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Posttranscriptional Inhibition of Gene Expression by Mycobacterium tuberculosis Offsets Transcriptional Synergism with IFN-γ and Posttranscriptional Up-Regulation by IFN-γ

Yaming Qiao,* Savita Prabhakar,* Antony Canova,* Yoshihiko Hoshino,† Michael Weiden,† and Richard Pine2*†

Host defense against Mycobacterium tuberculosis requires the cytokine IFN-γ and IFN regulatory factor 1 (IRF-1), a transcription factor that is induced to high levels by IFN-γ. Therefore, we chose to study regulation of IRF-1 expression as a model for effects of M. tuberculosis on response to IFN-γ. We found that IRF-1 mRNA abundance increased far more than transcription rate in human monocytic THP-1 cells stimulated by IFN-γ, but less than transcription rate in cells infected by M. tuberculosis. IFN-γ stimulation of infected cells caused a synergetic increase in IFN-1 transcription, yet IRF-1 mRNA abundance was similar in uninfected and infected cells stimulated by IFN-γ, as was the IRF-1 protein level. Comparable infection by Mycobacterium bovis bacillus Calmette-Guérin failed to induce IRF-1 expression and had no effect on the response to IFN-γ. We also examined the kinetics of transcription, the mRNA half-life, and the distribution of IRF-1 transcripts among total nuclear RNA, poly(A) nuclear RNA, and poly(A) cytoplasmic RNA pools in cells that were infected by M. tuberculosis and/or stimulated by IFN-γ. Our data suggest that infection by M. tuberculosis inhibits RNA export from the nucleus. Moreover, the results indicate that regulated entry of nascent transcripts into the pool of total nuclear RNA affects IRF-1 expression and that this process is stimulated by IFN-γ and inhibited by M. tuberculosis. The ability of infection by M. tuberculosis to limit the increase in IRF-1 mRNA expression that typically follows transcriptional synergism may contribute to the pathogenicity of M. tuberculosis. The Journal of Immunology, 2004, 172: 2935–2943.
EFFECTS OF M. tuberculosis ON IFN-γ-REGULATED GENE EXPRESSION

Ref. 23. The involvement of IRF-1 is at least partly that it is required to mediate IFN-γ induction of some of the genes important for host defense against M. tuberculosis (24–27). Although IFN-γ induces IRF-1 transcription (20), it is not known whether regulation of IRF-1 expression in response to IFN-γ is solely transcriptional, because IRF-1 expression can be regulated posttranslationally (28–30). Infection by M. tuberculosis induces IRF-1 (31, 32), but the nature of the regulation involved is not known. Moreover, whether infection alters IFN-γ regulation of IRF-1 expression is also not known.

We determined the effect of infection by M. tuberculosis on IRF-1 expression and whether it affected response to IFN-γ. The integrated effect of infection by M. tuberculosis and stimulation by IFN-γ was synergistic activation of IRF-1 transcription without the increases at the mRNA and protein level that would typically follow.

Materials and Methods

Bacterial strains, cells, and cell treatments

All manipulations with viable M. tuberculosis were performed under biosafety level 3 containment. M. tuberculosis TN913, a prevalent, drug-sensitive clinical isolate of the C strain from New York City tuberculosis outbreak of the 1990s (33), and M. bovis bacillus Calmette-Guérin (BCG) Pasteur were obtained from the Public Health Research Institute Tuberculosis Center. They were cultured, as previously described (34).

THP-1 cells (35), obtained from the American Type Culture Collection (Manassas, VA), were grown, treated with PMA for 1 day, infected or left uninfected, and harvested 3 days later, as previously described (36, 37). Treatment of these cells with PMA induces differentiation to a macrophage-like state (38). Mycobacterial infection of differentiated THP-1 cells is a good model for infection of alveolar macrophages, which are the predominant target in vivo (36, 37, 39). The multiplicity of infection (–1) and extent of infection by either M. tuberculosis or M. bovis BCG were confirmed, as previously described (37). Cells were unstimulated or were stimulated by 1 ng of IFN-γ (Amgen, Thousand Oaks, CA) per ml for 2 h or for various times before harvest, as indicated, and/or cells were harvested at various times after addition of actinomycin D (Calbiochem, La Jolla, CA) to a concentration of 10 μg/ml.

Nuclear extract preparation and EMSA

Native whole cell or nuclear protein extracts were prepared, as previously described, using buffers containing 0.5% Nonidet P-40 (31). Extracts from infected cells were sterilely filtered. Extracts were quickly frozen in crushed dry ice, then stored at −80°C. EMSA was performed with ~10 μg of extract protein (3 μl of extract) and an IFN-activated response element oligonucleotide as the probe, as previously described (40), using specificity of competition with excess unlabeled oligonucleotides and of reaction with antisera to confirm the identity of the IRF-1-protein-DNA complexes (data not shown). The IFN-stimulated response element and nonspecific oligonucleotides, as well as specific and nonspecific antisera, have been described previously (20, 41, 42). Radioactivity in protein-DNA complexes was visualized and quantified with a STORM 860 phosphor imager and ImageQuant software (Amersham Pharmacia Biotech). Coomassie blue staining of residual protein in the gels confirmed, as previously described (37). Cells were unstimulated or were stimulated by 1 ng of IFN-γ (Amgen, Thousand Oaks, CA) per ml for 2 h or for various times before harvest, as indicated, and/or cells were harvested at various times after addition of actinomycin D (Calbiochem, La Jolla, CA) to a concentration of 10 μg/ml.

Immunoblot analysis

Protein concentrations in the samples were determined using the Bio-Rad (Hercules, CA) Bradford reagent. Equal amounts of nuclear extracts (10 μg) were resolved on 10% SDS-polyacrylamide gels and electroblotted to polyvinylidene difluoride membranes (Hybond P; Amersham Pharmacia Biotech). Coomassie blue staining of residual protein in the gels confirmed that equal amounts of protein had been analyzed for each sample on a gel (data not shown). A previously described rabbit Ab against IRF-1 (20) was used, and chemiluminescent or chemiluminescent detection kits were used, as recommended by the respective vendors (Amersham or Kirkegaard & Perry Laboratories, Gaithersburg, MD). Fluorescence was detected with the blue laser of the STORM 860 phosphor imager, and luminescence was detected with x-ray film.

Nuclear run-on transcription rate assays

Nuclei were recovered and incubated to radiolabel nascent RNA with modified modifications of the procedure described method (43). The modifications included addition of 0.1% Nonidet P-40 to the cell lysis buffer, lysis by mixing vigorously instead of by using a Dounce homogenizer, incubating 10 min at 37°C instead of 15 min at 30°C, and purifying RNA by using TRIReagent, as recommended by the vendor (Molecular Research Center, Cincinnati, OH). Hybridization of a constant amount of radiolabeled RNA to excess plasmid probes fixed on nitrocellulose was performed, as previously described (20). The IRF-1 probe was a previously described cDNA clone in pBluescript KS− (20). Specificity of hybridization was determined with pGem1 (Promega, Madison, WI) as a probe. A human GAPDH probe, clone 754537 (Research Genetics, Huntsville, AL), was used as a positive control and as an internal standard. The results were quantified by subtracting the radioactive signal from the other signals as a background correction and then normalizing the corrected IRF-1 signal to the corrected GAPDH signal. Images were obtained and data were quantified with a phosphor imager (Amersham Pharmacia Biotech).

Quantitative RT-PCR (qRT-PCR)

Cells were recovered and total cellular RNA was prepared from the cell pellets. Alternatively, recovered cells were lysed and nuclei were recovered, as described above for the transcription rate assay. Total cytoplasmic RNA was prepared from the postnuclear supernatants. Nuclei were further purified by two washes in incubation buffer without NTPs (43), followed by centrifugation at 10,000 × g for 4 min through a cushion of that buffer modified to contain 60% glycerol. Total nuclear RNA was prepared from the nuclear pellets. In each case, RNA was obtained using TRIReagent, as recommended by the vendor. RNA recovery was quantified by spectroscopy. Oligo(dT) or random nonamer primers were used to prepare cDNA from polyadenylated or total RNA with Maloney murine leukemia virus reverse transcriptase, as recommended by the vendor (Promega). The PCR was performed with TaqDNA-polymerase (AmpliTaq Gold) under reaction conditions recommended by the vendor (Roche Molecular Systems, Branchburg, NJ), using primers to amplify sequences from exon 10 of IRF-1 (5′-GTCAGGGACTTGGACAGGAG-3′) and 5′-GCCAGCCAGTG-3′) or exon 9 of GAPDH (5′-GACCTCTACGTGGGGATG3′ and 5′-ACTGTTAGGAGGAGATTC-3′). PCR product was measured in real time by including a molecular beacon oligonucleotide (44) specific for IRF-1 (5′-FAM-CCGGCGGTGGGGGACTGAGTGTGGCGTCC-dabcyl-3′) or for GAPDH (5′-FAM-GGCGCCGTAATGTTGGTCC-dabcyl-3′). The specificity of RNA detection was confirmed for each RNA preparation by the failure to detect products after PCR amplification of mock cDNA samples prepared by omitting reverse transcriptase from the reverse-transcription reaction. Copy number for IRF-1 and GAPDH mRNAs was determined based on a standard curve for each primer pair with varying amounts of genomic DNA as the template. The abundance of GAPDH in each RNA pool and its distribution among the pools, relative to the nominal amount of RNA template used for reverse transcription as determined by OD, were not systematically affected by any of the experimental conditions (data not shown). Thus, GAPDH abundance is an appropriate internal standard, to which the abundance of IRF-1 was normalized.

To determine the t1/2 of IRF-1 mRNA under various conditions, its abundance relative to GAPDH mRNA was measured at various times after addition of actinomycin D. The abundance of GAPDH decreased by less than 5% in 2 h after addition of actinomycin D (data not shown). The t1/2 was calculated from exponential decay curves fit to the data. Average t1/2 was calculated with values from data having a correlation coefficient greater than 0.95 for the fit to exponential decay.

Statistical analysis

The paired or unpaired Student’s t test was used for statistical analysis, as appropriate. A p value of <0.05 was taken to indicate a statistically significant difference.

Results

M. tuberculosis specifically induces IRF-1 DNA-binding activity, but has little effect on its induction by IFN-γ

We compared induction of IRF-1 in cells infected by M. tuberculosis and by M. bovis BCG to determine whether the response we previously observed was specific for the pathogenic mycobacteria and whether either would affect induction of IRF-1 by IFN-γ.

THP-1 macrophages were infected equivalently by M. tuberculosis...
or by *M. bovis* BCG and cultured for 3 days. To assess regulation of IRF-1 expression at a functional level, we measured its DNA-binding activity. *M. tuberculosis*, but not *M. bovis* BCG, induced IRF-1 (Fig. 1A, lanes 1–3). Stimulation of uninfected and infected cells with IFN-γ for the final 2 h before extraction of nuclear proteins induced IRF-1 to similar levels (lanes 4–6).

Quantification of replicate experiments established that infection by *M. tuberculosis* increased IRF-1 DNA-binding activity 2-fold (Fig. 1B). IFN-γ stimulation of infected cells caused a further 2-fold increase (a 4-fold induction relative to the DNA-binding activity in uninfected, unstimulated cells), and produced a 3-fold increase in uninfected cells. The changes that underlie these comparisons were statistically significant, while the 26% difference in the comparison between the DNA-binding activity in uninfected and infected cells stimulated by IFN-γ was not. Quantification also confirmed that *M. bovis* BCG did not induce IRF-1 DNA-binding activity and did not affect the response to IFN-γ. In every case, the level of IRF-1 protein detected by immunoblot analysis correlated with the DNA-binding activity (Fig. 1C). Thus, *M. tuberculosis* induction of IRF-1 at the protein level is specific, and neither it nor *M. bovis* BCG affects subsequent induction by IFN-γ.

![FIGURE 1. *M. tuberculosis* infection specifically induces IRF-1 DNA-binding activity, but does not affect its induction by IFN-γ.](image)

To further characterize IFN-γ-regulated gene expression in uninfected cells and in cells infected by *M. tuberculosis* or *M. bovis* BCG, we assayed the abundance of IRF-1 mRNA, using qRT-PCR with molecular beacons for detection. Infection by *M. tuberculosis* increased the abundance of IRF-1 mRNA 3-fold, from 4.8 to 14 U (Fig. 2A). Stimulation by IFN-γ increased IRF-1 mRNA abundance 46-fold, to 221 U. These changes were statistically significant. Cells that were infected and then stimulated contained 276 U of IRF-1 mRNA. This induction was statistically significant compared with unstimulated cells, whether infected or not, but the difference between uninfected and infected cells stimulated by IFN-γ was not statistically significant. Infection by *M. bovis* BCG did not lead to an increase in IRF-1 mRNA (Fig. 2B). In these experiments, IFN-γ stimulation resulted in a statistically significant 35-fold increase in the abundance of IRF-1 mRNA, from 4 to 140 U, which was essentially unchanged by prior *M. bovis* BCG infection. Thus, infection by *M. tuberculosis* specifically induced IRF-1 mRNA, but neither *M. tuberculosis* nor *M. bovis* BCG affected induction by IFN-γ.

We observed that the increase in IRF-1 mRNA abundance was far greater than the increase in its DNA-binding activity in cells...
stimulated by IFN-γ, whether they were uninfected, infected by *M. tuberculosis*, or infected by *M. bovis* BCG (Fig. 3A, from the data in Figs. 1 and 2). We examined the induction kinetics of IRF-1 mRNA and protein in cells stimulated by IFN-γ to confirm that the difference in activity was due to a difference in total protein and to determine whether the difference between these levels of expression at a single time point is due to a lag after mRNA accumulation.

Throughout the period of IFN-γ stimulation, the increase at the protein level was almost identical with the increase in DNA-binding activity (data not shown) and was far less than the increase in mRNA abundance (Fig. 3B). Thus, the limited increase in DNA-binding activity and protein abundance after 2 h, relative to the accumulation of mRNA, is not merely a reflection of having measured them at the same time.

**IRF-1 transcription is specifically activated by *M. tuberculosis* and synergistically activated by IFN-γ stimulation of *M. tuberculosis*-infected cells**

We next measured the effect of IFN-γ on the transcription rate of the IRF-1 gene in uninfected cells and in cells infected by *M. tuberculosis* or *M. bovis* BCG, using a nuclear run-on assay that results in nascent transcripts being radiolabeled as they are elongated in isolated nuclei. The probe for the assay was a full-length cDNA; thus, the net effect on synthesis of transcripts due to a change in initiation and to any change in elongation was determined. The transcription rate of IRF-1 was greater in cells that were infected and stimulated than in cells that were only infected or only stimulated, although in each of those cases increased transcription was clear (Fig. 4A). Quantification of replicate experiments demonstrated that the average increases were 40-, 5-, and 7-fold, respectively, compared with uninfected, unstimulated cells (Fig. 4B). These increases in IRF-1 transcription were statistically significant, as were the differences in comparisons of cells that were either infected or stimulated with cells that were infected and stimulated. Infection by *M. bovis* BCG did not increase the transcription rate of IRF-1 and had little effect on stimulation by IFN-γ (Fig. 4C). Thus, infection by *M. tuberculosis* specifically induces transcription of IRF-1 in unstimulated cells and specifically has a synergistic effect on transcription of IRF-1 stimulated by IFN-γ.

**Changes in transcription rate fail to fully account for changes in mRNA abundance for IRF-1**

In uninfected cells stimulated by IFN-γ, the IRF-1 transcription rate increased 7-fold, while mRNA abundance increased 46-fold. IFN-γ stimulation of infected cells caused a 6-fold increase in transcription rate, but only a 25% increase in mRNA abundance compared with the respective measures in cells that were only infected. We tested whether the kinetics of induction for IFN-γ-stimulated IRF-1 transcription was the basis for these disproportionate changes in transcription rate and mRNA abundance. If the transcription rate in cells stimulated by IFN-γ fell greatly from an earlier peak, the increase observed 2 h after stimulation could be...
far less than the coincident increase in mRNA abundance. However, from 60 to 120 min after stimulation by IFN-\(\gamma\), the transcription rate was maintained at \(\sim 50\%\) of the peak level that occurred near 15 min after the start of stimulation (Fig. 5). If the transcription rate in cells infected by \textit{M. tuberculosis} and stimulated by IFN-\(\gamma\) increased sharply shortly before it was measured 2 h after stimulation, a lag in mRNA accumulation could account for the limited coincident increase in mRNA abundance. Yet, in those cells, the transcription rate reached a maximum at 90 min that was maintained at 120 min (Fig. 5). Thus, transcriptional regulation does not account for the disproportionately large increase or the limited increase in mRNA abundance relative to the respective changes in transcription rate in uninfected or infected cells stimulated by IFN-\(\gamma\).

Changes in mRNA stability fail to account for changes in mRNA abundance for IRF-1

We further investigated the disproportionate changes in IRF-1 mRNA abundance and transcription rate in uninfected and infected cells stimulated by IFN-\(\gamma\) by determining whether altered IRF-1 mRNA stability was a factor. The turnover of constitutively expressed IRF-1 mRNA after inhibition of transcription by addition of actinomycin D occurred with a \(t_{1/2}\) of 43 min (Fig. 6, A and E). The \(t_{1/2}\) of the IRF-1 mRNA in cells infected by \textit{M. tuberculosis} was 45 min (Fig. 6, B and E). In uninfected and infected cells stimulated by IFN-\(\gamma\), the \(t_{1/2}\) of IRF-1 mRNA was 37 min (Fig. 6, C and E) and 50 min (Fig. 6, D and E), respectively. The difference in \(t_{1/2}\) between uninfected and infected cell stimulated by IFN-\(\gamma\) was statistically significant; other differences were not. Thus, either there were no changes in mRNA \(t_{1/2}\) or the changes were the opposite of what would contribute to the differences between changes in mRNA accumulation and transcription rate.

Total nuclear RNA accumulation increases more than transcription rate for IRF-1 in cells stimulated by IFN-\(\gamma\), and the increase is limited in cells infected by \textit{M. tuberculosis}

Because neither regulation of transcript synthesis nor of mRNA degradation explained differences between changes in transcription rate and changes in mRNA accumulation, we tested whether the relation between them was governed by regulation of additional processes, such as nuclear transcript maturation and translocation in uninfected or infected cells stimulated by IFN-\(\gamma\). To obtain evidence for such regulation, we measured the abundance of IRF-1 RNA in three cellular pools. Using random primers for cDNA synthesis from isolated nuclear RNA and a probe for the last exon, we measured transcripts that were complete, or nearly so, at any stage of processing. Using oligo(dT) primer instead, we measured those transcripts if they were polyadenylated. We refer to the respective pools as total and poly(A) nuclear RNA. With oligo(dT) primer, cytoplasmic RNA template, and the last exon probe, we measured mRNA as cytoplasmic poly(A) RNA.

Total nuclear IRF-1 RNA and nuclear and cytoplasmic poly(A) IRF-1 RNA each increased 4- to 5-fold in cells infected by \textit{M. tuberculosis} (Fig. 7A; the transcription rates taken from Fig. 4B are included for comparison). In uninfected and infected cells stimulated by IFN-\(\gamma\), these RNA pools increased 18- to 22-fold and 26- to 30-fold, respectively, compared with cells that were uninfected and unstimulated. Thus, while the extent of induction varies among the experimental conditions, in each case the increase in total nuclear RNA is reflected in the other pools. This observation suggests that in uninfected and infected cells stimulated by IFN-\(\gamma\), the divergence between effects on transcription rate and on RNA accumulation involves posttranscriptional regulation that occurs within the nucleus.

To highlight the regulatory responses to stimulation by IFN-\(\gamma\) and/or infection by \textit{M. tuberculosis} that result in different degrees of induction at different levels of expression, we calculated a ratio for successive pairs of levels for each condition (Fig. 7B). The abundance of total nuclear IRF-1 RNA relative to the transcription rate measured at the same time was similar in uninfected and infected cells. In contrast, far more total nuclear IRF-1 RNA accumulated relative to the transcription rate in cells stimulated by IFN-\(\gamma\). This disproportionately large increase was not observed in cells infected by \textit{M. tuberculosis} and then stimulated by IFN-\(\gamma\). The ratio of poly(A) to total nuclear IRF-1 RNA was very similar under all conditions examined. The ratio of poly(A) cytoplasmic to poly(A) nuclear IRF-1 RNA was slightly increased by IFN-\(\gamma\)-stimulation. This ratio was reduced by infection, and the ratio was lower in cells that were infected and stimulated than in cells that were only stimulated. These comparisons suggest three conclusions: 1) that accumulation of total nuclear IRF-1 RNA is positively regulated posttranscriptionally in cells stimulated by IFN-\(\gamma\); 2) that this response to IFN-\(\gamma\) is counterregulated by infection; and 3) that infection limits translocation of poly(A) RNA from the nucleus to the cytoplasm.

Discussion

We examined regulation of gene expression in cells that were infected by \textit{M. tuberculosis} or \textit{M. bovis} BCG and compared IFN-\(\gamma\)-regulated gene expression in uninfected and infected cells by studying the transcription factor IRF-1, which is critical for host defense against \textit{M. tuberculosis} (21, 22). We found effects of infection by \textit{M. tuberculosis}, effects of stimulation by IFN-\(\gamma\), and effects of infection on response to IFN-\(\gamma\) that were unexpected. Our data show specificity for the effects of \textit{M. tuberculosis} on gene expression, demonstrate transcriptional synergism between \textit{M. tuberculosis} and IFN-\(\gamma\), and reveal posttranscriptional up-regulation in the response to IFN-\(\gamma\) that is offset by \textit{M. tuberculosis}. These effects are summarized schematically in Fig. 8. \textit{M. bovis} BCG was unable, or far less able, to induce IRF-1 expression at any level and did not affect IFN-\(\gamma\)-regulated gene expression. The fact that these responses are specific to \textit{M. tuberculosis} suggests that its ability to evoke them may contribute to pathogenicity.

We found that infection by \textit{M. tuberculosis} induced IRF-1 transcription 5-fold. The increases in total and poly(A) nuclear RNA were proportional. Cytoplasmic poly(A) RNA increased only half as much, although the mRNA \(t_{1/2}\) was unchanged. These results suggest inhibition of RNA export from the nucleus. The increase
at the protein level was proportional to the increase in cytoplasmic poly(A) RNA. As expected, IRF-1 transcription increased in cells stimulated by IFN-γ/H9253. The increases in nuclear and cytoplasmic RNA abundance were disproportionately large, again without a significant change in mRNA t1/2, suggesting in this case that unexpected posttranscriptional up-regulation within the nucleus contributes to the induction. Compared with the RNA induction, expression at the protein level was limited. Transcription of IRF-1 was assayed, as described in Materials and Methods. The average transcription rate of IRF-1 normalized to the transcription rate of GAPDH with the SD, and statistical significance (from Fig. 3, n = 6) is shown for comparison (tx rate). The abundance of IRF-1 transcripts relative to GAPDH transcripts is shown for total nuclear RNA (total nuc), poly(A) nuclear RNA (poly(A) nuc), and poly(A) cytoplasmic RNA (poly(A) cyto). The error bars for RNA abundance measurements show the SD of the averages from duplicate assays of four independent experiments (*, p < 0.05 or **, p < 0.001 relative to uninfected, unstimulated cells; #, p < 0.05; or, ###, p < 0.001 relative to infected, IFN-γ-stimulated cells). B, The values from A were used to calculate the ratio of expression at each successive level. The ratios of total nuclear IRF-1 RNA to IRF-1 transcription rate (total nuc: tx rate), poly(A) nuclear IRF-1 RNA to total nuclear IRF-1 RNA (poly(A) nuc: total nuc), and poly(A) cytoplasmic IRF-1 mRNA to poly(A) nuclear IRF-1 RNA (poly(A) cyto: poly(A) nuc) are shown.

The average t1/2 of IRF-1 mRNA in unstimulated, uninfected cells (un−/−TB); unstimulated, infected cells (un+/−TB); IFN-γ-stimulated, uninfected cells (IFN-γ/+TB); and IFN-γ-stimulated, infected cells (IFN-γ/+TB) was calculated from exponential decay curves fit to the data. Only values from experiments having a correlation coefficient >0.95 for the fit of the data to exponential decay were included in calculating the average t1/2. The averages shown are from four experiments for unstimulated, uninfected cells and from three experiments for each of the other conditions. The error bars show 1 SD (*, p < 0.05 compared with uninfected, IFN-γ-stimulated cells).

FIGURE 6. IRF-1 mRNA stability is not decreased in cells infected by *M. tuberculosis* or increased in cells stimulated by IFN-γ. THP-1 macrophages were uninfected (A and C) or infected by *M. tuberculosis* for 3 days (B and D) and were not stimulated (A and B) or were stimulated by IFN-γ for 2 h (C and D) before addition of actinomycin D. The abundance of IRF-1 mRNA relative to GAPDH mRNA was measured by real-time qRT-PCR at the start of actinomycin D treatment and at various times thereafter, and is shown as the fraction remaining 1 and 2 h after addition of actino-

FIGURE 7. IRF-1 total nuclear RNA accumulation increases more than the transcription rate in cells stimulated by IFN-γ, and the increase is limited in cells infected by *M. tuberculosis*. A, THP-1 macrophages were uninfected or infected by *M. tuberculosis* (TB) and unstimulated or stimulated for 2 h by IFN-γ, as indicated. Cytoplasmic and nuclear RNA were extracted, and IRF-1 was assayed, as described in Materials and Methods. The average transcription rate of IRF-1 normalized to the transcription rate of GAPDH with the SD, and statistical significance (from Fig. 3, n = 6) is shown for comparison (tx rate). The abundance of IRF-1 transcripts relative to GAPDH transcripts is shown for total nuclear RNA (total nuc), poly(A) nuclear RNA (poly(A) nuc), and poly(A) cytoplasmic RNA (poly(A) cyto). The error bars for RNA abundance measurements show the SD of the averages from duplicate assays of four independent experiments (*, p < 0.05 or **, p < 0.001 relative to uninfected, unstimulated cells; #, p < 0.05; or, ###, p < 0.001 relative to infected, IFN-γ-stimulated cells). B, The values from A were used to calculate the ratio of expression at each successive level. The ratios of total nuclear IRF-1 RNA to IRF-1 transcription rate (total nuc: tx rate), poly(A) nuclear IRF-1 RNA to total nuclear IRF-1 RNA (poly(A) nuc: total nuc), and poly(A) cytoplasmic IRF-1 mRNA to poly(A) nuclear IRF-1 RNA (poly(A) cyto: poly(A) nuc) are shown.
was synergistically induced in cells infected by *M. tuberculosis* and/or stimulated by IFN-γ. This is unanticipated because the levels of IRF-1 mRNA and protein were similar in uninfected and infected cells stimulated by IFN-γ. Despite transcriptional synergism, infection limits the effect of IFN-γ on the pool of total nuclear IRF-1 RNA, because the increase in this pool was less than proportional to the increase in transcription. The limited increase at the protein level relative to the induction of cytoplasmic poly(A) RNA in cells stimulated by IFN-γ also occurred in cells that were infected and stimulated. Similarly detailed analysis of other genes will likely reveal more instances of these multiple modes of regulation.

The finding that IFN-γ-regulated IRF-1 expression includes limited accumulation of DNA-binding activity relative to the accumulation of mRNA is ancillary to the major focus of this work; nonetheless, it was corroborated with independent measures. The immunoblot of IRF-1 protein was quantified by using chemiluminescent detection, and mRNA abundance was quantified by hybridizing oligo(dT)-primed, 32P-labeled cDNA reverse transcribed from total cellular RNA to excess target sequences fixed on nitrocellulose (data not shown). Moreover, we confirmed that the measurements of response to IFN-γ were not skewed by differences in the dynamic range or the signal-to-noise ratio among the assays (data not shown). There are at least three possible causes for limited induction of IRF-1 DNA-binding activity that are not mutually exclusive. Translational control and regulation of protein τ1/2 are general mechanisms that could be regulated by IFN-γ to affect IRF-1. If alternative splicing that produces IRF-1 mRNA encoding a protein defective in DNA binding occurs in THP-1 cells, as it does in some preleukemic and leukemic monocytic cells (28, 29), it could be increased by IFN-γ. At present, we have not studied these alternatives.

We have examined the basis for the differences between changes in IRF-1 transcription rate and RNA accumulation. The 5-fold increase in transcription rate and 3-fold increase in mRNA abundance 3 days after infection, without a decrease in mRNA τ1/2, could primarily result from the decreased nuclear RNA export that is indicated by the reduced ratio of cytoplasmic to nuclear poly(A) RNA. It is not necessary to invoke a greater transcription rate and/or a shorter mRNA τ1/2 earlier during the course of infection to account for the data, although such perturbations are not excluded. In uninfected and infected cells stimulated by IFN-γ, the kinetics of IRF-1 transcription was typical of that seen in several other cell types, in which IFN-γ stimulation causes prolonged, relatively stable transcription of the IRF-1 gene with only modest further increases or subsequent decreases after an initial rapid induction (40, 45). The observed profile directly rules out the possibility that the kinetics of transcriptional activation accounted for the lack of correlation between the changes in mRNA abundance and transcription rate measured in uninfected and infected cells after 2 h of stimulation by IFN-γ. A role for altered mRNA τ1/2 in the differences we found was also excluded.

Posttranscriptional regulation that does not involve mRNA stability still could affect cytoplasmic RNA abundance. For cells stimulated by IFN-γ, a regulated increase in polyadenylation alone, in contrast to an increase proportional to the increased abundance of the total nuclear RNA pool, could contribute to an increase in cytoplasmic poly(A) RNA that exceeded the increase in transcription, but it would also increase the ratio of poly(A) to total nuclear RNA. This was not observed. An IFN-γ-regulated increase in nuclear RNA export alone would increase both the abundance of cytoplasmic poly(A) RNA and the ratio of cytoplasmic poly(A) RNA to nuclear poly(A) RNA. The data also were inconsistent with this alternative. For infected cells, analogous consideration of the ratios between successive levels of expression (total nuclear IRF-1 RNA to transcription rate, nuclear poly(A) IRF-1 mRNA to total nuclear RNA, and cytoplasmic poly(A) IRF-1 mRNA to nuclear poly(A) IRF-1 mRNA) demonstrates that limited entry of nascent transcripts into the pool of total nuclear RNA and reduced RNA export from the nucleus are the explanations that are consistent with the experimental outcome.

Observations consistent with the existence of mechanisms that regulate conversion of nascent transcripts to mRNA have been reported previously for IFN-γ-stimulated expression of several genes, including FcγR1 (46), gp91phox (47, 48), HLA-A2 (49), and transferrin receptor (50), but little is known about what they may be. The regulation of total nuclear IRF-1 RNA accumulation by IFN-γ reported in this work may involve increased splicing of nascent transcripts (Y.Q. and R.P., unpublished observations). The molecular basis for that increase remains unknown. In addition to demonstrating that IFN-γ posttranscriptionally up-regulates IRF-1 mRNA accumulation, this is the first study definitively showing that *M. tuberculosis* can posttranscriptionally inhibit IFN-γ-stimulated mRNA accumulation. It remains to be determined whether infection directly inhibits the effect of IFN-γ on accumulation of IRF-1 mRNA or acts through an independent pathway. However the interaction occurs, regulation of IRF-1 expression will almost certainly serve as a model for the regulated expression of many other genes affected by infection and stimulation.

In murine and human macrophages, Toll-like receptor (TLR) 2 contributes to inhibition by *M. tuberculosis* or its 19-kDa lipoprotein of IFN-γ-stimulated MHC class II Ag presentation (51, 52). The effect on Ag presentation correlated with inhibition of IFN-γ-stimulated class II transactivator and MHC class II mRNA accumulation. However, the inhibition of mRNA accumulation was not shown to involve TLR2. Moreover, it is uncertain whether *M. tuberculosis* inhibits IFN-γ-stimulated expression of these genes transcriptionally or posttranscriptionally. Negative regulation of
IFN-γ-stimulated gene expression can be mediated by suppressor of cytokine signaling (SOCS) 1 or 3; these proteins can inhibit activation of STAT-1 and transcriptional activation of gene expression (reviewed in Ref. 53). Incubation of macrophages with LPS induces SOCS-3 (54), presumably through activation of TLR4. Infection by M. tuberculosis also induces SOCS-3 (S.P. and R.P., unpublished observations), and the 19-kDa M. tuberculosis lipoprotein induces SOCS-1 and -3. However, there does not seem to be a role for either in the inhibition of IFN-γ-stimulated MHC class II Ag presentation by the 19-kDa M. tuberculosis lipoprotein. Overall, there is no evidence that TLR signaling or SOCS induction affects the posttranscriptional regulation reported in this work, but the possibility that one or both might, at least for some genes, cannot be ruled out.

The opposed transcriptional and posttranscriptional regulation of IFN-1 gene expression in cells infected by M. tuberculosis and stimulated by IFN-γ resulted in little net change in IFN-1 DNA-binding activity; consequently, the role of IFR-1 as a transcription factor would be similar in uninfected and infected cells stimulated by IFN-γ. This contrasts with the substantial increase in IFN-1 DNA-binding activity that results from transcriptional synergism between TNF-α and IFN-γ (40, 55). By nearly preventing an increase in mRNA and protein abundance despite a synergistic increase in transcription, the specific effect of infection by M. tuberculosis on IFN-γ-stimulated IFN-1 expression and on any similarly regulated genes may be an important aspect of pathogenicity.

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References