Double-stranded (ds) RNA, a common component of virus-infected cells, is a potent inducer of the type I interferon and other cellular genes. For identifying the full repertoire of human dsRNA-regulated genes, a cDNA microarray hybridization screening was conducted using mRNA from dsRNA-treated GRE cells. Because these cells lack all type I interferon genes, the possibility of gene induction by autocrine actions of interferon was eliminated. Our screen identified 175 dsRNA-stimulated genes (DSG) and 95 dsRNA-repressed genes. A subset of the DSGs was also induced by different inflammatory cytokines and viruses demonstrating interconnections among disparate signaling pathways. Functionally, the DSGs encode proteins involved in signaling, apoptosis, RNA synthesis, protein synthesis and processing, cell metabolism, transport, and structure. Induction of such a diverse family of genes by dsRNA has major implications in host-virus interactions and in the use of RNA technology for functional ablation of specific genes.

Double-stranded RNA is not a major constituent of mammalian cells, but many viruses produce it during their replication cycle as either an essential intermediate for RNA synthesis or a byproduct generated by annealing of complementary mRNAs encoded by the opposite strands of a DNA virus genome. In addition, some viruses encode RNA species, such as VA RNA or EBER RNA, which have considerable ds structures. Virtually nothing is known about how dsRNA affects viral and cellular gene expression and functions in a virally infected cell, although the role of PKR, the dsRNA-activated protein kinase, in inhibiting protein synthesis has been studied in cells infected with a variety of viruses.

In the host-virus interaction context, dsRNA is closely associated with the interferon (IFN) system. dsRNA is a potent inducer of type I IFN synthesis and is believed to be the primary viral gene product that causes IFN production by infected cells. dsRNA has important roles in IFN actions as well. It is the obligatory activator of two classes of IFN-induced enzymes: PKR, the IFN-induced protein kinase, and 2–5′A synthetases, whose products activate the latent ribonuclease, RNaseL. Moreover, transcription of some IFN-stimulated genes (ISGs) is also induced by dsRNA. That this induction is direct and not mediated by induced IFN was convincingly demonstrated in IFN unresponsive cells and in cells that are devoid of the IFN gene locus. Direct induction of some ISGs by dsRNA suggests that the encoded proteins will be induced in virally infected cells without any involvement of IFNs. Thus regulation of viral gene expression by these proteins is relevant for all infected cells, even in the absence of IFN treatment.

Several transcription factors such as NF-κB, IRF-3, and ATF-1, are known to be activated by dsRNA. Their activation is mediated by protein kinases including PKR, p38, JNK2, and IKK (7, 8) although the pathways of activation are not completely understood. For genes that are induced by either IFN or dsRNA, the same cis-element regulates their induction by both reagents. But entirely different signaling pathways and transcription factors are used by the two inducers.

There has not been any attempt to systematically define the full repertoire of dsRNA-regulated genes. Identification of these genes is required not only for revealing the nature of all signaling pathways used by dsRNA but also for defining the set of proteins that are induced by dsRNA or virus infection. In the current study, we started this investigation using a cDNA microarray hybridization analysis of RNA isolated from dsRNA-treated and -untreated GRE cells that are devoid of the type I IFN locus and cannot synthesize IFNs. Using this approach, in the current study we have identified more than a hundred DSGs, only a few of which were previously known to be dsRNA-inducible. Furthermore we also identified multiple down-regulated genes. These genes were induced or repressed by dsRNA strongly, rapidly, and transiently. The encoded proteins are involved in a broad range of cellular functions and metabolic pathways.

EXPERIMENTAL PROCEDURES

Cell Culture—Conditions for culturing GRE cells and treatment with poly(I)poly(C) (Amersham Pharmacia Biotech) have been described previously (5). Where indicated, cells were treated with 50 μg/ml cycloheximide for 30 min prior to and during dsRNA treatment.

RNA Isolation and Northern Blot—GRE cells were grown in 150-mm plates until 80% confluent, and then cells were treated with poly(I)poly(C) or cytokines for 6 h. Total RNA was isolated by use of RNAzol reagent (Tel-test, Inc.) following the manufacturer’s protocol. Poly(A)+ RNA was extracted from total RNA samples using Oligotex poly(A)+ RNA purification kit (Qiagen) with one round of extraction from the oligo(dT)+ spin column. For Northern blot analysis, the total RNA was separated by 1.2% formaldehyde–agarose gel and transferred to Hybond-N+ (Amersham Pharmacia Biotech) nylon membrane by capillary blotting. Hybridization was carried out with 32P-labeled...
probe prepared from PCR-amplified cDNA of human I.M.A.G.E. clones (Research Genetics).

Microarray Analysis—Fluorescent-labeled cDNA probes were generated as described by Geiss et al. (9). Human cDNA I.M.A.G.E. clones were purchased from Research Genetics (UG Build 19F5.0, plate 1–48). The cDNA inserts were PCR-amplified and deposited on glass microscope slides as described previously (9). Hybridization conditions and washing conditions were as described previously. Slides were scanned by Avalanche dual laser scanner (Molecular Dynamics); signals were obtained for each spot. Quantitation of the signals produced at the optimum time for induction of 561 mRNA that encodes the 56 kDa protein, P56 (5). The two sets of RNA from dsRNA-treated and untreated cells were then used for microarray analysis. The microarray consisted of 4600 human cDNAs and ESTs. Each slide contained duplicate sets of samples, and the colors of the two cDNA probes were reversed in duplicate assays.

Two selected fields are shown in Fig. 1, A and B. The same four stimulated genes scored red in Fig. 1A and green in Fig. 1B. The mRNAs whose levels were similar in the two samples are depicted as yellow signals in both sets. The entire screen was carried out twice so that four independent values were obtained for each spot. Quantitation of the signals produced two kinds of information: the intensity of the signal was proportional to the abundance of the corresponding mRNA, and the degree of redness or greenness indicated the fold induction or repression of mRNA by dsRNA treatment of the cells. Our analyses revealed that 175 mRNAs were induced by 2-fold or more and 95 mRNAs were repressed by 2-fold or more (with minimum threshold of intensity of 400).

Eight dsRNA-stimulated genes and two dsRNA-repressed genes were selected for further characterization. These genes include IFIT1 (P56), which as a known DSG serves as a positive control. The others were chosen because of interesting properties of the encoded proteins. For example, ELF-3 (10) and ATF-3 (11) are transcription factors, TNF AIP3 is a DNA-binding protein (12), and TFPI2 (13) and PLAU (14) are modulators of proteolytic pathways. Another consideration for choosing these genes was that they represented the whole spectrum of genes from the very strongly induced (IFIT1: 52-fold) to weakly induced (ATF3: 2.6-fold). For independent validation of our screen, the levels of the mRNAs of the selected genes in dsRNA-treated and untreated cells were quantitated by Northern blotting. As shown in Fig. 1C, all of the eight candidate DSGs were strongly induced by dsRNA, although the level of actin mRNA was unchanged. Similarly, the steady-state levels of two candidate dsRNA-repressed genes were lower in the treated cells. Quantitation of the Northern signals revealed that the fold changes observed in the microarray analysis were in general underestimates. Thus, even a relatively small difference noted in the array analysis could be physiologically significant.

Evidence for Multiple dsRNA Signaling Pathways—Many extracellular stimuli are known to activate multiple and independent signaling pathways leading to transcriptional activa-
tion of different families of genes. Members of such different families can often be initially identified by noting differences in their induction kinetics and dependence on ongoing protein synthesis. Such analyses revealed that the identified DSGs can indeed be classified into distinct subsets, each of which is probably induced by a distinct dsRNA-elicited signaling path-

![dsRNA-regulated Gene Expression](http://www.jbc.org/)

**TABLE I**

Partial list of cellular genes induced by dsRNA treatment

| Functional class | HUGO symbol | Gene product | Fold induction | Standard deviation |
|------------------|-------------|--------------|----------------|--------------------|
| Interferon-stimulated genes | | | | |
| IFIT1 | interferon-induced protein with tetratricopeptide repeats 1 (p56) | 52.5 | 32.4 |
| GBP1 | guanylate-binding protein 1, interferon-inducible, 67kD | 5.5 | 2.5 |
| NMI | N-myc (and STAT) interactor | 4.0 | 1.8 |
| IRF1 | interferon regulatory factor 1 | 3.8 | 2.3 |
| ADAR | adenosine deaminase, RNA-specific | 3.5 | 2.2 |
| TNF-induced signaling/apoptosis | | | | |
| TNFAIP2 | tumor necrosis factor, alpha-induced protein 2 (B94) | 18.1 | 2.8 |
| TRAF1 | TNF receptor-associated factor 1 | 13.3 | 12.2 |
| TNFAIP6 | tumor necrosis factor, alpha-induced protein 6 | 6.9 | 2.9 |
| BIRC2 | baculoviral IAP repeat-containing 2 | 6.0 | 5.0 |
| TNFAIP3 | tumor necrosis factor, alpha-induced protein 3 (A20) | 4.8 | 1.4 |
| Cytokine and Growth Factors | | | | |
| IGFBP6 | insulin-like growth factor binding protein 6 | 4.9 | 0.5 |
| SCYA4 | small inducible cytokine A4 (homologous to mouse Mip-1b) | 4.1 | 1.2 |
| FGFR2 | fibroblast growth factor 2 (basic) | 3.3 | 2.5 |
| SCYA2 | small inducible cytokine A2 (monocyte chemotactic protein 1) | 2.3 | 0.3 |
| RNA synthesis | | | | |
| ELF3 | E74-like factor 3 (ets domain transcription factor, epithelial-specific) | 4.5 | 2.7 |
| ATF3 | activating transcription factor 3 | 2.6 | 1.0 |
| KLFR4 | Kruppel-like factor 4 (gut) | 2.6 | 0.8 |
| NFkBIA | nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha | 2.3 | 0.3 |
| HMG2 | high-mobility group (nonhistone chromosomal) protein 2 | 2.2 | 0.3 |
| Extracellular matrix/cell adhesion | | | | |
| BTNSA3 | butyrophilin, subfamily 3, member A3 | 5.4 | 3.9 |
| PLAUR | plasminogen activator, urokinase receptor | 4.8 | 1.4 |
| PRG1 | proteoglycan 1, secretory granule | 3.6 | 0.7 |
| ANXA1 | annexin A1 | 2.6 | 0.8 |
| CD44 | CD44 antigen (homing function and Indian blood group system) | 2.4 | 0.6 |
| Protein synthesis and degradation | | | | |
| EIF2S3 | eukaryotic translation initiation factor 2, subunit 2 (gamma, 52kD) | 4.0 | 3.3 |
| PSMA3 | proteasome (prosome, macropain) subunit, alpha type 3 | 2.4 | 0.6 |
| ubiquitin 1 | 3.2 | 2.2 |
| Metabolism and biosynthesis | | | | |
| FACCL3 | fatty-acid-Coenzyme A ligase, long-chain 3 | 9.5 | 6.6 |
| GCH1 | GTP cyclohydrolase 1 (dopa-responsive dystonia) | 6.0 | 0.9 |
| SUOX | sulfite oxidase | 2.3 | 0.1 |
| Transporters | | | | |
| ABCB2 | ATP-binding cassette, sub-family B (MDR/TAP), member 2 | 3.0 | 0.6 |
| SAT | spermidine/spermine N1-acetyltransferase | 2.8 | 1.4 |
| Cystoskeletal organization | | | | |
| IQGAP2 | IQ motif containing GTPase-activating protein 2 | 2.8 | 1.5 |
| CHN1 | chimerin (chimaerin) | 2.8 | 1.2 |
| AFAP | actin filament-associated protein | 5.3 | 1.3 |
When protein synthesis was inhibited by pretreating cells with cycloheximide, dsRNA could still induce TNFAIP3 mRNA effectively. In contrast, BTN3A3 mRNA induction by dsRNA was completely blocked in the presence of cycloheximide, although the basal level of BTN3A3 mRNA was maintained. These results demonstrated that a set of DSGs represented by TNFAIP3 is induced by signaling pathways that can use pre-existing cellular proteins, whereas the induction of another set of DSGs, represented by BTN3A3, requires new protein synthesis. The second set of genes is probably induced by the secondary effects of proteins encoded by specific DSGs induced in the first wave of induction. The observed low level of induction of the BTN3A3 mRNA after 6 h of dsRNA treatment (Fig. 1C) is consistent with the notion that it is the product of a secondary cascade. For the repressed gene Notch 3, blocking new protein synthesis had no effect (Fig. 1C); dsRNA still decreased the cellular level of this mRNA. Thus, Notch 3 repression is a direct effect of dsRNA treatment.

The characteristics of the underlying dsRNA signaling pathways were further explored by testing the response of DSGs to selected cytokines. Because dsRNA is viewed as an initiator of stress-response resulting from virus infection of mammalian cells, we wondered whether some of the DSGs are also induced by inflammatory cytokines, such as IFN-β, TNF-α, and IL-1, all of which are known to mediate host response to various types of tissue injury and inflammation. Results shown in Fig. 4 demonstrated that DSGs can be divided into at least four families according to their induction patterns. Genes, such as IFIT1, were induced by dsRNA or IFN-β but not by TNF-α or IL-1. BTN3A3 was induced by all inducers, and TNFAIP3 was induced by dsRNA, TNF-α, and IL-1 but not by IFN-β. In contrast, TPF2 was induced only by dsRNA. Similarly, only dsRNA repressed the Notch 3 gene; the three cytokines had no effect. These results suggest that dsRNA and different inflammatory cytokines may activate partially overlapping signaling pathways, thus providing opportunities for positive or negative cross-talks.

The partial overlaps between dsRNA, IFN, and viral signaling pathways are further illustrated by the comparison made in Fig. 5. In this analysis, induction profiles of several relevant genes were compared using primary data from this report and the literature. Four groups of genes were identified. Those in Group A are induced by dsRNA, IFN, or several viruses; genes in Group B are induced by IFN and viral infection; those in Group C are induced by dsRNA and viruses; and the Group D genes are induced by dsRNA and IFN. In addition, in cells transformed with HPV, expression of some of the same genes is repressed. These results indicate that different subsets of the same cellular genes are induced by the diverse signaling pathways activated by dsRNA, IFN, and virus infection.

**Functions of dsRNA-regulated Proteins—**Cellular functions of many but not all of the dsRNA-regulated genes identified by our screen are known. In Table I, the proteins encoded by these genes are grouped according to their functions. Several proteins known to be involved in IFN, TNF, and other cytokine- and growth factor signaling processes were induced by dsRNA. These proteins may mediate possible cross-talks between virus infection and cytokine actions. In addition, dsRNA induced a large number of cellular regulatory proteins affecting RNA and protein synthesis and processing, metabolism, transport, and cell structure. The abundance of several mRNAs encoding highly significant proteins was reduced as well (Table II). The repressed mRNA included those for VCAM1, TP53, PCK2, and STAM. Thus, it is clear that a short exposure of cells to dsRNA...
profoundly changes the cellular abundance of a large number of mRNAs whose products are essential to every aspect of cell physiology.

Because *IFIT1* was identified as the most strongly induced gene in response to treatment with dsRNA (Fig. 1) or IFN (15), we selected it for further functional studies. Cells were co-transfected with an expression vector for P56, the product of the *IFIT1* gene and an expression vector for the cell-surface protein CD20. Cells expressing CD20 were isolated by FACS and cultured for determining their doubling time. Cells expressing only CD20 and isolated in the same fashion served as controls. As shown in Fig. 6, cell growth was retarded in P56-expressing cells. The amount of P56 expressed in these cells was equivalent to that induced by 200 units/ml of IFN-β. It should be pointed out, however, that P56 was continuously expressed in the transfected cells whereas its expression in IFN or dsRNA-treated cells is transient. These results demonstrated that a major protein product of dsRNA signaling has a strong negative effect on the rate of cell proliferation.

**DISCUSSION**

We report here the results of the first systematic attempt for identifying genes regulated by double-stranded RNA. The most unexpected observation was that the expression of a large number of cellular genes was affected by dsRNA. Selected members of the set of DSGs identified here will be valuable tools for dissecting dsRNA signaling pathways. In this context, we are interested not only in the mechanisms regulating the transcriptional induction of these genes, but also in those that eventually shut off that process. The signaling pathways used by IFN, dsRNA, and viruses overlap partially because many genes are induced by two or three of these inducers (Fig. 5; Ref. 9, 16–19). Two dsRNA signaling pathways, although not fully characterized, are already known. One leads to NFκB activation and its binding to κB sites of genes and the other to activation of specific members of the IRF family, such as IRF-3 and IRF-7, and their binding to ISRE sites of genes (3, 5, 7).

Results presented here strongly indicate that additional pathways must exist because of non-coordinate up-regulation of different DSGs both kinetically and with respect to alternative inducers. For example, *TFIIF* was not induced by TNF-α or IL-1, although both of these cytokines activate NFκB. The same gene was also not induced by IFN-β, which activates transcription factors that induce transcription of ISRE-containing genes. Thus, further analysis of the promoter region of this gene will most likely reveal a new dsRNA signaling pathway that is mediated neither by κB elements nor by ISRE elements.

The current study also revealed that many genes are down-regulated by dsRNA. This novel observation is quite exciting, because nothing in the literature suggests such an effect of dsRNA.

Because dsRNA is often produced in virus-infected cells, the protein products of the dsRNA-regulated genes identified in our study are expected to play important functional roles in host-virus interaction. The dsRNA-induced transcription factors may directly affect virus gene transcription. On the opposite side, these gene products are certainly involved in the host response to virus infection. In the context of the equilibrium maintained between a virus and its host cells, some of these proteins may contribute to the host defense mechanisms whereas others may be used by the virus to evade the host defense. For example, P56, the product of the most strongly induced DSG *IFIT1*, is known to bind to the translation initiation factor eIF-3 and inhibit protein synthesis (20, 21). Here, we have shown that as a consequence, cell growth was inhibited (Fig. 6), which may, in turn, affect virus replication. Similarly, the efficacy of the spreading of viral infection *in vivo* may be affected by DSGs by altering the recognition pattern of the infected cell by cells of the immune system and modulating the synthesis of and response to antiviral cytokines such as IFN and TNF. One of the DSGs identified here encodes the DNA-binding zinc-finger protein TNFAIP3, which is known to block the NFκB signaling pathway activated by TNF (12).

Consequently, the null cells fail to down-regulate NFκB activation by TNF, and the corresponding mice die prematurely because of hypersensitivity to TNF and lipopolysaccharides (22). In the opposite scenario presented here, if TNFAIP3 is highly induced in virus-infected cells, these cells will be resistant to the apoptotic effects of TNF and LPS.

Functions of the DSGs are also relevant to the emerging field of RNAi, that uses gene-specific functional ablation by dsRNA. In bacteria, lower eukaryotes, plants and mice, expression of specific genes can be silenced by administering to the cells short dsRNAs corresponding to the genes (23). Thus, this strategy of gene ablation may soon become a major investigative and therapeutic tool for manipulating selective gene expression. In that context, the results presented here should alert us to the fact that any dsRNA, irrespective of its sequence content, will have additional global effects on cells by inducing the expression of a large number of cellular genes.

Similar considerations are also warranted for cells treated with antisense RNAs that are introduced by transfection or viral vectors.

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A Comprehensive View of Regulation of Gene Expression by Double-stranded RNA-mediated Cell Signaling

Gary Geiss, Ge Jin, Jinjiao Guo, Roger Bumgarner, Michael G. Katze and Ganes C. Sen

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