Escape from Transcriptional Shutoff during Poliovirus Infection: NF-κB-Responsive Genes IkBa and A20

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It has been known for a long time that infection of cultured cells with poliovirus results in the overall inhibition of transcription of most host genes. We examined whether selected host genes can escape transcriptional inhibition by thioridine marking newly synthesized host mRNAs during viral infection. Using cDNA microarrays hybridized to cDNAs made from thiolated mRNAs, a small set of host transcripts was identified and their expression verified by quantitative PCR and Northern and Western blot analyses. These transcripts were synthesized from genes that displayed enrichment for NF-κB binding sites in their promoter regions, suggesting that some NF-κB-regulated promoters can escape the virus-induced inhibition of transcription. In particular, two negative regulators of NF-κB, IkBa and A20, were upregulated during viral infection. Depletion of A20 enhanced viral RNA abundance and viral yield, arguing that cells respond to virus infection by counteracting NF-κB-induced proviral effects.

Infection of cells with poliovirus results in a dramatic inhibition of transcription (12) and translation (4) of host cell mRNAs. It has been known for a long time that 80 to 90% of host mRNAs cease to be transcribed around 2 h after infection of cultured cells with poliovirus (18, 42). To investigate the mechanism of inhibition of host transcription, Dasgupta and colleagues discovered that virus-encoded proteinases cleave several cellular transcription factors, including CREB, Oct1, and the TATA-binding protein TBP (5, 6, 36–39). Here, we investigate whether inactivation of cellular transcription factors results in the transcriptional inhibition of all cellular mRNAs or whether classes of cellular mRNAs can be transcribed during virus-induced inhibition of transcription.

The host response to poliovirus infection has been previously investigated using cDNA microarray analysis (19). This study revealed that the abundance of 12 mRNA species increased at 3 h after infection (19). However, this approach measured the steady-state abundance of mRNAs and, thus, could reflect altered turnover of preexisting mRNAs, synthesis of new mRNAs, or both. To distinguish between these possibilities, we employed an experimental system in which newly synthesized mRNAs can be identified at different times during viral infection. Specifically, HeLa cells that express the uracil-phosphoribosyltransferase (UPRT) gene from Toxoplasma gondii were infected with poliovirus. The addition of 4-thiouracil (4sU) to uninfected and infected HeLa-UPRT cells allowed the pulse-labeling of only newly synthesized RNAs that were infected with poliovirus. The addition of 4-thio-

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Materials and Methods

Cell culture and viral infection. HeLa cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) (GIBCO, Carlsbad, CA) supplemented with 10% fetal bovine serum (Omega Scientific, Tarzana, CA), 100 U/ml penicillin-streptomycin (GIBCO), and 2 mM L-glutamine (GIBCO). For infections, monkey type I poliovirus stocks were diluted in phosphate-buffered saline supplemented with 0.1 mg/ml CaCl2 plus 0.1 mg/ml MgCl2 (C-PBS). Cells were washed once with C-PBS and infected at a multiplicity of infection (MOI) of 5 to 50. Following incubation for 30 min at 37°C, antibiotic-free DMEM was added. Infections were allowed to proceed up to 5.5 h at 37°C. Mock infections, treated with C-PBS alone, were carried out in parallel. To label RNA, 4-thiouracil (4sU) (Acros, Geel, Belgium) stock solutions were made at a 200 mM concentration in dimethyl sulfoxide (DMSO), stored at −20°C, and thawed only once before dilution in the cell culture medium.

RNA and protein preparation. RNA and protein were prepared from 4 × 106 HeLa cells. RNA was purified using the TRIzol (Invitrogen, Carlsbad, CA) reagent. Poly(A)+ mRNA was purified from total RNA using Oligotex (Qiagen, Valencia, CA). Total RNA and poly(A)+ mRNA concentrations were determined with an ND-1000 spectrophotometer (NanoDrop). Protein lysates were prepared in RIPA buffer (1% deoxycholic acid, 1% Triton X-100, 0.1% SDS, 0.1 M Tris HCl, pH 7.4, and 0.15 M NaCl). A mini-tablet of complete protease inhibitors (Roche) was added to each 10-ml aliquot of RIPA buffer. Lysates were incubated on ice for 15 min and cleared by sedimentation at 14,000 rpm for 20 min at 4°C. Total protein concentration was determined using the standard Bradford protein assay (Bio-Rad) according to the manufacturer’s instructions.

Labeling and preparation of thioridine-containing RNA. The HeLa-UPRT cell line that stably expresses the uracil-phosphoribosyltransferase (UPRT) gene from Toxoplasma gondii was used in this study (7). This HeLa-UPRT cell line enabled the incorporation of a thio-labeled uracil into RNA. Briefly, cells were infected at 1 MOI of 50 with live poliovirus or virus that had been heat inactivated for 15 min at 65°C. Thiouracil (15 μM) was added to the medium, and RNA was extracted at different times after infection. The protocols for RNA extraction, biotinylation, purification on streptavidin beads, and preparation for Northern and cDNA microarray analyses were described by Cleary et al. (7).

cDNA microarrays. Three independent mRNA preparations were performed for each early (0.5 to 2.5 h) and late (2.5 to 5.5 h) time point. Briefly, 200 ng of poly(A)+-selected mRNA was converted to first-strand cDNA using Superscript II (Life Technologies, Carlsbad, CA) and labeled with Cy3-dUTP (Amersham Pharmacia Biotech) using the Klenow enzyme (Gibco-BRL) and random monomer. As a reference, Cy3-dUTP-labeled cDNA was prepared from a universal
human reference RNA (Stratagene) that hybridizes to all spots on the microarrays. The Cy5-labeled and Cy3-labeled samples were mixed, purified using YM-30 columns (Amicon), and hybridized for 20 h at 65°C to human cDNA microarray slides (Corning UltraGAPS-coated slides, SHGI standard 48-tip). These slides were obtained from the Stanford Functional Genomics Facility and contain 40,996 cDNA elements representing 23,228 unique putative genes. Controls included the DCV2.0 (Ambion/Stratagene) mRNA mix version, which is a mix of Methanococcus jannaschii mRNA controls prepared by in vitro transcription. These controls serve as controls for the efficiency of the labeling reactions and serve in data normalization.

Microarrays were scanned and gridded using GenePix 4000A and GenePix 5.1, respectively (Axon Instruments), and filtered using tools provided by the Stanford Microarray Database (SMD). Spots included in the analysis of microarrays met the following filtering criteria: regression correlation, >0.6; channel 1 intensity/median background intensity, >2.0; channel 2 normalized (intensity/median background intensity), >2.0; channel 1 net (median), >250; channel 2 net (median), >250, using only genes with >85% good data.

A total of 20,611 elements from the early time point arrays and 21,105 elements from the late time point arrays passed the filtering criteria and were entered into Me Viewer 3.1 (TIGR) for paired testing using alpha (overall threshold P value) 0.04. From the group of significant genes, those expressed at a >2-fold difference between infected and mock cells were chosen for further investigation. The complete data sets for these microarrays are available for analysis on the Stanford Microarray Database (SMD) website (http://genome-www5.stanford.edu/).

Detection of NF-κB binding sites. Genes were inspected for the presence of the NF-κB consensus motif GGGRNNNYCC (C, cytosine; G, guanine; N, any nucleotide; R, purine; Y, pyrimidine) in the enhancer/promoter regions (40).

The NF-κB consensus motif GGGRNNYYCC (C, cytosine; G, guanine; N, any nucleotide; R, purine; Y, pyrimidine) in the enhancer/promoter regions (40).

Results

Establishment of a protocol for the detection of newly synthesized mRNAs in living cells. To identify cellular transcripts that can escape poliovirus-induced inhibition of translation, a protocol was established that allows the purification of newly transcribed transcripts from virus-infected cells. Briefly, we used HeLa cells that stably express the uracil-phosphoribosyltransferase (UPRT) gene from Toxoplasma (7). These UPRT-HeLa cells are able to convert 4-thio-uracil (4S) to 4-thio-uridine, which can be used as a substrate by RNA polymerases. Modified RNA can then be extracted, biotinylated, and captured on streptavidin beads (Fig. 1A). This system was optimized so that 4S-containing mRNAs could be detected by Northern analysis after a 45-min pulse with 15 μM 4S (see Materials and Methods).

Identification of genes that are preferentially expressed during early and late infection of HeLa cells with poliovirus. To identify cellular mRNAs that can be newly synthesized during poliovirus infection, we compared the expression of cellular genes by cDNA microarray analysis using RNA samples from three sources: from mock-infected cells, from infected cells labeled from 0.5 to 2.5 h postinfection, or from infected cells labeled from 2.5 to 5.5 h postinfection (Fig. 1B). Extracted RNA was biotinylated, purified on streptavidin beads, and reverse transcribed into cDNA in the presence of fluorescent dUTP-Cy5. dUTP-Cy3-containing cDNA was generated from a human universal reference RNA (Stratagene) (Fig. 1B), which hybridizes to all spots on the human cDNA microarray, and was used for quality control. Both Cy5- and Cy3-labeled
cDNAs were then hybridized to human cDNA microarrays (Fig. 1B). Three pairs of arrays for each of these conditions were compared. Using a paired t test P value of 0.04, the number of significant signals on the arrays during early infection was 1,077. The number of significant signals on the chips during late infection was 1,838. Within the groups of significant signals, we chose to study the subsets of genes with a greater than 2-fold difference in abundance in infected compared to uninfected cells.

During early poliovirus infection, the newly synthesized mRNAs from nine genes were expressed at a more than 2-fold higher rate than mock-infected cells (Table 1). Later in poliovirus infection, newly synthesized mRNAs from 13 genes accumulated to a more than 2-fold higher abundance than mock-infected cells (Table 1). Two of the genes identified in the early expression set, A20 and CCL2, were also in the late expression set (Table 1).

Curiously, IxBa, IL-6, CCL2, A20, SOD2, and JUNB genes contain NF-κB binding sites, consisting of the κB consensus motif GGGRNNYYCC, in their promoter regions (Table 1). In contrast, IFIT1, IFIT2, and ISG15 do not contain NF-κB binding sites, but they are direct targets of beta and gamma interferon, whose genes contain NF-κB binding sites. Thus, a significant number of genes that are newly transcribed during poliovirus infection are likely to be regulated by NF-κB.

Analyses of cellular RNA transcripts synthesized during early and late stages of poliovirus infection. To substantiate the cDNA microarray expression pattern further, cells were infected with poliovirus and pulse-labeled with 4sU from 0.5 to 2.5 h or from 2.5 to 5 h after infection, and thiolated RNA was isolated and examined by quantitative PCR (qPCR). Figure 2A shows the qPCR data, displayed as fold increases in cDNA abundance in infected cells compared to that in mock-infected cells. As revealed by the cDNA microarray, IxBa, IER3, A20, MYC, IFIT1, IFIT2, SOD2, ISG15, JUN, JUNB, IL-6, and CCL2 were transcribed at a time when the transcription of control gene TAF7, HNRPH1, or ACT was inhibited (Fig. 2A and B). To examine whether identified mRNAs were expressed as full-length messages, the expression of selected genes was examined by Northern analysis. The Northern blots in Fig. 2B show that full-length IxBa mRNAs increased in abundance during early and late infection compared to mock-infected cells. It is noteworthy that IER3, A20, IFIT2, IL-6, and CCL2 mRNA abundances were barely detectable in mock-infected cells but that these mRNAs increased in abundance during viral infection (Fig. 2B). In contrast, the expression of TAF7 and ACT mRNAs greatly diminished during infection compared to their amounts in mock infection (Fig. 2B). We chose to study further selected genes with the presence of NF-κB sites in their promoter regions, because of their known
regulation by interferons (Table 1) and because we were intrigued by the preponderance of such genes in our collection.

To determine whether the enhanced expression of IkBa, IER3, IL-6, and CCL2 was dependent on viral gene expression or whether engagement of virus-receptor interactions could trigger enhanced transcription of these genes, we compared effects of live and inactivated virus. Northern blot analysis was performed on total RNA isolated from cells inoculated with live or heat-killed virus after 5 h of infection. Figure 3A shows that the mRNAs tested, IkBa, IER3, IL-6, and CCL2, accumulated to higher abundance in cells inoculated with live virus than in cells treated with heat-killed virus or in mock-infected cells. To examine whether cellular transcription required replicating viral RNA, the abundance of A20 mRNA was compared to cells inoculated with killed virus (Fig. 4B). Although A20 mRNA abundance was much higher in cells inoculated with live virus than with killed virus, especially during early viral infection (Fig. 4A). After 5 h of infection, the abundance of intracellular IL-6 declined rapidly. This is likely due to inhibition of IL-6 mRNA translation, IL-6 degradation, or both. Despite the intracellular accumulation of IL-6, the extracellular amount of IL-6 did not increase. This finding is likely explained by the inhibition of the host cell secretory pathway during poliovirus infection (8, 10, 11).

Next, we examined the intracellular protein abundances of A20 and IkBa using Western blot analysis. At 2 and 5 h after infection, A20 protein abundance was significantly higher in cells that were inoculated with live virus than in those inoculated with killed virus (Fig. 4B). Although A20 mRNA abundance increased during infection (Fig. 2A), the A20 protein abundance remained relatively unchanged throughout infection (Fig. 4B). IkBa, on the other hand, accumulated early, but not late, in infection. The concomitant decrease of IkBa mRNA (Fig. 2A) and protein (Fig. 4B) suggests that transcriptional downregulation of this gene late in infection could contribute to the decrease in IkBa protein abundance. Together, these findings suggest that mRNAs that are specifically synthesized during infection with poliovirus experience different posttranscriptional fates in the cytoplasm.

Depletion of A20 enhances poliovirus yield. Finally, we examined the function of A20, which is a negative transcriptional regulator of NF-kB and a target of NF-kB, in the viral life cycle. Figures 2 and 4 show that A20 mRNA and protein were expressed at higher abundances both early and late in infected cells. First, we depleted A20 RNA from HeLa cells by siRNA-mediated gene depletion. Figure 5A shows that at least 50% of

<table>
<thead>
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<th>Gene</th>
<th>Fold increase at time (hpi)</th>
<th>Presence of NFκB site</th>
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<tr>
<td></td>
<td>0.5–2.5</td>
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<tr>
<td>IkBa (NFκB inhibitor)</td>
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<td>IMAGE (395902)</td>
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<td>HLA-DQB2 (major histocompatibility complex II, DQ beta 2)</td>
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<td>ZC3HAV1 (zinc finger CCCH-type, antiviral 1)</td>
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* Listed are mRNAs with a t test P value of 0.04 and that were expressed greater than 2-fold in infected compared to uninfected cells.

* hpi, hours postinfection.

* Regulated by interferons.
A20 RNA and protein could be depleted. SiA20 RNA-treated and control siGFP RNA-treated cells were infected with poliovirus and the amount of intracellular virus was determined 5 h after infection. Figure 5B shows that depletion of A20 results in a small but significant increase in production of infectious virus. This finding argues that A20 has a negative effect on poliovirus growth.

To determine whether depletion of A20 affected viral RNA abundance, we measured intracellular viral RNA in siRNA A20- and siRNA GFP-treated cells. Figure 5C shows that viral RNA was increased more than 3-fold in siA20 RNA-treated cells than in siGFP RNA-treated cells. Because depletion of A20 did not alter the overall rate of cellular translation (data not shown), we speculate that A20 displays a specific antiviral effect. Because A20 is a negative regulator of NF-κB, we examined the abundances of NF-κB during poliovirus infection when A20 was depleted by siA20 RNAs. The results showed that A20 depletion resulted in a slight accelerated degradation of NF-κB after 4 h of infection compared to that in control-treated cells (data not shown). Thus, proteinase 3C-mediated

FIG. 3. Effects of viral gene expression on cellular transcription. (A) Northern blot analyses on RNA isolated from mock-, live-virus-, and killed-virus-inoculated cells. (B) Northern blot analyses on RNA isolated from poliovirus-infected cells (MOI = 50) in the absence or presence of 2 mM guanidine hydrochloride. See legend for Fig. 2 for gene designations. A20 RNA and protein could be depleted. SiA20 RNA-treated and control siGFP RNA-treated cells were infected with poliovirus and the amount of intracellular virus was determined 5 h after infection. Figure 5B shows that depletion of A20 results in a small but significant increase in production of infectious virus. This finding argues that A20 has a negative effect on poliovirus growth.

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Degradation of NF-κB (26) and, possibly, nuclear localization of NF-κB are affected by A20 abundance.

**DISCUSSION**

Previous cDNA microarray analyses have revealed that the abundance of 18 cellular mRNAs was increased in poliovirus-infected HeLa cells (19). However, it was not known whether these mRNAs were newly synthesized or stabilized during infection. Because most host mRNAs cease to be transcribed around 2 h after infection (18, 42), changes in cellular mRNA abundance had been attributed to changes in mRNA stability. In this study, we have marked newly transcribed RNA with thiouridine. Thiolated mRNAs can be modified with biotin and physically isolated on streptavidin beads. cDNA microarray analysis revealed that at least 20 host genes can continue to be transcribed during the early or late stages of poliovirus infection.

**NF-κB promoter-containing genes that can escape poliovirus-induced transcriptional inhibition.** Although only 1% of known human genes contain NF-κB binding sites in their promoters (14), six of the identified host genes that were transcribed during poliovirus infection (IκBa, A20, CCL2, IL-6, SOD2, JUNB) contain such sites. Three additional transcribed genes, IFIT1, IFIT2, and ISG15, do not contain NF-κB binding sites but are regulated by interferons whose genes contain NF-κB binding sites.

NF-κB proteins are eukaryotic transcription factors that regulate the expression of genes that modulate inflammatory responses and innate immune responses to viral infection (15, 27). In its inactive form, NF-κB is sequestered in the cytoplasm by NF-κB inhibitor IκBa (3, 16). Most NF-κB activators converge on the IKK kinase (IKK) pathway. IKK phosphorylation of IκBa targets it for ubiquitination and degradation by the proteasome (32, 35). NF-κB can then enter the nucleus to activate target gene expression (25, 31, 41). Interestingly, poliovirus (26), rhinovirus (29), and Theiler murine encephalomyelitis (28) have been reported to induce NF-κB-activated host gene expression. Evidence was provided that the NF-κB complex translocates to the nucleus 2 to 3 h after poliovirus infection, with concomitant degradation of IκBa (26). However, later in infection, the p65-RelA component of the NF-κB complex is cleaved by viral protease 3C (26). Similar events occur during infection with ECHO-1 and rhinovirus infection, suggesting that regulation of NF-κB response by p65-RelA cleavage is shared between some picornaviruses (13, 26). Our studies provide additional evidence that NF-κB plays a role in the expression of specific genes that escape the general inhibition of host transcription during poliovirus infection.

Why can certain genes escape the transcriptional inhibition during poliovirus infection? As mentioned above, virus infection induces the proteolysis of several transcription factors by viral proteinase 3C (6, 21, 22). Curiously, some NF-κB-responsive promoters, such as the one in A20, are constitutively occupied by TATA binding protein (TBP) and associated tran-
It is thought that permanent occupancy may allow a more rapid transcriptional induction in response to NF-κB (1). It is tempting to speculate that TBP is resistant to cleavage by viral proteinase /H9251 not only to combat inflammation caused by bacteria (17) but also to modulate immune responses to virus infection (24, 30, 33). In particular, it has been noted that A20 blocks retinoic acid-inducible gene I (RIG-I)- and interferon-regulated factor 3 (IRF3)-mediated activation of NF-κB in response to RNA virus infection (24, 30, 33). We show here that A20 mRNA and protein continue to be abundant during poliovirus infection and limit viral production.

ACKNOWLEDGMENTS

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REFERENCES


FIG. 5. Effects of A20 depletion on poliovirus yield and viral RNA abundance. (A) siRNA-mediated depletion of A20. siGFP denotes siRNA directed against GFP. One representative of three Northern blots and three Western blots is shown. (B) Viral yield from siA20- or siGFP-treated cells. Error bars represent the standard errors of results from three independent, replicate experiments. (C) Quantitative PCR of viral RNA at different times after infection of siA20- or siGFP-treated cells. Results are displayed as poliovirus cDNA enhancement in siA20-treated cells relative to siGFP-treated cells. Mean results and standard errors of results from three independent experiments are shown.
Correction for Doukas and Sarnow, “Escape from Transcriptional Shutoff during Poliovirus Infection: NF-κB-Responsive Genes IκBa and A20”

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Volume 85, no. 19, p. 10101–10108, 2011, https://doi.org/10.1128/JVI.00575-11. It has recently come to our attention that there were errors in the assembly of Fig. 2B. We apologize for these errors and make the following changes.

Page 10103, column 2, lines 18 to 19: “IER3, A20, IFIT2, IL-6, and CCL2” should read “IER3, A20, IL-6, and CCL2.”

Page 10105, Fig. 2B, row OCIAD1: Northern blot data represented by the four panels of row OCIAD1 are correct, but there appears to have been a duplication in the figure assembly. We have a paper copy of the original Northern blot data, but we do not have a digital image file of OCIAD1. The four panels of row OCIAD1 have been deleted.

Page 10105, Fig. 2B, row ACT: The 2nd panel of row ACT was incorrectly reversed during figure assembly. This panel has been reversed so that the orientation is correct.

Page 10105, Fig. 2B, row A20: Northern blot data represented by the four panels of row A20 are correct, but there appears to have been a duplication in the figure assembly. The four panels of row A20 have been replaced using an original digital image file of the A20 Northern blot.

Page 10105, Fig. 2B, row IFIT2: Northern blot data represented by panels 3 and 4 of row IFIT2 are correct, but we cannot locate a Northern blot digital image file for panels 1 and 2. The four panels of row IFIT2 have been deleted.

Figure 2B should appear as shown below.
The errors in and our corrections to Fig. 2B and the text do not affect the scientific results or conclusions. The main conclusion that IkBa and A20 transcripts and proteins are upregulated during infection with poliovirus is independently supported by microarray (Table 1), RT-PCR (Fig. 2A), and Western blot (Fig. 4B) analyses.