20 ng of nuclear RNA, 1× reverse transcription buffer (Roche Molecular Biochemicals) 1 mM deoxynucleoside triphosphates (PE Applied Biosystems, Foster City, Calif.), 24 U of RNasin (Promega), 11 U of avian myeloblastosis virus reverse transcriptase AMV-RT (Roche Molecular Biochemicals), and either 3.75 μM oligo(dT), random hexamers, or IFN-γ gene-

specific primers. The reverse transcription reactions were carried out at 42°C for 60 min, and reaction mixtures were held at 4°C for no more than 15 min before real-time PCR was performed. Control reactions without avian myeloblastosis virus reverse transcriptase were performed to demonstrate that the quantitative real-time PCR measurements were the result of reverse transcription of the RNA template and not due to DNA contamination of the input RNA.

Quantitative real-time PCR was performed with an ABI Prism 7700 sequence detector (PE, Applied Biosystems). The PCR primers (5′-CTAATTAGGCAA GGCTATGTGATT-3′ and 5′-CATCAAGTGAAATAAACACACAA-3′) and the Taqman probe (5′-CCAACTAGGCAGCCAACCTAAGCA-3′) were designed to target the 3′ untranslated region (UTR) of the IFN-γ gene. The Taqman probe was synthesized by PE, Applied Biosystems and was labeled with reporter dye 6-carboxyfluorescein (FAM) and quencher dye 6-carboxy-tetramethylrhodamine (TAMRA) on its 5′ and 3′ ends, respectively. PCRs were performed in triplicate for each sample. Each reaction was performed on one-fourth of the reverse-transcribed product with 1× Taqman universal PCR master mixture (PE, Applied Biosystems), a 900 nM concentration of each primer, and 900 nM Taqman probe. The thermal cycle conditions were 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, 50°C for 30 s, and 72°C for 1 min. The release of the fluorescent reporter dye resulting from the nucleolytic cleavage of the Taqman probe by the *Taq* polymerase was measured over the course of the PCR run. Plasmid D2107, which contains 8.6 kb of human IFN-γ genomic DNA, was used as the reference DNA for the quantitative PCR. The plasmid DNA was prepared as a series of 10-fold serial dilutions prior to PCR amplification. The change in fluorescence of the sample compared to that produced by an internal reference dye (ROX) was referred to as the normalized sample fluorescence. When the value of the sample fluorescence increased to 10 standard deviations above the background (ROX) fluorescence, a threshold fluorescence cycle (C_t) was reached. From a log plot of the initial target copy number for the DNA standard versus C_t, the amount of target DNA in the unknown samples could be accurately determined. The linear dynamic range of the primer-probe set was from 10^3 to 10^{10} copies of DNA with a correlation coefficient of >0.98.

RESULTS

IL-12 induces nuclear accumulation of the IFN-γ mRNA. The mechanism(s) responsible for posttranscriptional control of IFN-γ gene expression is unknown. Stimulation of NK cells by IL-2 and IL-12 provides a good cellular model for the study of posttranscriptional control of IFN-γ expression; however, these cytokines also contribute to transcriptional activation of the IFN-γ gene (8, 57). Thus, we began by analyzing the impact of these cytokines on the transcriptional activity of the IFN-γ gene. Using PhosphorImager SI and ImageQuant analysis programs, we first quantitated the fold change in transcriptional activity in NK cells following stimulation with IL-2 and IL-12 alone and in combination. Nuclear run-on assays of nuclei isolated from NK 92 cells after treatment with IL-2 demonstrated an 11-fold peak in transcriptional activity after 3 h of treatment; activity then fell to undetectable levels (Fig. 1). Stimulation of NK 92 cells with IL-12 resulted in a greater-than-100-fold increase in transcription after 3 h of stimulation (Fig. 1). Moreover, transcriptional activity remained high for at least 6 h following treatment. The response of NK 92 cells to costimulation by IL-2 and IL-12 was initially very similar to that seen with IL-12 stimulation in that a greater-than-100-fold increase in transcription was observed at 3 h of stimulation (Fig. 1). At later times, transcriptional activity deviated from that seen with IL-12 alone in that it was far more sustained. This was demonstrated after 12 h of IL-2 and IL-12 costimulation, whereby transcriptional activity of the IFN-γ gene was still approximately 75% of the peak rate. In contrast, by 12 h, IL-12-induced transcription was reduced to 20% of that seen at the 3-h peak.

While transcriptional control of gene expression is a nuclear event, posttranscriptional control is not limited to a singular cellular compartment and may occur in either the nucleus or the cytoplasm (14, 16, 18, 23, 61). Thus, our next studies were designed to identify the cellular location responsible for posttranscriptional regulation of IFN-γ gene expression. To simultaneously measure mRNA accumulation in the nuclear and cytoplasmic compartments of NK 92 cells, RPAs were performed using probes that crossed exon-intron boundary regions. The use of the exon-intron-containing sequences allowed us to monitor both the unprocessed and processed nuclear forms of the IFN-γ mRNA as well as cytoplasmic mRNA with a single probe (Fig. 2).

In IL-2-treated cells, accumulation of IFN-γ mRNA in both

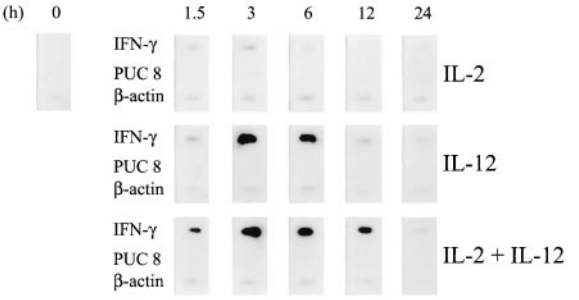

FIG. 1. Nuclear run-on analysis of nuclei isolated from NK 92 cells treated with IL-2, IL-12, and IL-2 plus IL-12. NK 92 cells were rested in medium without IL-2 and IL-15 for 12 h and then stimulated with IL-2 (1,000 U/ml), IL-12 (10 U/ml), or IL-2 (1,000 U/ml) plus IL-12 (10 U/ml) for the times indicated. Nuclei were isolated from cells following stimulation and incubated in a transcription mixture containing [^{32}P]UTP for 20 min at 26°C. Nuclei were lysed, and nuclear RNA was isolated with Trizol LS. The radioactive transcripts were hybridized at 42°C for at least 48 h to filters containing cDNAs for human IFN-γ, PUC 8, and human β-actin. The filters were washed and exposed in a phosphorimager cassette for 24 to 48 h. The data are representative of those obtained from three independent experiments.

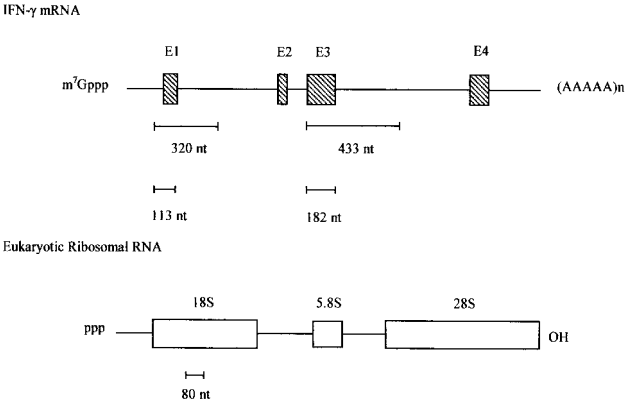

FIG. 2. Probe design for RPA. Two IFN-γ probes were designed to cross exon-intron boundary regions. The E1-I1 probe protects a 320-nt exon-intron mRNA fragment. The exon-specific portion of the probe protects a 113-nt fragment present in the spliced form of the message. The E3-I3 probe protects a 433-nt exon-intron mRNA fragment. The exon-specific portion of this probe protects a 182-nt fragment. A eukaryotic 18S rRNA probe was also selected as an internal control for sample variation in the RPAs. This probe protects an 80-nt fragment of the 18S rRNA.

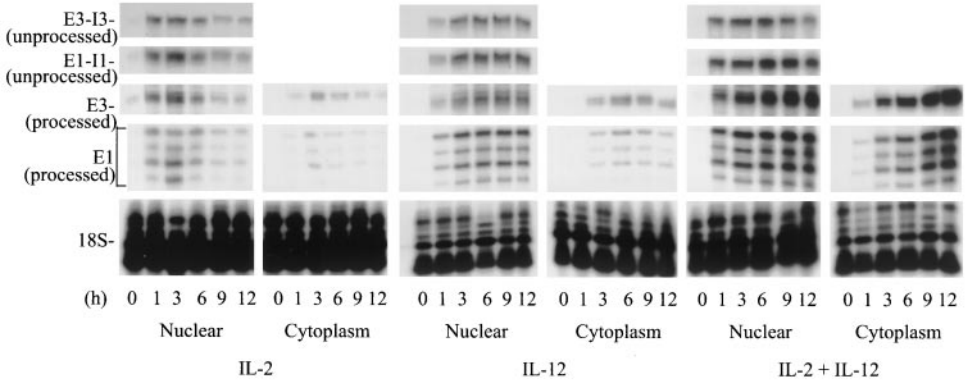

FIG. 3. RPA analysis of IFN-γ mRNA accumulation in the nuclei and cytoplasm of NK cells. NK 92 cells were rested in medium without IL-2 and IL-15 for 12 h. Cells were stimulated with IL-2 (100 U/ml) or IL-12 (10 U/ml) alone or IL-2 (100 U/ml) plus IL-12 (10 U/ml) for the times indicated. Cells were lysed, and nuclear and cytoplasmic RNAs were isolated. An RPA was performed using [^{33}P]UTP-labeled E1-I1, E3-I3, and 18S rRNA riboprobes. IFN-γ mRNA accumulation in the nuclei and cytoplasm of NK 92 cells, treated as indicated, is shown. For all RPA samples, a probe that detected changes in 18S RNA expression was used as a control for differences in mRNA input in the hybridization reactions and also to control for gel loading differences. This RPA is one of three independent assays, all with similar results.

the nucleus and cytoplasm was detected by 3 h of IL-2 stimulation and declined thereafter (Fig. 3). This indicated that changes in the nuclear and cytoplasmic accumulation of IFN-γ mRNA mirrored the changes in the transcriptional activity of the IFN-γ gene. Thus, our data supported earlier reports that demonstrated that IL-2, in the absence of other cytokines, regulated IFN-γ expression entirely by alterations in transcriptional activity (8, 57).

Interestingly, nuclear accumulation of the IL-12-induced IFN-γ mRNA did not parallel the transcriptional activity of the IFN-γ gene. Instead, IL-12 induced an elevated and sustained accumulation of both the precursor and processed forms of the IFN-γ mRNA (Fig. 3). The elevation in nuclear IFN-γ mRNA expression was visible by 1 h, plateaued after 3 h of stimulation, and continued to be high throughout the remainder of the 12-h observation period. There was no selective accumulation of either the precursor or processed forms of the mRNA in response to IL-12 stimulation, demonstrating that alterations in nuclear accumulation of the IFN-γ mRNA were not specific to any given form. Surprisingly, the cytoplasmic IFN-γ mRNA accumulation pattern in these cells failed to follow the nuclear IFN-γ mRNA accumulation pattern but was similar to transcriptional initiation in that it was transient. This transitory pattern of cytoplasmic expression was demonstrated by a significant increase in the amount of IFN-γ mRNA after 3 h of IL-12 stimulation, which peaked by 6 h and then declined at later times (Fig. 3).

RPA analysis demonstrated that costimulation with IL-2 plus IL-12 resulted in large increases in the amounts of processed nuclear and cytoplasmic IFN-γ mRNAs. Interestingly, in the nucleus, the expression patterns of precursor mRNA and processed mRNA were dramatically different (Fig. 3). There, the precursor IFN-γ mRNA peaked by 6 h of stimulation with IL-2 plus IL-12 and then declined; however, the processed IFN-γ mRNA plateaued after 6 h of treatment and remained elevated (Fig. 3). Increases in the amount of cytoplasmic mRNA could also be seen after 1 h of costimulation, and the amount continued to rise throughout the 12-h time course. This cytoplasmic buildup of the IFN-γ mRNA in cells stimulated with IL-2 plus IL-12 was in direct contrast to that observed in cells treated with IL-12 alone, where mRNA expression was transient.

IL-2 and IL-12 together stabilize nuclear IFN-γ mRNA. The sustained accumulation of the processed nuclear IFN-γ mRNA in the NK cells treated with IL-2 plus IL-12 led us to speculate that together these cytokines may specifically promote stabilization of this form of the mRNA. To test this, experiments were conducted on actinomycin D-treated NK 92 cells that had been prestimulated with IL-2, IL-12, or IL-2 plus IL-12 for 3 h. RPA analysis of the nuclear IFN-γ mRNA demonstrated that IL-2 plus IL-12 had a profound effect on nuclear stabilization of the processed form of the mRNA (Fig. 4). After 1 h of actinomycin D treatment, only trace amounts of the precursor mRNA remained in the cells treated with IL-2 plus IL-12 (Fig. 4A). In contrast, the processed form of the mRNA underwent minimal amounts of decay, with approximately 95% of the mRNA still present after 3 h of actinomycin D treatment (Fig. 4B). The IL-2 and IL-12 alone were not as effective in blocking decay of the processed IFN-γ mRNA. After 3 h of actinomycin D treatment there was visual evidence of mRNA breakdown (Fig. 4A), with only approximately 65 and 85% of the processed mRNA remaining in the IL-2- and IL-12-treated cells, respectively (Fig. 4B). The IL-2-plus-IL-12 stabilization effects were observed by using the E3-I3 probe, which protects at the 3′ end of the IFN-γ mRNA. The 5′ end-specific E1-I1 probe also demonstrated analogous mRNA decay patterns with respect to all cytokine treatments (data not shown).

Polyadenylation as a regulator of nuclear IFN-γ mRNA accumulation. Although the combination of IL-2 plus IL-12 had profound effects on nuclear IFN-γ mRNA accumulation, our RPA data demonstrated that IL-12 alone could promote sustained nuclear expression of all forms of the IFN-γ mRNA at times when cytoplasmic IFN-γ mRNA levels had dropped. The IL-12 induced effects on the unprocessed and processed IFN-γ mRNAs paralleled one another, suggesting that splicing was not a regulated step. Moreover, the IL-12-induced increase in the half-life of the processed IFN-γ mRNA over that

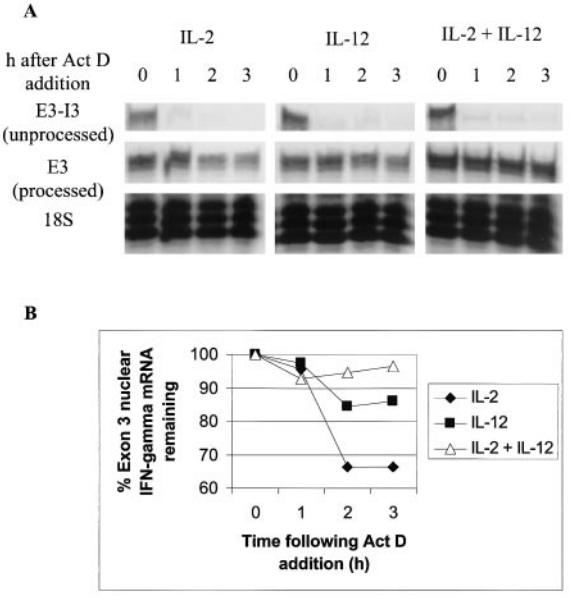

FIG. 4. RPA analysis of IFN-γ mRNA decay in cytokine-stimulated NK cells. NK 92 cells were rested in media without IL-2 and IL-15 for 12 h. The cells were stimulated with IL-2 (100 U/ml) and IL-12 (10 U/ml) alone or in combination for 3 h. Actinomycin D (Act D; 5 μg/ml) was added to the media, and the cells were incubated for the times indicated. Nuclei were isolated, and mRNA was extracted. (A) Accumulation of the nuclear IFN-γ mRNA following actinomycin D treatment as measured by RPA using the E3-I3 probe. An 18S probe was also used to control for variations in RNA input during the RPA and gel loading. (B) Graphical representation of the quantitation performed by ImageQuant analysis of the image shown in panel A. The experiment is one of three separate experiments, all with similar results.

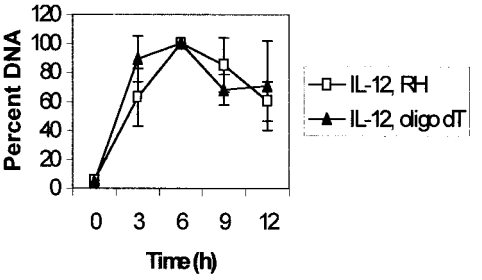

FIG. 5. Quantitative analysis of IFN-γ poly(A) in nuclei of NK cells. NK 92 cells were rested in the absence of IL-2 and IL-15 for 12 h and then stimulated with IL-12 (10 U/ml) for the times indicated. Nuclear RNA was isolated and reverse transcription-PCR was performed with random hexamers and oligo(dT) as primers for reverse transcription. Quantitative real-time PCR was performed on the resulting cDNAs with an IFN-γ-specific Taqman probe and primers specific for the 3′ UTR of the IFN-γ gene. The amount of DNA synthesized from the random hexamer (RH)-generated cDNA (open squares) was compared to the amount produced from the oligo(dT)-generated cDNA (solid triangles). Data for each time point represent the mean values of three separate experiments ± two standard deviations.

seen in the IL-2-treated cells could not explain the concurrent and discordant expression of IFN-γ mRNA in the nuclear and cytoplasmic compartments. These observations led us to speculate that nuclear retention of the IFN-γ mRNA may be primarily a control mechanism in IL-12-stimulated NK cells.

Because polyadenylation is involved in mRNA stability, processing, nucleocytoplasmic transport, and translation (2, 40, 44, 45), we speculated that an IL-12-induced nuclear blockage of IFN-γ mRNA may be due to a partial or complete loss of mRNA polyadenylation. To test this assumption, quantitative real-time PCR was used to monitor the polyadenylation state of the IFN-γ mRNA. The amounts of nonpolyadenylated and polyadenylated IFN-γ mRNAs in nuclei of IL-12-treated NK 92 cells were compared by reverse transcribing nuclear RNA with random hexamers or oligo(dT) as primers. Random hexamers were used to prime reverse transcription of all mRNA species regardless of their polyadenylation state, whereas oligo(dT) primers were used to initiate reverse transcription of only polyadenylated mRNAs. NK 92 cells were stimulated with IL-12 for the times indicated in Fig. 5, and the amounts of cDNA produced from random hexameric and oligo(dT) priming were quantitated and expressed as percentages of total DNA synthesized per unit time (Fig. 5). In three separate experiments, the quantitative PCR results demonstrated no difference in the amounts of DNA synthesized from the random hexamer and oligo(dT) reverse transcription-PCRs at any time following IL-12 stimulation. From this data, we conclude that essentially all of the nuclear IFN-γ mRNA is polyadenylated. Thus, nuclear retention of the IFN-γ mRNA is independent of the polyadenylation state of the mRNA.

The role of IL-2 as a secondary effector of IFN-γ mRNA expression. The reason for the sustained nuclear accumulation of IFN-γ mRNA in IL-12-stimulated NK cells is unknown; however, the nuclear stores of IFN-γ mRNA may enable NK cells to mobilize this mRNA very quickly as a response to secondary stimulatory signals. To examine this assumption, we stimulated NK 92 cells with IL-12 alone for 6 h. At 6 h cytoplasmic IFN-γ mRNA accumulation peaks in response to IL-12 treatment and then declines. By adding a secondary cytokine stimulus after 6 h of IL-12 stimulation and then comparing the cytoplasmic IFN-γ mRNA accumulation to that in IL-12-stimulated cells we were able to identify any alterations in mRNA expression that were specific to the secondary cytokine. For the secondary activator, we chose to test IL-2 because, when used as single activator, it has no effect on nuclear retention of IFN-γ mRNA (Fig. 3). A comparison of the cytoplasmic IFN-γ mRNA accumulation in NK 92 cells stimulated with IL-12 alone to that in cells with the IL-2 secondary stimulus revealed that, after 1 h of IL-2 addition, there was a significant increase in the amount of cytoplasmic IFN-γ mRNA (Fig. 6A). Moreover, the increase in IFN-γ mRNA was still apparent after 3 h of IL-2 secondary stimulation, whereas, in IL-12-stimulated cells, the amount of cytoplasmic IFN-γ mRNA was greatly diminished. The effects of IL-2 on nuclear IFN-γ mRNA accumulation resulted in unequal increases in the amounts of precursor and processed IFN-γ mRNAs (Fig. 6B). After 1 h of the secondary IL-2 stimulation, no increase in the amount of precursor IFN-γ mRNA was evident (Fig. 6B and C); however, a 30% increase in the amount of processed mRNA was observed. After 3 h of secondary IL-2 stimulation, there were only slight alterations in this effect, with approximately 12 and 40% increases in IFN-γ precursor and processed

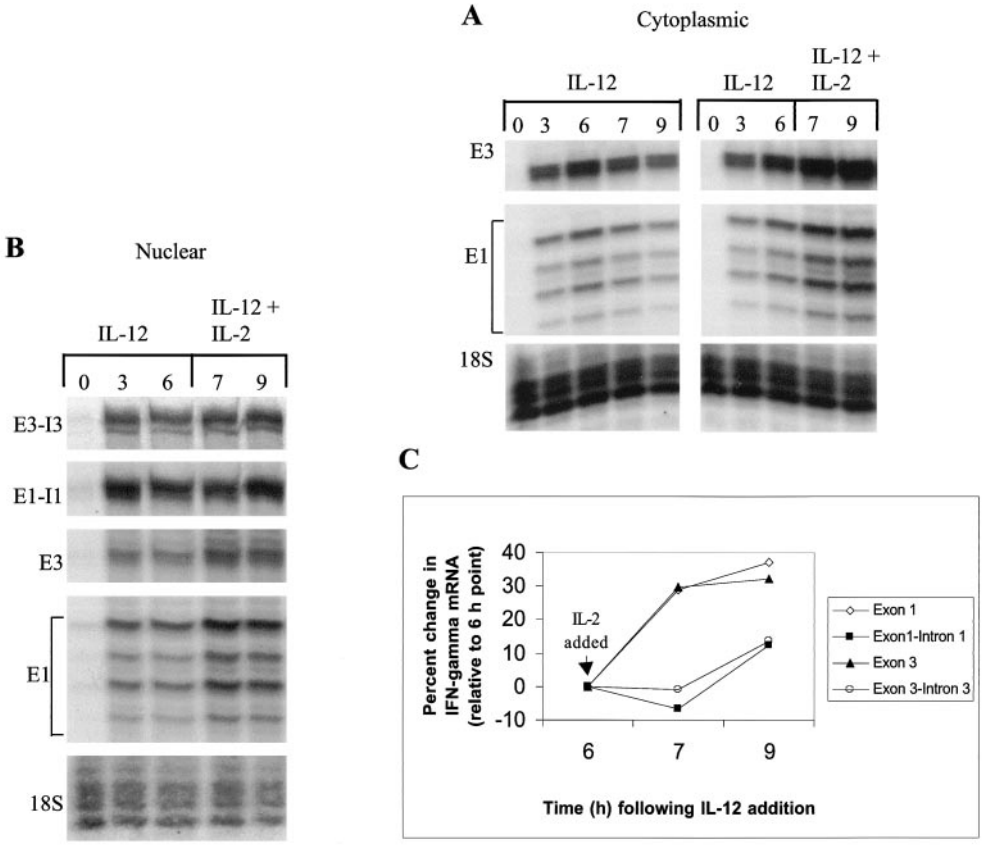

FIG. 6. Cytokine-induced mobilization of nucleus-retained IFN-γ mRNA to the cytoplasm of NK cells. (A) (Left) RPA analysis of cytoplasmic RNA isolated from NK 92 cells stimulated with IL-12 alone for the times indicated. (Right) RNA accumulation in NK 92 cells stimulated with IL-12 (10 U/ml) alone for 6 h and then costimulated with IL-2 (100 U/ml). (B) Nuclear accumulation of IFN-γ precursor and processed mRNAs in the same NK 92 cells stimulated with IL-12 and then with IL-2 as the secondary stimulus. The mRNA accumulation was measured by RPA using E1-I1, E3-I3, and 18S probes. In all reactions, an 18S ribosomal RNA probe was used to control for sample variability and gel loading differences. (C) Graphical illustration of the percent change in the processed form of the IFN-γ mRNA as compared to the unprocessed mRNA following IL-2 addition to the IL-12-prestimulated NK 92 cells. The experiment is one of three independent experiments, all having similar results. Quantitative values were calculated using ImageQuant analysis of the image shown in panel B.

mRNAs, respectively. Thus, IL-2 appears to specifically alter the accumulation of the processed form of the IFN-γ mRNA.

The IL-2-induced nuclear effect had little impact on precursor IFN-γ mRNA; however, we and others have demonstrated that IL-2 can induce transcriptional activation of the IFN-γ gene (8, 57). Therefore, we wanted to determine if IL-2 could induce transcription-independent transport of the IFN-γ mRNA out of the nucleus. To accomplish this, NK 92 cells were stimulated with IL-12 alone for 6 h and actinomycin D was added to the cells in the presence and absence of IL-2. Alterations in IFN-γ mRNA accumulation were measured by RPA using the E3-I3 probe. The inhibitory effect of actinomycin D on transcription was evident within 30 min following cotreatment with IL-2 and actinomycin D. At this time a greater-than-60% decrease in precursor IFN-γ mRNA expression occurred (Fig. 7A and C), and by 2 h the precursor IFN-γ mRNA was virtually absent from the nuclei. In contrast, after 2 h of treatment with IL-2 plus actinomycin D approximately 50% of the processed IFN-γ mRNA remained. This demonstrates that only the processed form of the IFN-γ mRNA is stabilized in response to IL-2 plus IL-12 when given sequentially (Fig. 7A) or together (Fig. 4).

Examination of nucleocytoplasmic transport of the IFN-γ mRNA revealed a rapid increase in cytoplasmic mRNA accumulation after 30 min of IL-2 and actinomycin D treatment (Fig. 7A and D). However, at later times, nucleocytoplasmic shuttling was abated. This may be due to the cytotoxic effects of actinomycin D on nuclear processing and shuttling (6). Nevertheless, the movement of IFN-γ mRNA at times immediately following actinomycin D addition strongly suggests that IL-2 acts posttranscriptionally to induce transcription-independent nucleocytoplasmic movement of the IFN-γ mRNA. To further test our findings, we conducted the same experiment but in the presence of a second transcriptional blocker, 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB). These experiments corroborated our actinomycin D results, in that IL-2 induced nucleocytoplasmic transport of the IFN-γ mRNA in the presence of DRB and IL-12 (data not shown).

To ensure that these effects were specific to IL-2 and independent of any secondary actions by actinomycin D, alterations

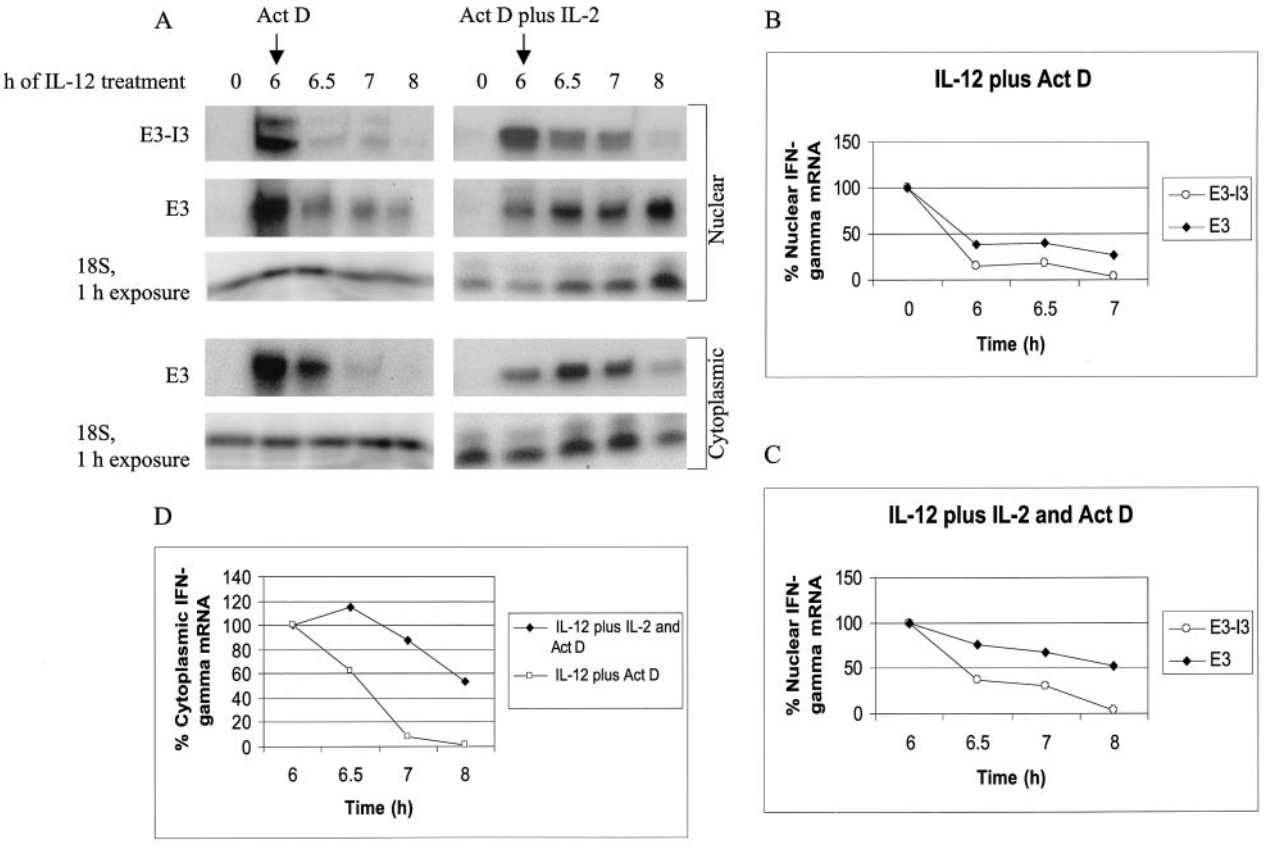

FIG. 7. RPA analysis of nuclear and cytoplasmic IFN-γ mRNA accumulation in NK 92 cells stimulated with IL-12 and actinomycin D (Act D) in the presence and absence of IL-2. (A) NK 92 cells were stimulated with IL-12 (10 U/ml) alone for 6 h and then treated with actinomycin D (2.5 μg/ml) with and without IL-2 (100 U/ml) for the times indicated. Nuclear and cytoplasmic RNAs were isolated and used in RPAs to evaluate changes in nuclear and cytoplasmic accumulation of the IFN-γ mRNA. A probe that detected 18S ribosomal RNA was used to control for differences in mRNA content during hybridization and also to control for loading between lanes. (B) Graphical illustration of the percentages of nuclear precursor and processed IFN-γ mRNA remaining following actinomycin D addition to IL-12-pretreated NK 92 cells. (C) Nuclear IFN-γ mRNA accumulation in IL-12-pretreated NK 92 cells following IL-2 and actinomycin D treatment. (D) Percent IFN-γ cytoplasmic mRNA accumulation in the IL-12- and actinomycin-treated NK 92 cells in the presence and absence of IL-2. For panels B to D values are percentages of mRNA relative to that at time 0. The 0-h value in panel B and the 6-h values in panels C and D are arbitrarily set at 100%. The calculated values represent ImageQuant analysis of the RPA shown in panel A. Results are representative of at least two independent experiments, all with similar results.

in nuclear IFN-γ mRNA accumulation and transport in IL-12-prestimulated NK 92 cells in the presence of actinomycin D alone were measured. Our observations clearly demonstrated decreases in both unprocessed and processed forms of the IFN-γ mRNA and a lack of nucleocytoplasmic shuttling (Fig. 7A and D). These data further support the ability of IL-2 to induce transcription-independent movement of IFN-γ mRNA.

IFN-γ synthesis and release in IL-12-activated NK cells. The increase in cytoplasmic IFN-γ mRNA accumulation following IL-2 addition to IL-12-pretreated NK 92 cells does not predict biological function, as all IFN-γ-mediated effects occur following cellular secretion of the cytokine. To evaluate IFN-γ protein production and release from NK 92 cells, enzyme-linked immunosorbent assays were performed on supernatants from NK 92 cells treated with IL-2 and IL-12 alone. IFN-γ release from these cells was compared to that observed in cells concurrently stimulated with the cytokines or stimulated with IL-12 alone for 6 h and then with IL-2 as a secondary stimulus. Addition of IL-2 or IL-12 alone to cells activated IFN-γ secretion; however, the amount of protein released was moderately low (<5,000 pg/ml at 24 h) (Fig. 8). In contrast, concurrent addition of IL-2 and IL-12 produced a robust induction of IFN-γ release from cells, which was approximately 14-fold higher than that seen with IL-2 or IL-12 alone after 24 h of stimulation. Interestingly, the addition of IL-2 to NK cells that been pretreated with IL-12 resulted in a dramatic increase in IFN-γ secretion. With this paradigm, IL-12 induction of IFN-γ was modest following 6 h of stimulation. Addition of IL-2 at this time produced a rapid induction of IFN-γ secretion, which was notable after 3 h of costimulation (9-h time point). Moreover, by 18 h of costimulation (24-h time point) the amount of IFN-γ secretion rapidly approached that seen in the cells concomitantly treated with IL-2 and IL-12. These data reveal that IL-12-preactivated cells can respond quickly and robustly to IL-2 as a secondary stimulus. This further supports a model whereby transcription-independent release of nuclear IFN-γ

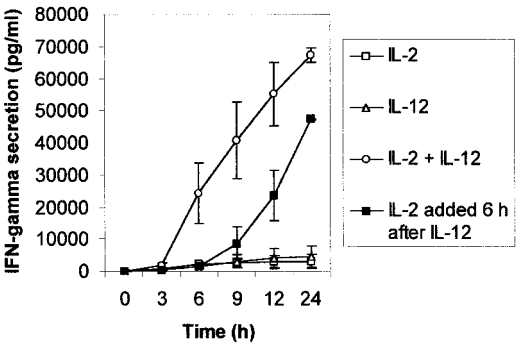

FIG. 8. IFN-γ production in NK 92 activated cells. NK 92 cells were stimulated with IL-2 and IL-12 alone or IL-12 plus IL-2 in combination or were pretreated with IL-12 for 6 h followed by IL-2 as a secondary stimulus for the times indicated. Supernatants were taken from cells and analyzed by enzyme-linked immunosorbent assay to determine the amount of IFN-γ secretion in response to the various cytokine treatments. Each value is a mean cytokine production± the range for two independent experiments. These data are representative of six separate experiments, all displaying similar patterns of IFN-γ secretion from NK 92 cells.

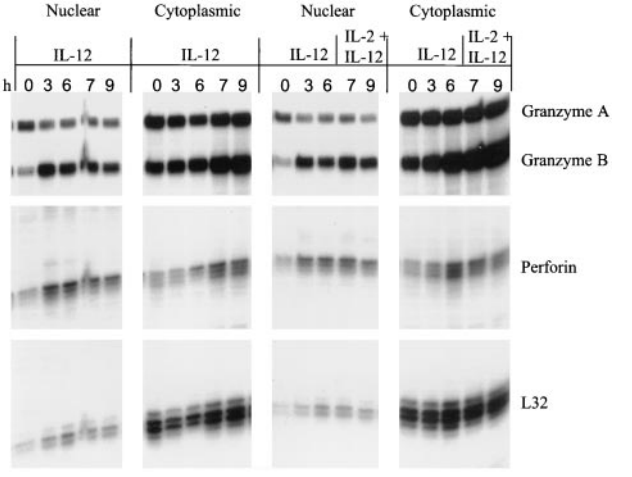

FIG. 9. Accumulation of IL-12-inducible mRNAs in NK 92 cells. Cells were stimulated with IL-12 alone for 6 h and then costimulated with IL-2 for the times indicated. Nuclear and cytoplasmic mRNAs were isolated and used in a multiprobe RPA to measure changes in mRNA accumulation. The transcription template chosen for the multiprobe assay was the hAPO-4 probe set from BD Pharmingen. This probe set includes an L32 ribosomal protein probe that is used as an internal control to measure for differences in mRNA input during hybridization reactions and to account for differences in RNA loading of the gel. Granzyme B and perforin mRNA accumulation are known to be responsive to IL-12 treatment. Granzyme A mRNA expression is independent of IL-12 treatment and is used a negative control. This RPA is representative of three independent experiments all with similar results.

mRNA stores results in rapid production and release of the IFN-γ protein from the cells, thus permitting cells to fine tune their ability to respond to multiple stimulatory signals that are received at different times.

Specificity of the IL-12 effects on nuclear mRNA retention in NK cells. IL-12 modulates mRNA expression of several other genes in NK cells. These genes include the granzyme B, perforin, and IFN-regulating factor 1 (IRF-1) (15, 46) genes. Regulation of these genes by IL-12 appears to be mainly due to changes in transcription, and, for IRF-1, transcriptional control is mediated by transcription factor STAT 4 (15). To compare the effects of IL-12 on IFN-γ mRNA expression to those on other IL-12-regulated genes, we performed RPA analysis on RNA purified from NK 92 cells treated with IL-12 alone or with IL-2 added 6 h following IL-12 stimulation. We monitored the kinetics of mRNA accumulation for the IL-12-inducible granzyme B and perforin genes and also the granzyme A gene, an IL-12-insensitive gene (46). We found that IL-12 induced the nuclear accumulation of perforin and, to a greater extent, granzyme B mRNAs (Fig. 9). Furthermore, this increase in nuclear mRNA accumulation was followed by increased cytoplasmic mRNA accumulation for both genes (Fig. 9). As an additional control for IL-12 specificity for these genes we examined changes in granzyme A mRNA accumulation. As previously reported (46), we found that cytoplasmic granzyme A mRNA accumulation was not affected by IL-12. However, IL-12 did appear to slightly downregulate nuclear expression of this gene.

Addition of IL-2 to IL-12-pretreated NK 92 cells did not significantly alter nuclear or cytoplasmic accumulation of granzyme B or perforin mRNA (Fig. 9). Although IL-2 alone has been reported to increase expression of these mRNAs (46), it is possible that addition of IL-12 prior to IL-2 can alter the effectiveness of IL-2 induction of granzyme B or perforin.

The examination of mRNA changes for granzymes A and B and perforin in NK 92 cells revealed that alterations in cytoplasmic mRNA accumulation were predicted by similar changes in nuclear expression. This is in direct contrast with our observations for IFN-γ mRNA expression in IL-12-treated NK cells. The differences in expression among granzyme B and perforin and IFN-γ genes in these cells demonstrate the ability of the IL-12 signaling pathways to discriminate and alter expression of genes in different ways. Furthermore, the lack of congruence between IFN-γ mRNA expression and that of other genes provides additional support for IL-12 to selectively modulate nuclear IFN-γ mRNA expression.

Effects of IL-12 on the intranuclear localization of precursor and processed forms of IFN-γ mRNA. To further examine the specificity of the effect of IL-12 on nuclear IFN-γ mRNA expression, we performed in situ hybridization assays. Unlike biochemical assays, in situ hybridization allows us to visualize colocalization of the unprocessed and processed forms of the IFN-γ mRNA. We speculated that, in IL-12-stimulated NK cells, a high degree of mRNA colocalization may predict that these mRNAs have a common regulatory mechanism. In situ hybridization assays were performed on NK 92 cells using intron-only and cDNA antisense probes to differentiate between the unprocessed and processed forms of the IFN-γ mRNA, respectively. The first probe was biotin labeled and detected with a steptavidin-rhodamine conjugate. This probe was complementary to intron 1 and detected only the unprocessed forms of the IFN-γ mRNA. The second probe was labeled with fluorescein and detected with an antifluorescein antibody conjugated to fluorescein. This probe was generated from an IFN-γ cDNA sequence, and, although it contained an exon sequence which could allow it to hybridize to unprocessed

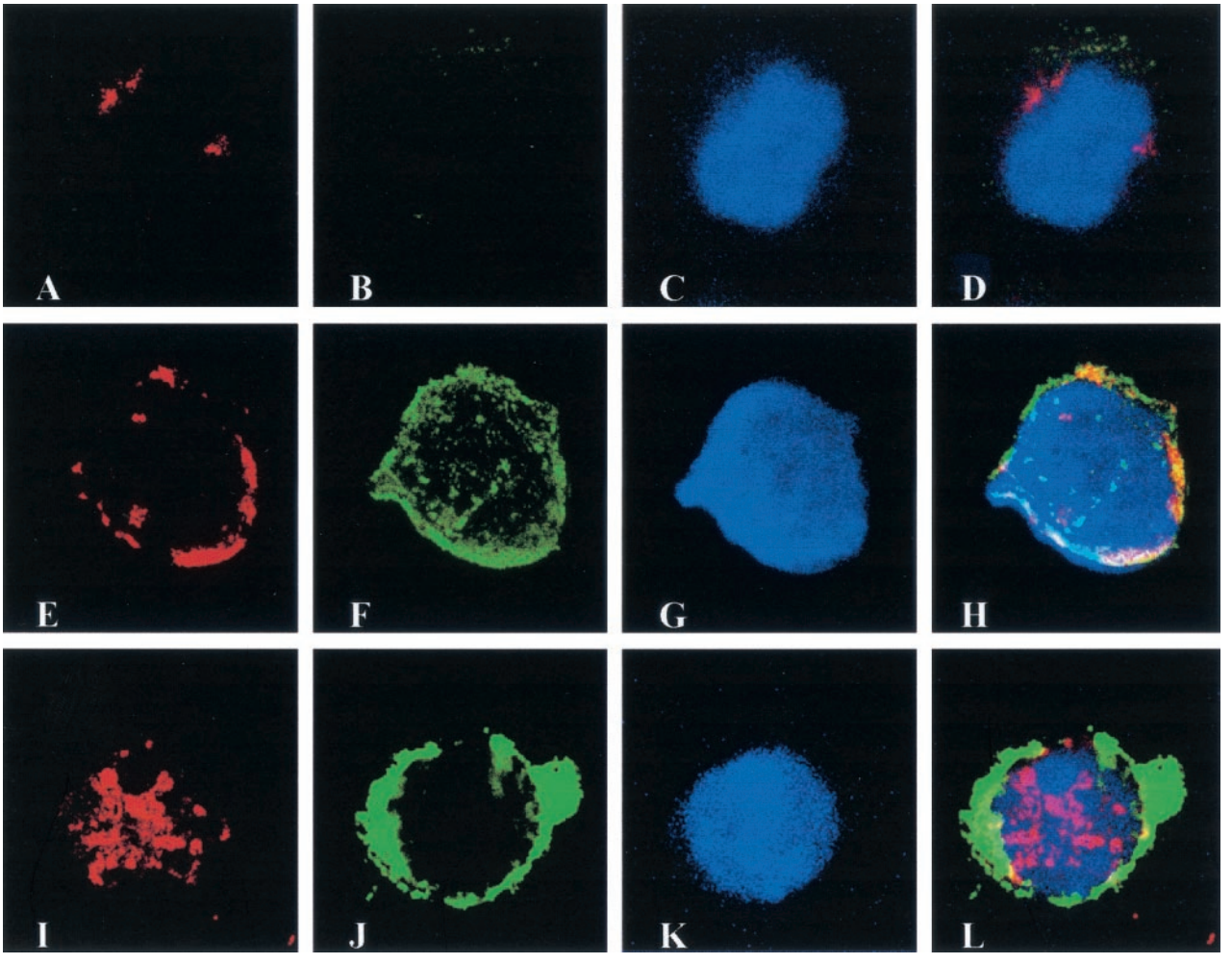

FIG. 10. Localization of precursor and processed forms of IFN-γ mRNA by in situ hybridization. IFN-γ cDNA and intron 1-specific probes were hybridized to NK cells that were unstimulated (A to D), stimulated with IL-12 alone (10 U/ml) for 12 h (E to H), or stimulated with IL-12 for 6 h and then costimulated by the addition of IL-2 (100 U/ml) for an additional 6 h (I to L). (A, E, and I) Hybridization of cellular RNA to the IFN-γ intron 1-specific probe. (B, F, and J) mRNA hybridization to the IFN-γ cDNA-specific probe. (C, G, and K) DAPI staining of the nuclei. (D, H, and L) Composites of the other panels.

mRNA, it primarily hybridizes to fully processed IFN-γ mRNA (Fig. 10J). This specificity is likely due to sequence complementarity across multiple splice junctions. For detection purposes, both probes were added to a single hybridization reaction mixture and a two-color fluorescence detection system was used to determine the distribution of the unprocessed and processed forms of the IFN-γ mRNA. In untreated cells, the IFN-γ mRNA expression was at very low levels in the nucleus and was virtually absent in the cytoplasm (Fig. 10A to D). We then examined nuclear IFN-γ mRNA accumulation in NK cells that had been stimulated with IL-12 for 12 h. This time was chosen because transcriptional activity no longer contributes to IFN-γ mRNA accumulation and thus any mRNA present should represent mRNA that is acted upon in a post-transcriptional manner. In cells treated with IL-12 for 12 h, there was a dramatic increase in the amount of nuclear IFN-γ mRNA (Fig. 10E to H). Moreover, the unprocessed and processed forms of the mRNA in the nucleus appeared to have a high degree of colocalization. In all cells, both the intron-containing and processed IFN-γ mRNAs were primarily located close to the nuclear periphery (Fig. 10E and F). However, the processed IFN-γ mRNA was more widely distributed throughout the nucleus (Fig. 10F). We also observed only a small amount of processed mRNA outside the DAPI-stained region of cells (Fig. 10H), indicating that the majority of the IFN-γ mRNA was nuclear.

Addition of IL-2 to cells that had been previously stimulated with IL-12 resulted in a significant redistribution of IFN-γ precursor mRNA throughout the nucleus (Fig. 10I). The redistribution pattern may indicate that the precursor mRNA is associating with splicing factor-rich domains termed "nuclear speckles" (13). These nuclear splicing domains would process and ready the IFN-γ mRNA for transport out of the nucleus. This hypothesis is consistent with our biochemical data that demonstrate that IL-2 addition to IL-12-pretreated cells results in a significant increase in the amount of processed IFN-γ mRNA.

In contrast to our observations made by using an intron-

specific probe on the costimulated cells, we found that the cDNA probe did not detect a widespread distribution of the IFN-γ mRNA in the nuclei of the costimulated cells (Fig. 10J). Instead, the majority of the processed IFN-γ mRNA appeared at the nuclear periphery and in the cytoplasm of the NK 92 cells (Fig. 10J and L). These data further demonstrate that IL-2 can mobilize the nuclear mRNA in such a way that it undergoes nucleocytoplasmic shuttling.

The appearance and colocalization of the unprocessed and processed IFN-γ mRNAs in IL-12-stimulated cells further support a model whereby there is a block in nucleocytoplasmic transport of the IFN-γ mRNA following IL-12 treatment. The block in nucleocytoplasmic transport may result in a concomitant block in the processing of IFN-γ mRNA. To overcome these processing and nucleocytoplasmic transport blockades, secondary cytokine activators are needed to send the proper signal to the nucleus to allow for reactivation of the processes necessary for IFN-γ mRNA maturation and transport.

DISCUSSION

Control of IFN-γ gene expression is complex and involves both transcriptional and posttranscriptional regulation. The complexity of this regulation is common among many early-response genes that encode proteins for transcriptional modulators, structural proteins, and cytokines (22, 26, 38, 43). Our results demonstrate that cytokines IL-2 and IL-12 alone and in combination impact the transcriptional activity of the IFN-γ gene and the amount of IFN-γ mRNA produced. We have found that regulation of IFN-γ gene expression by IL-12 is unique in that IL-12, as a single cellular stimulator, can cause an elevation in nuclear IFN-γ mRNA stores at times when transcriptional activity of the gene has declined. Previous reports by our laboratory and others have demonstrated that IL-12 does not stabilize cytoplasmic IFN-γ mRNA (8, 57), but its impact on nuclear mRNA accumulation has not been examined. The ability of IL-12 to increase nuclear accumulation of all forms of IFN-γ mRNA represents a novel form of posttranscriptional regulation. Our observations support two models: (i) IL-12 acts to promote nuclear retention of IFN-γ mRNA and (ii) IL-12 acts to stabilize the nuclear forms of the IFN-γ mRNA. These models could function independently or together to induce elevated amounts of IFN-γ mRNA in the nucleus. However, nuclear retention seems more plausible. This is because a stabilizing effect should not impede nucleocytoplasmic transport of the mRNA, thus permitting a continual buildup of cytoplasmic mRNA. Yet, in IL-12-treated cells, cytoplasmic accumulation of the IFN-γ mRNA is transient, suggesting that nucleocytoplasmic transport of the IFN-γ mRNA is absent. Interestingly, the nuclear retention effect of IL-12 of IFN-γ mRNA can be overcome by IL-2, resulting in rapid, transcription-independent shuttling of the IFN-γ mRNA. In contrast to IL-12 alone, the combination of IL-2 plus IL-12 appears to stabilize the processed form of the mRNA, which accounts for a disproportionately larger amount of processed IFN-γ mRNA than of unprocessed IFN-γ mRNA in the nuclei of NK 92 cells.

We conclude that IL-12 alone or in combination with IL-2 mediates nucleus-specific effects on IFN-γ mRNA accumulation. Furthermore, the signaling pathways directed by these two treatments target IFN-γ mRNA accumulation in very different ways. Studies to evaluate the IFN-γ mRNA structure as well as nuclear factors that mediate these various control mechanisms are under way. One potential group of regulatory proteins is the heterogeneous nuclear ribonucleoproteins (hnRNPs). During transcription, these proteins bind to a precursor mRNA and remain with that mRNA during processing and nuclear shuttling (36, 37). There are many hnRNPs, and it is possible that a particular hnRNP or related protein may associate with the IFN-γ mRNA in IL-12-stimulated cells, thus handicapping the processing and nucleocytoplasmic transport of the mRNA. Addition of IL-2 to IL-12-pretreated NK cells may allow for disassociation of this factor to overcome an IL-12-induced nuclear blockade of the IFN-γ mRNA.

Another potential regulator of nuclear IFN-γ mRNA expression is the TAP-p15 heterodimer complex. In humans, nuclear mRNA transport is dependent on mRNA interaction with this complex. One model for mRNA transport involves recruitment of the TAP-p15 complex to nuclear mRNA that is already complexed with hnRNPs (12). Once at the nuclear pore, some hnRNPs dissociate but others aid the TAP-p15 complex by acting as mRNA chaperones during cytoplasmic shuttling of the mRNA. Interestingly, microinjection of TAP and p15 into *Xenopus* oocytes stimulates export of mRNAs that are otherwise inefficiently transported (7), thus bypassing nuclear retention of these mRNAs. The ability of the TAP-p15 complex to overcome a nuclear transport blockage suggests that these proteins may be involved in the IL-2-induced transport of IFN-γ mRNA.

The dramatic increase in processed IFN-γ mRNA following IL-2 addition to IL-12-prestimulated NK cells is a novel cytokine-induced nuclear effect on posttranscriptional control of IFN-γ. Our current data demonstrate that there is stabilization of the processed form of the IFN-γ mRNA in cells stimulated with IL-2 plus IL-12; however, we have not yet investigated the effects of splicing on the accumulation of this form of the mRNA. Although splicing is not an absolute requirement for nucleocytoplasmic transport of a mRNA, efficient nuclear export appears to strongly depend on this process. Others have demonstrated that microinjected fully spliced naked mRNA or spliced mRNA that was subsequently complexed with hnRNPs prior to injection was not effectively transported from the nucleus of *Xenopus* oocytes (28). In contrast, a microinjected precursor form of the mRNA or mRNA that had undergone splicing in vitro before injection was efficiently transported. These data suggest that mRNA splicing events recruit specific nuclear factors that play a critical role in the nucleocytoplasmic transport of the processed mRNAs.

The nuclear proteins involved in posttranscriptional control of IFN-γ expression should mediate their effects by interacting with specific structures or sequences contained in the IFN-γ mRNA. Thus, nuclear retention of the IFN-γ mRNA in the IL-12-stimulated cells may be directed by an internal mRNA sequence element. This concept is supported by the identification of a sequence-directed nuclear mRNA retention element in the U2AF mRNA (29) that prevents nucleocytoplasmic transport of that mRNA. The IFN-γ mRNA contains several putative regulatory AUUUA pentameric repeats in its 3′ UTR. These elements are important regulators of mRNA stability (27, 50, 56). However, their role in nuclear retention

of mRNAs has not been established. Thus, we are currently investigating the potential of the AUUUA sequences as well as other mRNA elements to play a role in nuclear retention and release of IFN-γ mRNA.

The capacity of IFN-γ to modulate immune responses is well known (3, 4). Moreover, the ability of factors to regulate IFN-γ is critical for the maintenance of a healthy immune system. IL-12 is a potent activator of IFN-γ and can induce an initial burst of IFN-γ release from NK cells; however, we find that IL-12 can also induce accumulation and storage of nuclear IFN-γ mRNA in NK cells. This novel form of regulation may allow NK cells to respond more rapidly to other more latent cytokines that interact with NK cells at times further along in the immune response pathway. Consistent with this model, a report has demonstrated that preformed IL-12 is released from the dendritic cells within minutes after contact with intracellular parasites (42). This creates an environment where, at the site of a localized innate immune response, the rapidly released IL-12 may cause NK cells to increase their content of IFN-γ mRNA. As T cells enter the inflammatory site, they secrete IL-2. The interaction of IL-2 with its target receptors on NK cells triggers large quantities of IFN-γ protein production, further enhancing the localized immune response.

The combinatorial effects of IL-12 and IL-2 on IFN-γ production enable NK cells to be potent cellular regulators of immune function. However, IFN-γ production is not restricted to signaling pathways activated by these receptors. NK cells contain a large repertoire of receptors, two of which, the killer cell-activating receptors (KAR) in humans and their murine counterparts, Ly49s, can initiate signaling pathways that result in IFN-γ production (30, 31, 39). The potential for IL-12-preactivated NK cells to respond to KAR or Ly49 signaling pathways, thus leading to enhanced production of IFN-γ, has not been investigated. It is possible to envision that two such activating pathways could synergize to allow enhanced production of IFN-γ in NK cells. Thus, the ability of IL-12 to interact with a variety of signaling pathways could position this cytokine as a central and key modulator of NK function.

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